CYTOTOXIC AND ANTI-ESTROGENIC ACTIVITIES OF FIVE THAI MEDICINAL PLANTS CALLED 'HUA-KHAO-YEN' AND THEIR ISOLATED COMPOUNDS

BY

MISS PAKAKRONG THONGDEEYING

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN MEDICAL SCIENCES

FACULTY OF MEDICINE

THAMMASAT UNIVERSITY

ACADEMIC YEAR 2016

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ENTITLED

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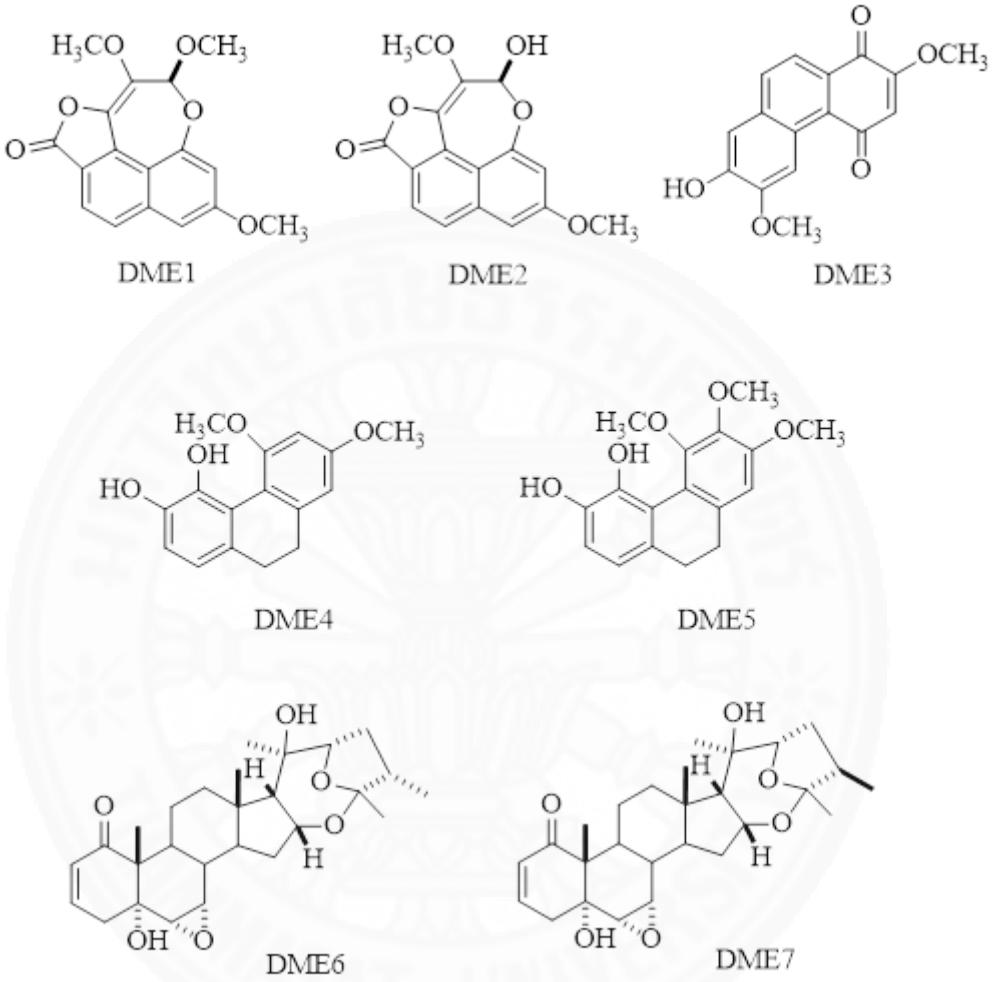
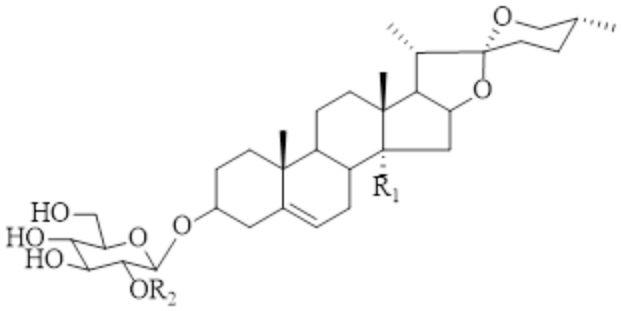
ABSTRACT

The rhizomes of Hua-Khoa-Yen have been used in Thai traditional medicine as an ingredient formula for cancer treatment especially liver and breast cancer which is the most cancer found in Thailand. Based on *in vitro* cytotoxicity of the ethanolic and water extract of Hua-Khoa-Yen, which are *Dioscorea birmanica*, *Dioscorea membranacea*, *Smilax corbularia*, *Smilax glabra* and *Pygmaeopremna herbacea* against hepatocellular carcinoma (HepG2), cholangiocarcinoma (KKU-M156) and breast cancer (MCF-7 and T47D) cells, both extracts of *D. membranacea* showed high potency specific. Aim of the study is to investigate the cytotoxic and estrogenic/anti-estrogenic activities of ethanolic and water extracts and constituents of *D. membranacea* to support its traditional use. The SRB assay was used to determine the cytotoxic activity against hepatocellular carcinoma (HepG2), cholangiocarcinoma (KKU-M156), breast cancer (MCF-7 and T47D) cells and one normal human keratinocyte immortal cells (HaCaT) with its extract and isolated compounds. The bioassay-guided fractionation of the rhizome of ethanolic extract of *D. membranacea* led to the isolation of two napthofuranoxepins [dioscorealide A (DME1) and dioscorealide B (DME2)], phenanthraquinone [dioscoreanone (DME3)], two phenanthrenes [5,6-dihydroxy-2,4-dimethoxy-9,10-dihydrophenanthrene (DME4) and

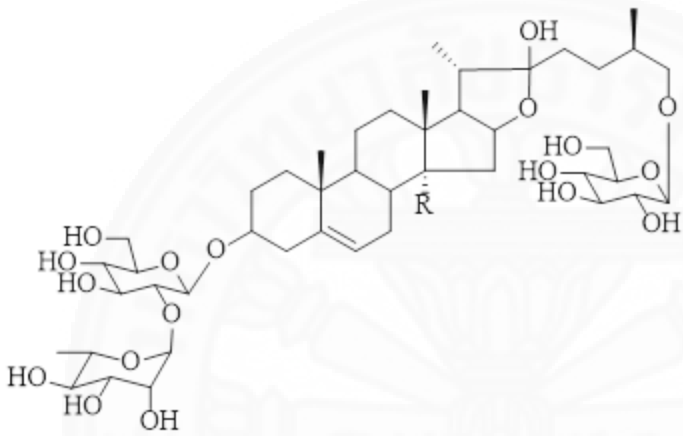
2,5,6-trihydroxy-3,4-dimethoxy, 9, 10-dihydrophenanthrene (DME5)], a known steroid [panthogenin B (DME6)] and a novel steroid [epipanthogenin B (DME7)]. The crude water extract of *D. membranacea* was purified by HPLC technique to give thirteen compounds namely six known steroids saponins; three spirostane steroids [25 (*R*)-spirost-5-en-3β,14α-diol-3-*O*-β-D-glucopyranoside or polygodoside E (DMW1), 25 (*R*)-spirost-5-en-3β, 14α-diol -3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-β-D-glucopyranoside or 25 (*R,S*)-dracaenoside F (DMW2) and diosgenin-3-*O*-α-L-rhamnosyl (1→2)-β-D-glucopyranoside or prosapogenin A of dioscin (DMW3)] and three furostane steroids [26-*O*-β-D-glucopyranosyl- 25(*R*)-furost-5-en-3β,14α,22, 26-tetrol -3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside or 25(*R,S*)-dracaenoside N (DMW4), 26-*O*-β-D-glucopyranosyl-22-hydroxyfurost-5-ene-3β,26-diol-3-*O*-α-L-rhamnopyranosyl (1 → 2)-β-D-glucopyranoside (DMW5) and 3β, 26-dihydroxy-25 (*R*)-furosta-$\Delta^{5,20(22)}$-diene-3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-β-D-glucopyranoside (DMW6)], in addition to seven compounds which were isolated from ethanolic extract (DME1-DME7). Their structures were elucidated by spectroscopic methods. The cytotoxicity of all isolated compounds against cholangiocarcinoma (KKU-M156), hepatocellular carcinoma (HepG2) and breast cancer (MCF-7 and T47D) were tested. Dioscorealide B (DME2) showed the highest potency against KKU-M156 (IC_{50} = 1.67 ± 0.21 μM), HepG2 (IC_{50} = 2.87 ± 0.10 μM), MCF-7 (IC_{50} = 2.17 ± 0.01 μM) and T47D (IC_{50} = 1.70 ± 0.17 μM) cells and had no toxicity against normal cell (HaCaT) with IC_{50} >100 μM but less toxic on normal cell with the SI value of >14.9 and >58.82, respectively. DME3 also showed high cytotoxicity against these four cell lines but toxic on immortal keratinocyte cell (HaCat). For the estrogenic and anti-estrogenic activities against E2-enhance T47D cell proliferation, no extracts showed estrogenic activitity through the concentration of 0.01-10 μg/mL. The crude ethanolic extract (DME) showed anti-estrogenic activity at 0.1 μg/mL, and crude water extract (DMW) was recognized the anti-estrogenic activity at 100 times less concentration (< 0.01 μg/mL). The dihydrophenanthrene (DME4) and steroid spirostane saponin (DMW1) showed strong inhibition against T47D cell at low concentrations with their $iEqE_1$ values of <0.1 μM and the highest activity than tamoxifen ($iEqE_1$ = 9 μM). Thus, this study supports Thai traditional use of this plant

for treatment cancer patients. DME2 should be devoped as anti-cancer drug for liver and bile duct cancer patients. In addition, this compound should be as anti-cancer drug for breast cancer because it displayed anti-estrogen.

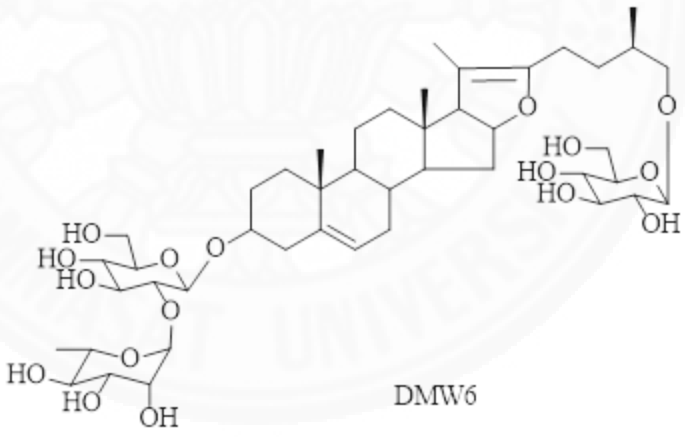




	R_1	R_2
DMW1	OH	H
DMW2	OH	Rhamnose
DMW3	H	Rhamnose



	R
DMW4	OH
DMW5	H



DMW6

Keywords: *Dioscorea membranacea* Pierre, cytotoxic activity, SRB assay, anti-estrogenic activity, hepatocellular carcinoma, cholangiocarcinoma, breast cancer

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Miss Pakakrong Thongdeeying

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LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
CC	Column chromatography
$CHCl_3$	Chloroform
EtOAc	Ethyl acetate
EqE100	A concentration of sample that stimulated the cell proliferation to the equivalent level of 100 pM of estradiol (E2)
EqE10	A concentration of sample that stimulated the cell proliferation to the equivalent level of 10 pM of estradiol (E2)
HMQC	Heteronuclear multiple quantum coherence
HMBC	Heteronuclear multiple bond correlation
HPLC	High performance liquid chromatography
IC_{50}	Concentration required for 50% inhibitionA concentration of sample that
iEqE50	inhibited 50% of the cell proliferation enhanced by 100 pM of E2 (effect to the equivalent level of 50 pM at 50% inhibition)
iEqE10	A concentration of sample that inhibited 90 % of the cell proliferation enhanced by 100 pM of E2 (effect to the equivalent level of 10 pM at 90% inhibition)
IR	Infrared spectroscopy
MeCN	Acetonitrile
MeOH	Methanol
L	Liter
mL	Milliliter

NMR	Nuclear magnetic resonance
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser effect spectroscopy
t_R	Retention time
TLC	Thinlayer chromatography
UV	Ultraviolet spectroscopy
µM	Micromolar
pM	Picomolar
VLC	Vacuum liquid chromatography

CHAPTER 1
INTRODUCTION

Cancer is one of those serious diseases currently affecting lives and society. There has not been any way to heal effectively because of the cancer cells display uncontrolled growth, ultimately spreading throughout the body and interfering with the function of healthy tissues and organs (Cooper & Hausman, 2009). Cancer is caused by both external factors (chemical, radiation, tobacco and infectious organisms) and internal factor (inherited mutations, hormones, and immune condition). Treatments on cancer such as surgery, radiation, chemotherapy, hormone therapy, immune therapy, and targeted therapy (drugs that interfere specifically with cancer cell growth) were observed (American Cancer Society, 2016).

Cancer is also the leading cause of death in Thailand (The Bureau of Policy and Strategy, 2017). Thai cancer data from the National Cancer Institute reported that the most common cancer in women is breast cancer and the most common cancer in men are lung, colon, liver and bile duct cancer (Pongnikorn *et al*., 2015).

Breast cancer is a significant public health problem in female worldwide. It is suggested that estrogens cause breast cancer by stimulating cell growth and proliferation through receptor-mediated processes and via their genotoxic metabolites (Mense *et al*., 2008). These functions are thought to be mediated through estrogen binding to two types of estrogen receptors, ERα and ERβ, which act as transcriptional factors regulating estrogen responsive gene expressions (Osborne, 1998). Moreover, estrogen also plays an important role in maintaining bone density, cognitive functions and lowering cholesterol in female. The major treatments for breast cancer are chemotherapy and anti-estrogen drugs. Tamoxifen is drug commonly used, but the most common side-effects include hot flashes (50% of women), vaginal discharge, and irregular menses in pre/perimenopausal women (Ali *et al*., 2011). The phytoestrogens such as isoflavones have been proposed as being a chemopreventive factor against breast cancer in Asian populations (Rettberg *et al*., 2011). Many phytoestrogens appear to have a biphasic effect on cell proliferation, stimulating

growth at low concentrations and suppressing growth at high levels. At low concentrations, resveratrol and quercetin dose-dependently promoted growth in ER-positive MCF-7 cells, but it inhibited proliferation and induced cell death at high concentrations (Mense *et al*., 2008).

Liver and bile duct cancer is a major health problem in the northeast Thailand because its high incidence rate and the most cancer patients are male (Srivatanakul *et al*., 2004; Wiangnon *et al*., 2012; Pongnikorn *et al*., 2015). Bile duct cancer or cholangiocarcinoma is a malignant tumor arising from the intrahepatic or extrahepatic bile duct epithelial cells. It is one of the most serious diseases in the northeast Thailand because of the prevalence of *Opisthorchis viverrini* infection was high, (Khuhaprema *et al*., 2007; Watanapa *et al*., 2002; Intuyod *et al*., 2012). Liver fluke infections occur in some Asian countries when people eat raw or poorly cooked fish which are infected with these tiny parasitic worms. In humans, these flukes live in the bile ducts which bring to chronic inflammation and contributed to cholangiocarcinogenesis. The main types of treatment for bile duct cancer is chemotherapy, but the principle of treatment using chemotherapy is finding a drug that can kills cancer cells and has little effect on normal cells (Halliwell & Gutteridge, 1988). Many anticancer agents have been discovered, but to be useful, they must be related to novel molecular targets; i.e. they should be effective against specific types of cancer cells but non- toxic to normal cells, or have a unique mechanism of action for specific types of cancer (Pezzuto, 1997). While some cancer patients can receive treatment with a plethora of orthodox cancer treatment procedures, a large percentage still relies on Thai traditional medicines (Jiradhammo, 2008). Many anticancer drug formulations have been used for the treatment of cancer patients namely Yod-ya-mareng and Ya-tan-mareng Singburi (Poonthananiwatkul *et al*., 2015) which were introduced by Pra Paponpat Pibanpaknitee, the abbot of Khampramong temple. These formulations are used to treat various types of cancer, especially liver, lung, prostate, cervical and breast cancer. Thai herbal medicine called ‘Hua-Khao-Yen’ is the main ingredient of anticancer drug formulations in Yod-ya-mareng of Kumpramong temple. Hua-Khao-Yen-Neua and Hua-Khao-Yen-Tai [*Smilax corbularia* Kunth. (Smilacaceae) and *Dioscorea membranacea* Pierre (Dioscoreaceae), respectively] are used in this formulation (Itharat *et al*., 2004; Poonthananiwatkul *et al*., 2015).

Thai medicinal plants locally known as “Hua-Khao-Yen” have mostly been used in Thai traditional medicines (Ancient Medicine Association, 1962, 1978; Mutita, 1989; Pongbunrod, 1976; Traditional Lanna Thai Medicine, 1982). About 2449 preparations of Hua-Khao-Yen have been registered at the Ministry of Public Health of the Thai government (Division of Medical Research, 1986). These preparations have been used to treat leprosy, venereal diseases, inflammations, bacterial infections, and cancers. However, in a survey of 23 Thai traditional doctors nationwide, discovered that using “Hua-Khao-Yen” with 5 species which are *Dioscorea birmanica*, *Dioscorea membranacea*, , *Pygmaeopremna herbacea*, *Smilax corbularia* and *Smilax glabra*, to treat cancers, AIDS, septicemia and lymphatic diseases (Itharat *et al*., 1998). In the previous work, only crude ethanolic extract of the rhizomes of *D. membranacea* exhibited many activities such as anti-allergic activity (Tewtrakul & Itharat, 2006), anti-inflammatory activity (Tewtrakul & Itharat, 2007; Reanmongkol *et al*., 2007), anti-HIV-1 integrase- and HIV-1 protease (Tewtrakul *et al*., 2006) and also stimulate immune (Panthong *et al*., 2014). Additionally, the ethanolic extract of this plant also showed anti-cancer activity whereas, there are several studies to support Thai traditional use. These report showed that the ethanolic extract of this plant showed cytotoxic activity against five human cancer cell lines, including breast (MCF-7), large cell lung carcinoma (COR-L23), colon (LS-174T), liver (HepG2), and prostate (PC3), using the SRB assay, but had no toxicity on two type of normal cells (human keratinocytes SVK-14 and lung fibroblast MRC-5) (Itharat *et al*., 2004; Itharat *et al*., 2014). Ethanolic and water extracts of *D. membranacea* were tested for toxicity at Department of Medical Sciences, Ministry of Public Health and showed no acute toxicity to rats (Itharat & Ooraikul, 2007). In the previous report, the ethanolic extract of *D. membranacea* rhizome has been phytochemical studied, yielded two napthofuranoxepins [dioscorealide A and B] (Itharat *et al*., 2003), a phenanthraquinone [dioscoreanone] (Itharat *et al*., 2003), three phenanthrenes [2,4-dimethoxy-5,6-dihydroxy-9,10-dihydrophenanthrene, 5-hydroxy-2,4,6-trimethoxy-9,10-dihydrophenanthrene and 2,5,6-trihydroxy-3,4-methoxy, 9,10-dihydrophenanthrene] (Itharat *et al*., 2014), three steroids [β-sitosterol, stigmasterol and sitosterol-β-D-glucoside] and two steroid saponins [diosgenin-3-*O*-α-L-rhamnopyranosyl (1→2)-β-D-glucopyranoside and diosgenin-3-*O*-β-D-glucopyranosyl

(1→3)-β-D-glucopyranoside] (Itharat & Ooraikul, 2007). All of these compounds have been tested for *in vitro* cytotoxic activity using SRB assay (Itharat *et al*., 2003; Itharat & Ooraikul, 2007; Itharat *et al*., 2014). Moreover, the steroid saponins which is rich source of the rhizomes of *Dioscorea* genus have been reported for their anti-bacterial, anti-osteoporotic, anti-cancer, anti-inflamatory activities and stimulate immune (Sautour, 2007). In addition the steroid sapogenins can be used as a drug for oral contraceptive, hormones and cortisone (Maneenoon, 2013).

In addition, ethanolic extract from *S. corbularia* rhizome significantly suppressed the paw edema induced by carrageenin in rats (Reanmongkol *et al*., 2007b). Moreover, resveratrol is a phytoestrogen compound from *S. corbularia* which is one type of Hua-Khao-Yen shown to have anti-estrogenic activities when testing against the estrogen-responsive human breast cancer cell lines MCF-7 (Wungsintaweekul *et al*., 2011). However, for the five plants called Hua-Khao-Yen, there has been no report of their cytotoxic effect on liver and bile duct cancer, while for four types of Hua- Khao-Yen, there has been no report of their estrogenic effect.

Objectives

1. To study on cytotoxicity against liver and bile duct cancer including breast cancer cells and estrogenic and anti-estrogenic activities of extracts of five species of Hua-Khao-Yen.

2. To study on isolation and structure elucidation of five species of Hua-Khao-Yen.

3. To study on isolated compounds of active species on cytotoxicity against breast cancer, liver and bile duct cancer, estrogenic and anti-estrogenic activities of extracts of five species of Hua-Khao-Yen.

CHAPTER 2
REVIEW OF LITERATURE

2.1 General data of plants called Hua-Khao-Yen

Thai medicinal plants locally known as Hua-Khao-Yen have mostly been used as an ingredient in Thai traditional medicines (Ancient Medicine Association, 1962, 1978; Mutita, 1989; Pongbunrod, 1976; Traditional Lanna Thai Medicine, 1982). More than 2400 preparations having Hua-Khao-Yen have been registered at the Ministry of Public Health of Thailand (Division of Medical Research, 1986). These preparations have been used to treat leprosy, venereal diseases, inflammations, bacterial infections and cancers. Hua- Khao-Yen has been found as ingredients in almost every traditional drug formula for cancer (Vimolkhunakorn, 1979). Itharat *et al*., 1998 found that traditional doctors of Southern Thailand used Hua-Khao-Yen as one of the ingredients in their drug formula (60% of 30 formulas) for cancer treatment. Moreover, 23 Thai traditional doctors have used five species of Hua-Khao-Yen including *Dioscorea birmanica, Dioscorea membranacea, Smilax corbularia, Smilax glabra*, and *Pygmaeopremna herbacea* to treat cancers, AIDS, septicemia and lymphatic diseases (Itharat, 2002). The extracts of these species were usually prepared by boiling with water or soaking with ethanol (Pongbunrod, 1976; Tungtrongjit, 1978). Among the five species, *Dioscorea membranacea* Pierre, called Hua-Khao-Yen-Tai, showed the highest cytotoxic activity against human cancer cell lines but was less active for normal cells (Itharat, 2002) and it has also been widely used to prepare Thai traditional anticancer medicines.

2.2 *Dioscorea* species

Genus *Dioscorea* (Dioscoreaceae) consists of about 600 species in all tropical and subtropical regions, which includes 42 species found in Thailand. Some *Dioscorea* species were used as staple food in some countries of Africa but more recently, the food made from yam (*Dioscorea* species), due to its less economic value,

has been increasingly replaced by other food crops. *Dioscorea* species are also used as sources of steroidal saponin and medicines derived from them (Wilkin & Thapyai, 2009). In Thailand, Thai traditional doctors have used two species of *Dioscorea* species: *Dioscorea birmanica* and *Dioscorea membranacea*, to treat cancers, AIDS, septicemia and lymphatic diseases (Itharat, 2002).

2.2.1 *Dioscorea birmanica* Prain et Burkill (Dioscoreaceae)

Figure 2.1 *Dioscorea birmanica* Prain et Burkill (Itharat, 2002)

Scientific name: *Dioscorea birmanica* Prain et Burkill

Snynonym: *Dioscorea horrida* Buch-Ham, *D. rangunensis* R.Kunth, *D. spinosa* Wall

Family name: Dioscoreaceae

Local name: Kloi-Khao (กลอยเขา), Man Chuak (มันจั๊วก), Man Nok (มันนก), Hua-Khao-Yen (หัวข้าวเย็น)

It is distributed in northern central and lower Burma, Thailand, excluding the Peninsula. It is used as a male contraceptive and to treat Parkinson's disease (Prain & Burkill, 1936).

2.2.1.1 General description of *Dioscorea birmanica* Prain et Burkill

A description of *Dioscorea birmanica* Prain et Burkill is shown in **Figure 2.1** and **2.2** is: woody rhizome and has a hard skin cracking rather rectangularly like the carapace of a tortoise. The stem is reaching a length of 20 m or

more, pubescent or puberulous or glabrous, about if pubescent than as it ages glabrescent faintly grooved, armed with scattered prickles, most abundant lower down about the leaf bases. Bulbils never observed. Leaves scattered, cordate to broadly cordate, up to 15 cm in length by 16 cm in width, upper surface glabrous or pubescent towards the base, with the 7-11 nerves, and the largest commonly carrying one or more sharp recurved prickles; petiole with prickles, pubescent, puberulous or glabrous, 4.5-9 cm long. Male flowers group into few-flowered cymes with arranged along the axis of spike-like inflorescence, flower sessile, axis pubescent; cymes 1 cm long, perianth deeply campanulate, stamen 6. Female flowers, 20-40 on a spike perianth-segments, staminodes minute, ovary densely pubescent. Capsule crowed, 20 to 30 mm long, 10 to 15 mm wide (Prain & Burkill, 1936).

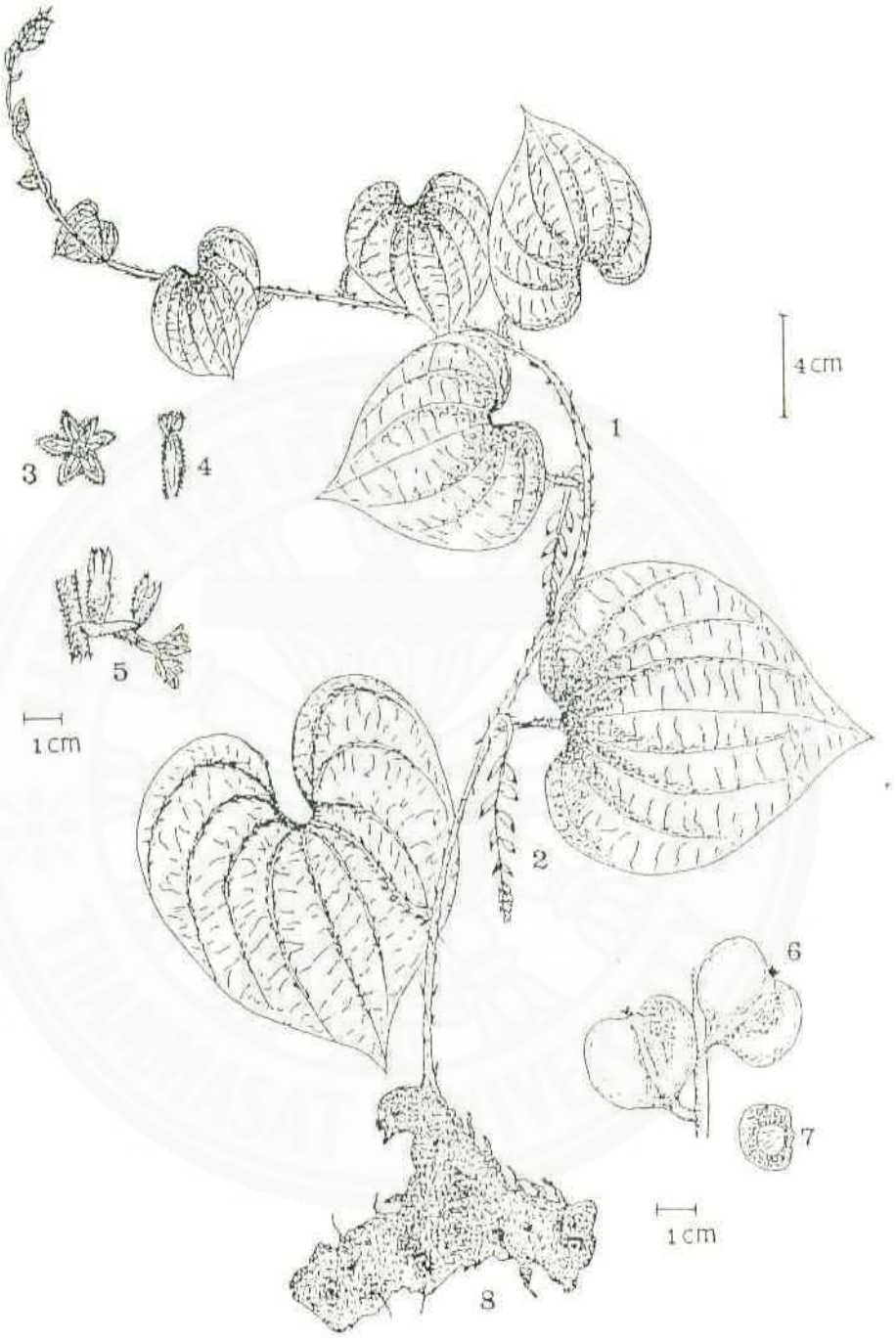


Figure 2.2 *Dioscorea birmanica* Prain et Burkill; 1.whole plant; 2.female inflorescence; 3. staminate flower; 4.pistillate flower; 5.male florescence; 6.fruits; 7.seed; 8. Rhizome (Itharat, 2002; Boonyaratanakornkit & Chantaptavan, 1993)

2.2.2 *Dioscorea membranacea* Pierre ex Prain & Burkill. (Dioscoreaceae)

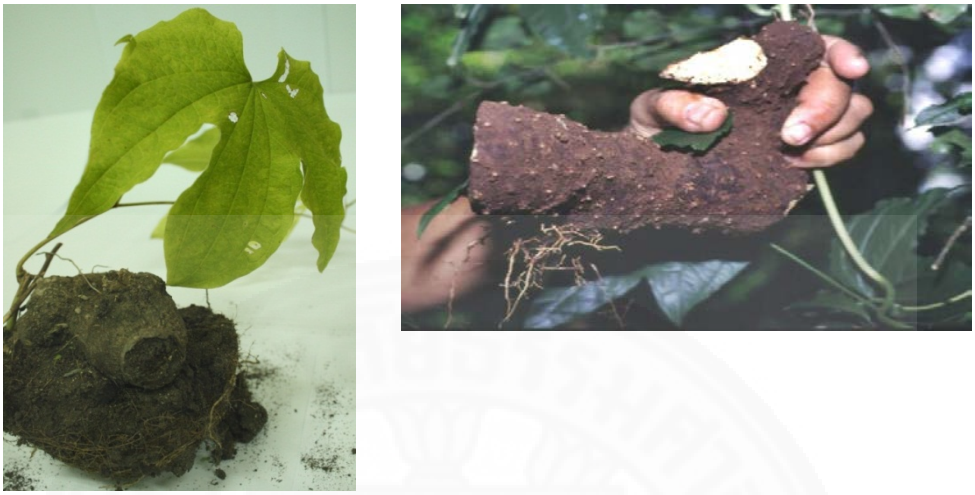


Figure 2.3 *Dioscorea membranacea* Pierre (Itharat, 2002)

Scientific name: *Dioscorea membranacea* Pierre ex Prain & Burkill.

Family name: Dioscoreaceae

Local name: Phak-Lum-Phua (ผักลืมผัว), Man Moo (มันหมู), Khao-Yen-Tai (ข้าวเย็นใต้) (Supatanakul *et al*., 1985)

It is distributed from Thailand westwards to north Myanmar and eastwards into Cambodia; southwards passing beyond the Isthmus of Kra into Malaysia. It grows on limestone at its southern limit (Burkill, 1951). Its rhizome is edible and medicinally used for long time by local people (Supatanakul *et al*., 1985). It is used to treat cancer (Subchareon, 1998; Vimolkhunakorn, 1979).

2.2.2.1 General description of *Dioscorea membranacea* Pierre

A description of *Dioscorea membranacea* Pierre is shown in **Figure 2.3**-**2.6** is; a wide running rhizome, perhaps even to 2 m, dark brown, with white flesh. Stem slightly ridged, unarmed. Leaves deeply trifid above a cordate base, shortly acuminate, nine nerves, two primary nerves reaching the forerunner tip along

with the midrib and the second pair reaching the tips of the leteral lobes; petiole 1/2-2/3 the length of the blade. Male flowers in small subsessile cymes with up to 4 flowers, tepals 1 mm long, long-ovate. Stamens all alike, the filaments inserted just below the tepals, 0.3 mm long; anther introse, small. Female flowers on down-wardly directed spike- like racemes. Tube of flower absent. Outer tepals obovate, inner ones lanceolate, a little shorter than the outer, style short, capsules 1-2 cm apart (Burkill, 1951).

Figure 2.4 *Dioscorea membranacea* Pierre A and B: inflorescence C: flower D: stem (Itharat, 2002)

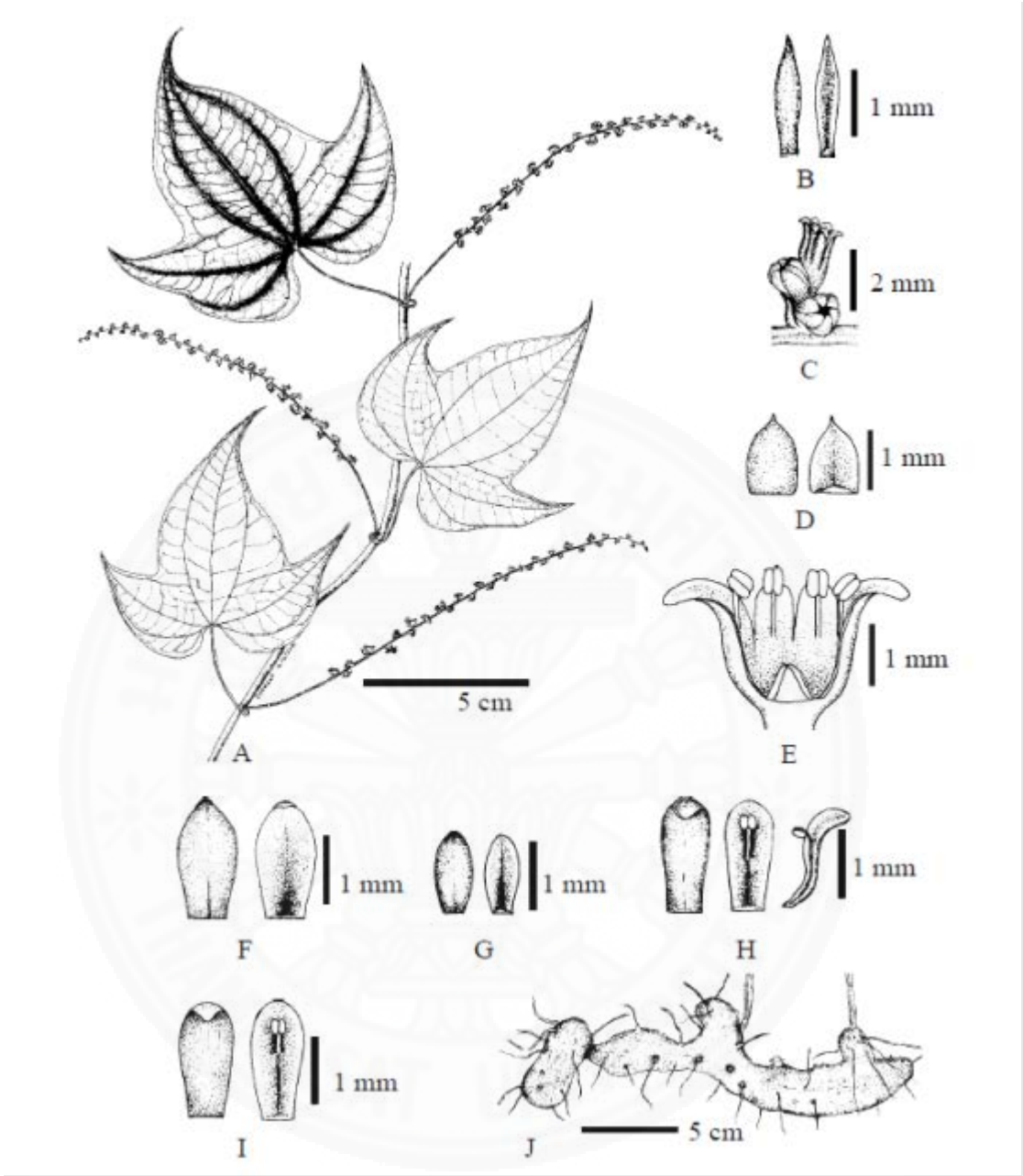


Figure 2.5 *Dioscorea membranacea* Pierre: male plant, A. habit with inflorescences; B. primary bract dorsal and ventral surfaces; C. part of inflorescence, showing flower in a cymule; D. cymular bract dorsal and ventral surfaces; E-I. male flower; E, I. section, showing stamens and pistillode; F, G. floral bract and bracteole dorsal and ventral surfaces respectively; H, I. outer and inner tepal dorsal and ventral surfaces respectively, showing position of stamen insertion, J. rhizome
(Ruangnoo, 2012; Wilkin & Thapyai, 2009; Thapyai, 2004)

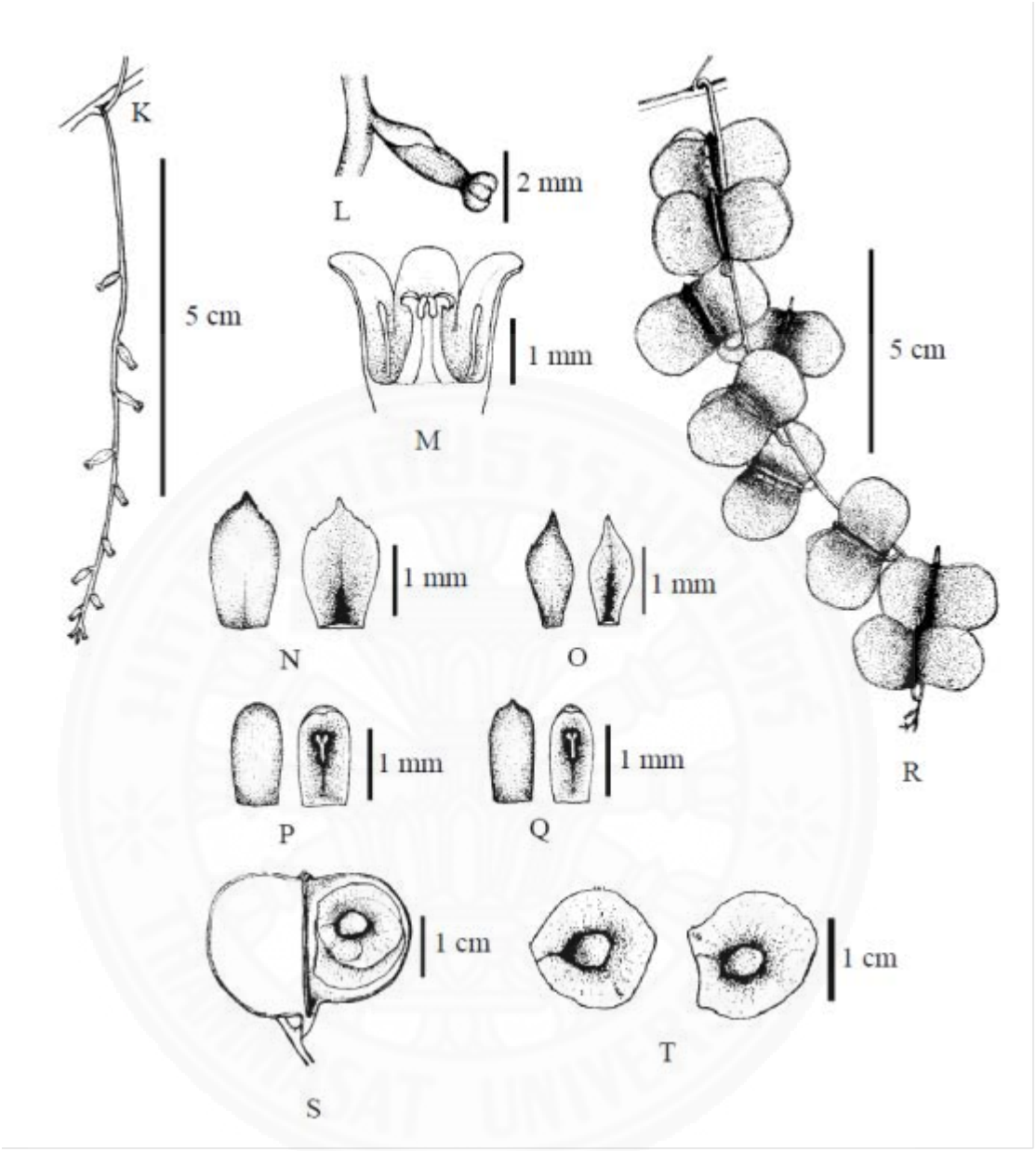


Figure 2.6 *Dioscorea membranacea* Pierre: female plant, K. inflorescence; L-Q. flower; L. side view; M. l. section (excluding ovary) showing staminodes, style and stigmas; N, O. floral bract and bracteole dorsal and ventral surfaces respectively; P – Q. outer and inner tepal dorsal and ventral surfaces respectively, showing position of staminode insertion; R. infructescence; S. mature capsule, l. section showing seed position; T. seed (Ruangnoo, 2012; Wilkin & Thapyai, 2009; Thapyai, 2004)

2.2.3 Biological activity of *Dioscorea* species

The investigations on the biological activity of *Dioscorea* species in Thailand are shown in **Table 2.1**.

Table 2.1 *Dioscorea* species found in Thailand and biological activity

***Dioscorea* species**	**Plant part**	**Activity**	**Result of biological activity**	**References**
D. birmanica	Rhizome	Anti-HIV	The water and ethanol extracts of *D. birmanica* exhibited high anti-HIV-1 IN activity with IC_{50} values of 4.5 vs 4.7µg/mL. Both extracts showed mild activity against HIV-1 PR.	Tewtrakul *et al.*, 2006
	Rhizome	Anti-inflammatory	Ethanol and water extracts from *D. birmanica*, exhibited low potent inhibitory activity (IC_{50} = 41.7 and 62.6 µg/mL, respectively) against LPS induced NO production in RAW 264.7 cell lines.	Tewtrakul & Itharat, 2007
	Rhizome	Anti-inflammatory	Four fractions from DBE were isolated using vacuum liquid chromatography (VLC) with ordering polarity of solvents and were coded as DB-Fr1 to DB-Fr4, respectively. Fraction DB-Fr2 ($CHCl_3$), DB-Fr3 ($CHCl_3$: MeOH, 8:2) and DB-Fr1 (Hexane : $CHCl_3$, 2:8) exhibited high inhibitory effect on PGE2 production (IC_{50} = 5.02 ± 0.31 µg/mL, 6.96 ± 0.04 µg/mL and	Jaiaree *et al.*, 2013

Table 2.1 (continued)

***Dioscorea* species**	**Plant part**	**Activity**	**Result of biological activity**	**References**
D. birmanica	Rhizome	Anti-inflammatory	11.04 ± 1.23 μg/ml, respectively) (IC_{50} of indomethacin or positive control as 1 μg/mL).	
	Rhizome	Antimicrobial	From bioassay- guided fractionation, all extracts had no antibacterial activity against gram-negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Shigella dysenteriae* but showed less antibacterial against gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis* (clear zone diameter = 7-8 mm.). For the antifungal activity, DB-Fr4-P1 showed the highest activity against *Candida albicans* (clear zone was equal to 10 ± 0.17 mm. and MIC =12.5 μg/mL).	Jaiaree *et al.*, 2013
	Rhizome	Cytotoxic	Ethanolic extract showed high activity against COR-L23 (IC_{50}=7.4 ±1.4 μg/mL) and MCF-7 (IC_{50}=16.3 ±0.8 μg/mL). Water extract inactive against Lung (COR-L23), Breast (MCF-7) and colon cancers (LS-174T).	Itharat *et al.*, 2004

Table 2.1 (continued)

***Dioscorea* species**	**Plant part**	**Activity**	**Result of biological activity**	**References**
D. birmanica	Rhizome	Cytotoxic	The ethanolic extract showed high cytotoxic activity against lung cancer cells; A 549 and COR-L23 (IC_{50} = 7.45 and 8.71 μg/mL, respectively) but no cytotoxic activity against normal cancer cells MRC-5 (IC_{50} = 94.76 μg/mL). Diosgenin-3-*O*-*α*-L-rhamnosyl (1→2)-β-D-glucopyranoside or prosapogenin A of dioscin, isolated compound had highest cytotoxic activity against those two types of lung cancer cells (IC_{50} = 1.81 and 1.84 μg/mL), respectively) but less cytotoxicity against normal lung cells MRC-5 (IC_{50} = 37.09 μg/mL).	Jaiaree *et al.*, 2010
D. membranacea	Rhizome	Anti-allergy	Ethanolic extract of *D. membranacea* exhibited potent inhibitory activity against β-hexosaminidase release as a marker of degranulation in RBL-2H3 cells (IC_{50} = 37.5 μg/mL).	Tewtrakul & Itharat, 2006

Table 2.1 (continued)

***Dioscorea* species**	**Plant part**	**Activity**	**Result of biological activity**	**References**
D. membranacea	Rhizome	Anti-allergy	The isolated compounds from crude ethanolic extract were tested for their anti-allergic activities. The results showed that dioscorealide B (**4**) possessed the highest activity (IC_{50} = 5.7 µM), followed by dioscoreanone (**5**, IC_{50} = 7.7 µM), dioscorealide A (**3**, IC_{50} = 27.9 µM), and diosgenin (**1**, IC_{50} = 29.9 µM).	Tewtrakul & Itharat, 2006
	Rhizome	Anti-inflammatory	Ethanolic extract of *D. membranacea* exhibited the most potent inhibitory activity (IC_{50} = 23.6 µg/mL) against lipopolysaccharide (LPS) induced nitric oxide (NO) production in RAW264.7 cell lines. Diosgenin-3-*O*-α-L-rhamnosyl (1→2)-β-D-glucopyranoside (IC_{50} = 3.5 µM), dioscoreanone (IC_{50} = 9.8 µM) and dioscorealide B (IC_{50} = 24.9 µM) isolated from ethanolic extract which are active principles for NO inhibitory activity of *D. membranacea*, and dioscoreanone (IC_{50} = 17.6 µM) also exhibited a potent inhibitory effect on TNF-α release.	Tewtrakul & Itharat, 2007

Table 2.1 (continued)

***Dioscorea* species**	**Plant part**	**Activity**	**Result of biological activity**	**References**
D. membranacea	Rhizome	Anti-inflammatory	Oral administration of the ethanol extract at the dose of 1600 mg/kg significantly decreased the paw edema induced by carrageenin in rats. The aqueous extract (1600 mg/kg) also significantly suppressed the carrageenin-induced paw edema in rats. The ethanol extract had no significant effects on an antinociceptive response in writhing, formalin and hot plate tests and antipyretic activities in yeast-induced fever in rats. No significant effects on writhing test and yeast-induced fever were observed after oral administration of the aqueous extract in experimental animals.	Reanmongkol *et al*., 2007a
	Rhizome	Anti-inflammatory	Dioscorealide B, isolated compound from an ethanolic extract of *D. membranacea*. The molecular mechanisms of DB on the inhibition of NO production and mRNA expression of iNOS, IL-1b, IL-6, and IL-10 were due to the inhibition of the upstream kinases activation, which	Hiransai *et al*., 2010

Table 2.1 (continued)

***Dioscorea* species**	**Plant part**	**Activity**	**Result of biological activity**	**References**
D. membranacea	Rhizome		further all eviated the NF-kB and MAPK/ERK signaling pathway in LPS-induced RAW264.7 macrophage cells.	
	Rhizome	Anti-inflammatory	Dioscoreanone, isolated from an ethanolic extract of *D. membranacea*. The mechanisms of dioscoreanone on the inhibition of NO production and mRNA expression of iNOS, IL-1b, and IL-6 were due to both the inhibition of NF-kB activation and the activation of ERK1/2 proteins.	Itharat & Hiransai, 2012
	Rhizome	Anti-HIV	The water and EtOH extract were apparently inactive (IC_{50} > 100 µg/mL) against HIV-1 IN, but EtOH extract showed appreciable activity (IC_{50} = 48µg/mL) against HIV-1 PR.	Tewtrakul *et al.*, 2006
	Rhizome	Antioxidant	The ethanolic extract of *D. membranacea* rhizome showed the highest antioxidant activity by lipid peroxidation of liposome assay (IC_{50} = 8.10 ±1.21 µg/mL).	

Table 2.1 (continued)

***Dioscorea* species**	**Plant part**	**Activity**	**Result of biological activity**	**References**
D. membranacea	Rhizome	Antioxidant	The DPPH test for antioxidant activity was employed and also tested for LDH release as an indicator of damage to the cell membrane. Dioscoreanone, isolated compound of ethanolic extract showed highest antioxidant activity.	Itharat *et al.*, 2007
	Rhizome	Cytotoxic	Dioscorealides A and B and dioscoreanone were isolated from ethanolic extract of which Dioscorealides A is slightly active against only MCF-7, whereas dioscorealides B exhibited the best potency, especially against MCF-7 and COR-L23, as well as the best selective discrimination among normal and cancer cells.	Itharat *et al.*, 2003
	Rhizome	Cytotoxic	Ethanolic extract showed high activity against COR-L23 (IC_{50} = 6.2 ±1.9 µg/mL) moderate for MCF-7 (IC_{50} = 12.0 ± 0.2 µg/mL) and LS-174T (IC_{50} = 16.7 ±10.4 µg/mL). While its water extract exhibited high cytotoxic activity against breast and colon cancer cell lines with the IC_{50} value of 5.5 and 15.6 µg/mL, respectively. Both extracts	Itharat *et al.*, 2004

Table 2.1 (continued)

***Dioscorea* species**	**Plant part**	**Activity**	**Result of biological activity**	**References**
D. membranacea	Rhizome	Cytotoxic	had no cytotoxic activity to SVK-14 keratinocyte normal cell line (IC_{50} >70 µg/mL).	
	Rhizome	Cytotoxic	The molecular mechanism of cytotoxic compounds of *D. membranacea* was investigated. It was found that in vitro cytotoxic activity of dioscorealide B against human breast cancer cells (MCF-7) was significant with IC_{50} = 0.94 µg/mL. To determine whether this active compound induces apoptosis in MCF-7, Annexin V assay was performed. The results showed that the numbers of apoptotic cells were increased to 7-12 folds of control after treatment with various concentrations of dioscorealide B (1, 2 and 4 µg/ml) for 24 hours. In addition, the data revealed that dioscorealide B induced the activation of caspase-7, -8, and -9. These data suggested that the apoptotic mechanism of this compound might be involved in both intrinsic and extrinsic apoptotic pathway. Moreover, dioscorealide B showed cytotoxic effect on MCF-7 (IC_{50} = 2.76 µM) and	Saekoo *et al.*, 2010.

Table 2.1 (continued)

***Dioscorea* species**	**Plant part**	**Activity**	**Result of biological activity**	**References**
D. membranacea	Rhizome	Cytotoxic	MDA-MB 468 (IC_{50} = 9.93 μM). The mRNA level for p53, p21 and Bax were increased while Bcl-2 was decreased after the treatment. MCF-7 treated with dioscorealide B showed the induction of apoptosis via the activation of caspase-9 and -7.	
	Rhizome	Cytotoxic	The cytotoxicity of dioscorealide B was evaluated in two human breast cancer cell lines, MCF-7 and MDA-MB 468 by SRB assay. RT-PCR and Caspase-Glo® assay were used to further elucidate its cytotoxic mechanism. Dioscorealide B showed cytotoxic effect on MCF-7 (IC_{50}=2.76 μM) and MDA-MB 468 (IC_{50} = 9.93 μM). The mRNA level for p53, p21 and Bax were increased while Bcl-2 was decreased after the treatment. MCF-7 treated with dioscorealide B showed the induction of apoptosis via the activation of caspase-9 and -7. The mechanisms of dioscorealide B might be involvedin p53 and the intrinsic apoptotic pathway.	Saekoo *et al*., 2010

Table 2.1 (continued)

***Dioscorea* species**	**Plant part**	**Activity**	**Result of biological activity**	**References**
D. membranacea	Rhizome	Cytotoxic	Two known dihydrophenanthrenes [2,4 dimethoxy-5,6-dihydroxy-9,10-dihydrophenanthrene (1) and 5-hydroxy-2,4,6-trimethoxy-9,10-dihydrophenanthrene (2)], and a new dihydrophenanthrene 5,6,2 -trihydroxy 3,4-methoxy, 9,10-dihydrophenanthrene (3) were isolated compounds showed the highest cytotoxic activity against COR-L23, MCF-7 and PC3 cell lines (IC_{50} = 14.89, 17.49 and 19.04 µM, respectively), and compound 1 showed selective cytotoxic activity against PC3 (IC_{50} = 23.54 µM). The new compound 3 showed selective cytotoxic activity against only MCF-7 cells (IC_{50} = 31.41 µM).	Itharat *et al*., 2014
	Rhizome	Toxicity	Ethanolic and water extracts had no acute toxicity in rats (LD_{50} = 9 g/kg and LD_{50} >25 g/kg, respectively).	Itharat & Ooraikul, 2007

Table 2.1 (continued)

***Dioscorea* species**	**Plant part**	**Activity**	**Result of biological activity**	**References**
D. membranacea	Rhizome	Cytotoxic	Dioscoreanone (DN) slowed down the cell division and arrested the cell cycle at the G2/M phase in treated A549 cells, leading to a dose- and time- dependent increase of the sub-G1 population (apoptotic cells). Consistently, early apoptotic cells (AnnexinV +/PI−) were detected in those cells that were treated for 24 h and increased progressively over time. Moreover, the highest activity of caspase-3 in DN-treated A549 cells was detected within the first 24 h, and pretreatment with the general caspase inhibitor z-VAD-fmk completely abolished such activity and also DN-induced apoptosis in a dose-dependent manner. Additionally, DN increased the Bax/Bcl-2 ratio in treated A549 cells with time, indicating its induction of apoptosis via the mitochondrial pathway.	Hansakul *et al*., 2014

Table 2.1 (continued)

***Dioscorea* species**	**Plant part**	**Activity**	**Result of biological activity**	**References**
D. membranacea	Rhizome	Immunomodulatory	Ethanolic extract of *D. membranacea* significantly stimulated NK cells activity against K562 cells line at lower concentrations of 10 and 100 ng/mL, but not at higher concentrations. The ethanolic extract showed no observable effect on lymphocyte proliferation. The crude water extract significantly increased NK cell activity at concentrations of 10 ng/mL, 100 ng/mL, 1 μg/mL, 10 μg/mL and 100 μg/mL, and also activated lymphocyte proliferation at concentration of 1 ng/mL, 10 ng/mL, 100 ng/mL, 1 μg/mL, 5 μg/mL, 10 μg/mL and 100 μg/mL.	Panthong *et al*., 2014
	Rhizome	Immunomodulatory	Dioscorealide B had no significant effect at lower concentrations (0–1 μg/mL and 0–0.1 μg/mL, respectively) on NK cell activity and lymphocyte proliferation. At higher concentrations (>10 μg/mL and >0.5 μg/mL) of DB cause a significant decrease in NK cell activity and lymphocyte proliferation.	Panthong *et al*., 2014

2.2.4 Chemical constituents of *Dioscorea* species

Chemical constituents isolated from *Dioscorea* species found in Thailand are summarized in **Table 2.2,** and **Figure 2.7**. Information from SciFinder Scholar copyright in 2014 will be presented and classified into steroid groups.

Table 2.2 Chemical constituents of *Dioscorea* species

***Dioscorea* species**	**Plant part**	**Chemical constituents**	**References**
D. birmanica	Rhizome	**Steroid saponin**: diosgenin (**1**), diosgenin-3-O-α-L-rhamnosyl (1→2)-β-D-glucopyranoside or prosapogenin A of dioscin (**2**)	Sudkhayan *et al*., 1985 Jaiaree *et al*., 2010
D. membranacea	Rhizome	**Naphthofuranoxepins**: dioscorealide A (**3**), dioscorealide B (**4**), **Phenanthraquinone**: dioscoreanone (**5**), **Steroids:** β-sitosterol (**6**), stigmasterol **(7**), diosgenin 3-*O*-β-D-glucopyranosyl (1→3)-β-D-glucopyranoside (**8**), β-sitosterol-3-*O*-β-D-glucopyranoside (**9**), diosgenin-3-*O*-α-L-rhamnopyranosyl (1→2)-β-D-glucopyranoside (**2**), diosgenin (**1**)	Itharat *et al*., 2003 Itharat, 2002
	Rhizome	**Dihydrophenanthrene**: 2,4-dimethoxy-5,6-dihydroxy-9,10-dihydrophenanthrene (**10**), 5-hydroxy-2,4,6-trimethoxy-9,10-dihydrophenanthrene (**11**), 5,6,2 -trihydroxy 3,4-methoxy, 9,10- dihydrophenanthrene (**12**)	Itharat *et al*., 2014

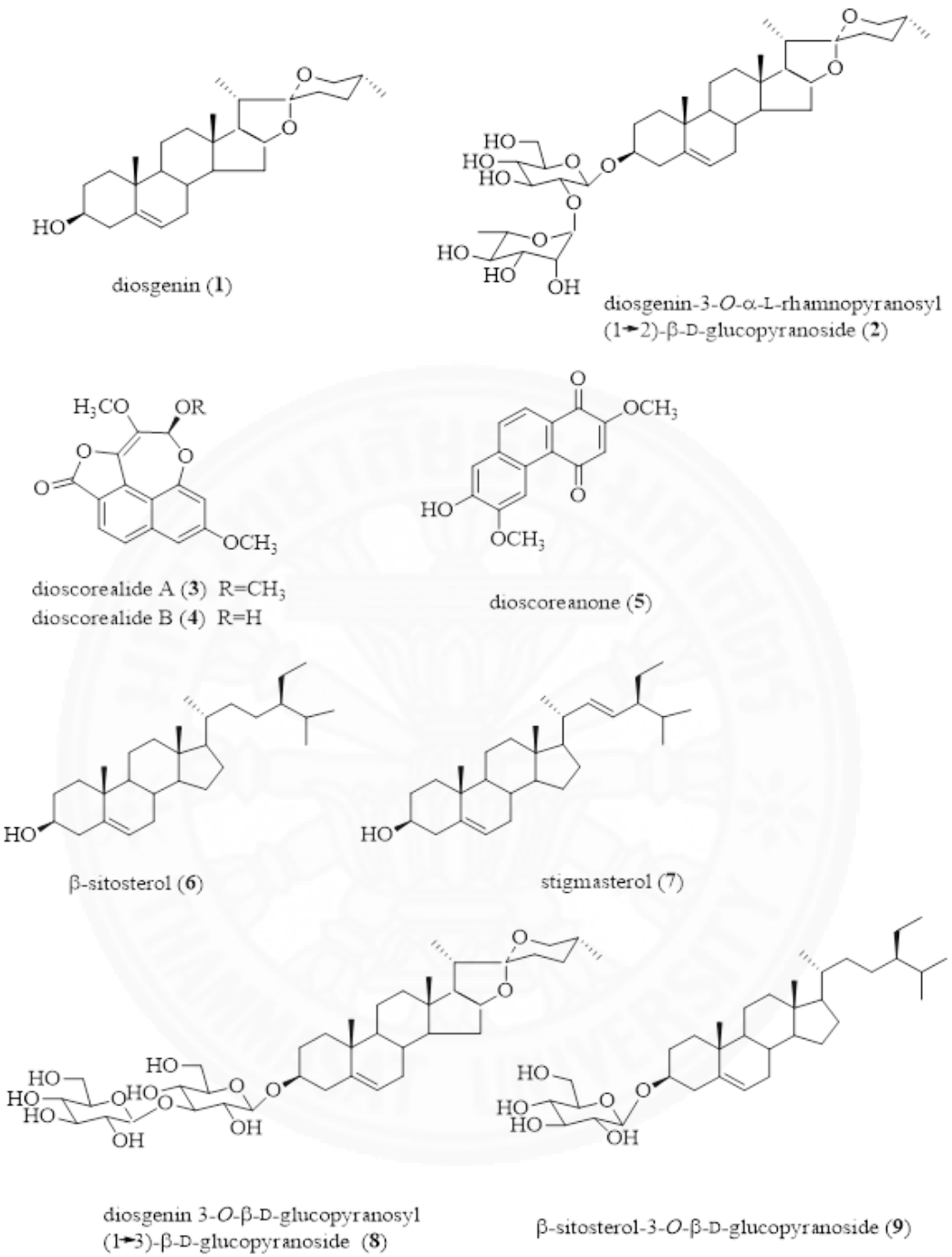
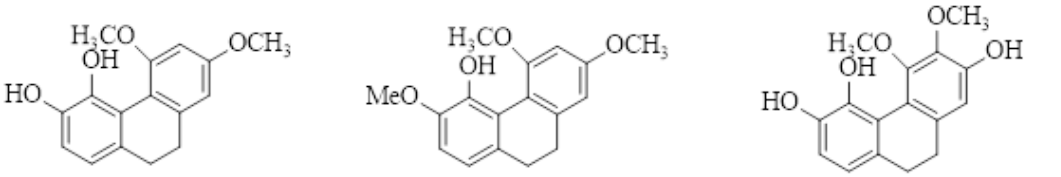


Figure 2.7 Chemical structure of compounds found in *Dioscorea* spp



5,6-dihydroxy-2,4-dimethoxy-9,10-dihydrophenanthrene (**10**)

5-hydroxy-2,4,6-trimethoxy-9,10-dihydrophenanthrene (**11**)

2,5,6-trihydroxy-3,4-dimethoxy-9,10-dihydrophenanthrene (**12**)

Figure 2.7 (continued). Chemical structure of compounds found in *Dioscorea* spp

2.3 *Smilax* species

2.3.1 *Smilax corbularia* Kunth (Smilacaceae)

Figure 2.8 *Smilax corbularia* Kunth (Ruangnoo, 2012)

Scientific name: *Smilax corbularia* Kunth.

Synonyms: *S. hypoglauca* Benth, *S. corbularia* Kunth var. hypoglauca (Benth) T. Koyama, *S. peguana* A. DC., *S. balansaeana* H. Bon ex Gagnep., *S. pseudochina* Lour.

Family name: Smilacaceae

Local name: Hua-Khao-Yen-Wok (หัวข้าวเย็นวอก), Hua-Khao-Yen-Nua (หัวข้าวเย็นเหนือ)

It is rather widely distributed in South-Eastern Asia from Southern China and upper Burma through Thailand and Indonesia southwards to the Malaysian Peninsula and South Eastwards to Borneo (Koyama, 1975). It is found in tropical evergreen and lower mountain forest from sea level to ca. 2000 m. In Thai traditional medicine, the rhizome has been used to treat venereal disease (Perry, 1980). *Smilax corbularia* Kunth. has been used in combination with another 4 plant species; *Ludwigia hyssopifolia* (G.Don) Exell., *Polygala chinensis* Linn., *Canna indica* Linn. and *Climacanthus siamensis* Brem. for the treatment of cancer in a Thai Traditional medicine preparation. Later, the Thai Traditional medicine preparation has been changed by the addition of 4 plant species; *Acanthus ebracteatus* Wall., *Hedyotis corymbosa* Lamk. *Mallotus oblongifolius* Muell. and *Hygrophila incana* Nees. (Vimonkunakorn, 1979; Pornsiriprasert, *et al*., 1986).

2.3.1.1 General description of *Smilax corbularia* Kunth

The description of *Smilax corbularia* Kunth is a small herb climber up 2 to 4 m long; with a woody stem and dense branches (**Figure 2.8** and **2.9**). The branches are straight with internodes 3-10 cm long. Leaves are highly variable in shape and thickness. The blades are elliptic, cuneate, rounded or shallowly cordate at the base with a coriaceous, acuminate tip at the apex, and about 3-10 cm long by 1.5-5 cm wide. They are fresh-green and shiny on the upper surface, and strongly glaucous and more or less white-powdery on the lower side. Petioles are short, about 7-15 mm long and the tendrils develop only on sterile branches and stems. Flowering branches are 5-20 cm long and upper leaves reduced to bracts. The umbels with peduncles are 5-12 mm long. The staminate umbels have 10 to 40 flowers and the pistillate umbels have 8 to 20 flowers. The stamens have reddish perianth with free petals. There are 6 nearly sessile stamens with the elliptic anther, 1.3 mm long. The pistillate has a greenish to yellowish perianth, 1.5-2 mm long, with oblique petals. The ovary is ellipsoid, contracted at the apex, 2 mm long, 1.5 mm wide, capped with 3-lobed stigma. There are 3 needle-like staminodes, 1.25 mm long. Berries are globose, purplish-black, 6-8 mm across with 1 to 3 seeds (Koyama, 1975).

Figure 2.9 *Smilax corbularia* Kunth.; 1.branch; 2.fruit; 3.rhizome
(Itharat, 2002; Booyarattanakornkit & Chantaptavan, 1993)

2.3.2 *Smilax glabra* Roxb. (Smilacaceae)

Figure 2.10 *Smilax glabra* Roxb. (Itharat, 2002)

Scientific name: *Smilax glabra* Roxb.
Synonym: *Smilax hookeri* Kunth.
Family name: Smilacaceae
Local name: Ya-hua (ยาหัว), Hua-Khao-Yen-Tai (หัวข้าวเย็นใต้) (Smithinan, 1980)

It is distributed in India, Burma, Indochina, Central and Southern Continental China and Taiwan. It grows in tropical evergreen forests at 300 to 1400 m above sea level. In Thailand this warm-temperate species is found at altitudes from 600-1300 m. It is used in China to treat dysuria with turbid discharge, chronic eczema of limbs, and muscle pain in syphilis (Hui-Lin *et al*, 1979).

2.3.2.1 General description of *Smilax glabra* Roxb.

Description of *Smilax glabra* Roxb., which is shown in **Figure 2.10** and **2.11** is: climbing stems 3-5 mm thick, branches hardly zigzag, leaf-blades lanceolate, 5-18 cm long, 2-7 cm wide, rounded at base, attenuate to acute or acuminate apex, coriaceous with nerved and thickened margins, upper surface shiny, pale or white-powdery on lower surface, 5 vein including a marginal pair, all divided to base; petioles 10-30 mm long. Umbels on the lower part of branches as well as on the distal part of stem; peduncles short, staminate umbels globose with 30 to 60 on

filiform rays, 1-2.5 cm long. Pistillate umbels turbinate to hemispherical 10 to 25 flowers on filiform rays, 1-1.5 cm long. Staminate, pale-greenish; tepals free slightly open, 1.5 mm long 1 mm wide. Stamen 6, anthers 0.7 mm long, white subsessile. Pistillate perianth pale, 1.5-2 mm long, 3-2.5 mm across; tepals free. Ovary ellipsoid, 2 mm long and wide. Stigmas, 3-lobed, Berries globose, 5-8 mm in diameter maturing blue-black, 1 to 3-seeded (Koyama, 1975).

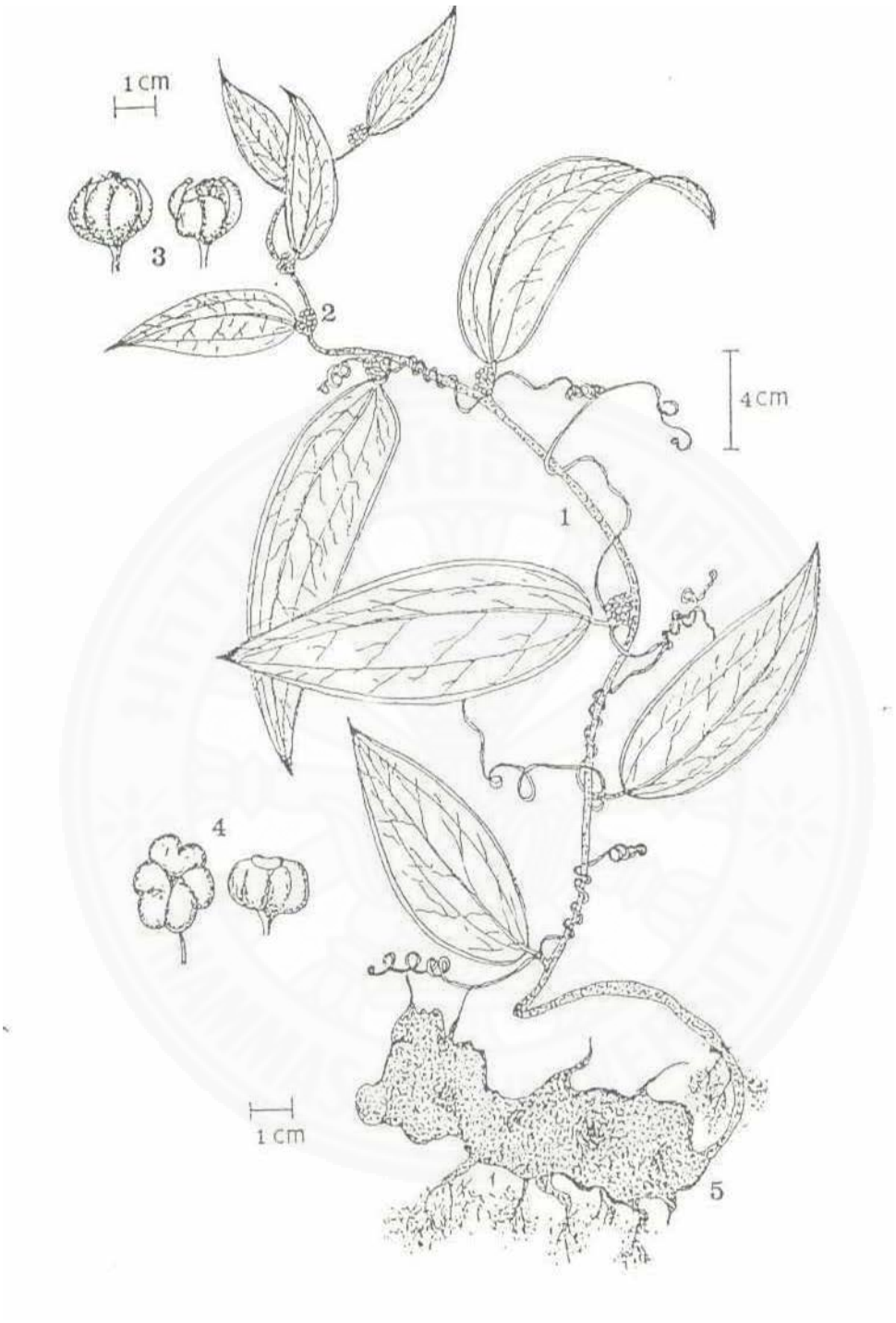


Figure 2.11 *Smilax glabra* Roxb.; 1 whole plant; 2.inflorescence; 3.pistillate flower; 4. staminate flower; 5.rhizome (Itharat, 2002; Boonyarattanakornkit & Chantaptavan, 1993)

2.3.3 Biological activity of *Smilax* species

The investigations on the biological activity of *Smilax* species in Thailand are shown in **Table 2.3**. Thai traditional doctors have used two species of Hua-Khao-Yen including *Smilax corbularia* and *Smilax glabra* to treat cancers, AIDS, septicemia and lymphatic diseases (Itharat, 2002).

Table 2.3 *Smilax* species found in Thailand and biological activity

***Smilax* species**	**Plant part**	**Activity**	**Result of biological activity**	**References**
S. corbularia	Rhizome	Anti-HIV	The ethanolic (EtOH) extract and water extract of *S. corbularia* exhibited anti-HIV-1 IN activity with IC_{50} = 1.9 and 5.4 μg/mL, respectively.	Tewtrakul *et al*., 2006
	Rhizome	Anti-inflammatory	Ethanolic and water extracts exhibited low potent inhibitory activity (IC_{50} = 61.0 and 61.2 μg/mL, respectively) against LPS induced NO production in RAW 264.7 cell lines.	Tewtrakul & Itharat, 2007
	Rhizome	Anti-inflammatory	The ethanolic extract exhibited little potent inhibitory activity on TNF-α and NO production, with an IC_{50} values 61.97 and 83.90 μg/mL, whereas the water extract had no activity (IC_{50} > 100 μg/mL).	Ruangnoo *et al*., 2012

Table 2.3 (continued)

***Smilax* species**	**Plant part**	**Activity**	**Result of biological activity**	**References**
S. corbularia	Rhizome	Anti-inflammatory	Engeletin, astilbin, and quercetin were isolated from the ethanolic extract of *S. corbularia*. Quercetin possessed the highest activity against NO production (IC_{50} = 33.12 μM) whereas engeletin and astilbin had no effect on nitric oxide production. Engeletin, astilbin and quercetin all possessed potent inhibitory activity against PGE2 release from RAW 264.7 cells with IC_{50} values of 33.1, 43.5 and 65.8 μM, respectively. Engeletin exhibited the most potent inhibitory effect on LPS-induced PGE2 release in RAW 264.7 cells. Quercetin possessed the most potent inhibitory activity against TNF-α release (IC_{50} = 4.14 μM). Quercetin showed higher IC_{50} values for inhibition of NO production and TNF-α release than indomethacin. However, quercetin showed lower inhibition of PGE2 release than indomethacin.	Ruangnoo *et al*., 2012

Table 2.3 (continued)

***Smilax* species**	**Plant part**	**Activity**	**Result of biological activity**	**References**
S. corbularia	Rhizome	Anti-inflammatory	Oral administration of the ethanolic extract of *S. corbularia* rhizomes (1600 mg/kg) significantly suppressed the paw edema induced by carrageenin in rats while the aqueous extract had no effect. Neither the ethanolic extract nor aqueous extract significantly affected the antinociceptive tests in mice and yeast-induced fever in rats.	Reanmongkol *et al*., 2007b
	Rhizome	Cytotoxic	A water extract of *Smilax corbularia* Kunth., *Ludwigia hyssopifolia* (G.Don) Exell.), *Polygala chinensis* Linn., *Canna indica* Linn. and *Clinacanthus siamensis* Brem. have been used in a combination for the treatment of cancer in a Thai traditional medicine preparation. This preparation was inactive against CA-9KB cell (*in vitro*) but active when an *in vivo* test by IP injection was used in the rat.	Pornsiriprasert *et al*., 1986

Table 2.3 (continued)

***Smilax* species**	**Plant part**	**Activity**	**Result of biological activity**	**References**
S. corbularia	Rhizome	Cytotoxic	The effect of water extract of Thai traditional formula against cancer including; *Smilax corbularia*, *Polygala chinensis*, *Ludwigia hyssopifolia*, *Cinnacanthus siamensis*, and *Canna indica* on the effect for cells involved in cancer immunity, natural killer (NK) cells and monocyte/macrophages. 13 breast cancer patients were studied for 2 weeks. Significantly increased number of natural killer (NK) cells and monocyte/macrophage. It showed cytotoxic activity against K562 erythroleukemic cells *in vitro* assay. It also increased the release of tumor necrosis factor–alpha from monocyte/macrophages.	Thisoda *et al*., 1995
	Rhizome	Cytotoxic	Ethanol and water extract were tested for cytotoxic activity using SRB assay exhibited less cytotoxic activity against lung, breast and colon cancer (IC_{50} >50 µg/mL).	Itharat *et al*., 2004

Table 2.3 (continued)

***Smilax* species**	**Plant part**	**Activity**	**Result of biological activity**	**References**
S. corbularia	Rhizome	Estrogenic and anti-estrogenic	All isolates had their estrogenic and anti-estrogenic activities determined using the estrogen-responsive human breast cancer cell lines MCF-7 and T47D. The major constituents were recognized as flavanonol rhamnosides by the suppressive effect on estradiol induced cell proliferation at a concentration of 1 µM. Meanwhile, flavanonol rhamnoside acetates demonstrated estrogenic activity in both MCF-7 and T47D cells at a concentration of 100 µM, and they enhanced the effects of co-treated E2 on T47D cell proliferation at concentrations of more than 0.1 µM.	Wungsintaweekul *et al*., 2011
S. glabra	Rhizome	Anti-HIV	The EtOH and water extract of *S.glabra* exhibited anti-HIV-1 IN activity with IC_{50} values of 6.7 and 8.5µg/mL, respectively.	Tewtrakul *et al*., 2006

Table 2.3 (continued)

***Smilax* species**	**Plant part**	**Activity**	**Result of biological activity**	**References**
S. glabra	Rhizome	Anti-inflammatory	Ethanolic and water extracts of *S. glabra* exhibited less potent inhibitory activities against LPS induced NO production in RAW 264.7 cell lines (IC_{50} = 66.4 and 68.5 µg/mL, respectively).	Tewtrakul & Itharat 2007
	Rhizome	Antimicrobial	Smiglabrone A was isolated from ethylacetate extract showed antimicrobial activity against *Candida albicans* with MIC value of 0.146 mM, which was stronger than cinchonain Ia with an MIC of 0.332 mM. Smilachromanone and smiglastilbene exhibited inhibitory activity against *Staphylococcus aureus* with MIC values of 0.303 and 0.205 mM, respectively.	Xu *et al*., 2013
	Rhizome	Cytotoxic	Ethanol and water extract were tested using SRB assay exhibited less cytotoxic activity against lung, breast and colon cancer (IC_{50} >50 µg/mL).	Itharat *et al*., 2004

2.3.4 Chemical constituents of *Smilax* Species

Chemical constituents isolated from *Smilax* species found in Thailand are summarized in **Table 2.4** and **Figure 2.12**. Information from SciFinder Scholar copyright in 2014 will be presented and classified into flavonoid groups.

Table 2.4 Chemical constituents of *Smilax* species

***Smilax* species**	**Plant part**	**Chemical constituents**	**References**
S. corbularia	Rhizome	**Flavonoids:** (2*R*,3*R*)-2"-acetyl astilbin (**13**), (2*R*,3*R*)-3"-acetyl astilbin (**14**), (2*R*,3*R*)-4"-acetyl astilbin (**15**), (2*R*,3*R*)-3"-acetyl engeletin (**16**), (2*R*,3*S*)-4"-acetyl isoastilbin (**17**), corbulain Ia (**18**), corbulain Ib (**19**), gnetumontanin E (**20**), gnetumontanin F (**21**), gnetumontanin G (**22**), 5,7,3',4'-tetrahydroxy-3-phenylcoumarin (**23**), astilbin (**29**), neoastilbin (**30**), isoastilbin (**28**), neoisoastilbin (**31**), engeletin (**32**), isoengeletin (**28**), (+) taxifolin (**24**), (+) dihydrokaempferol (**26**), naringenin (**25**), eriodictyol (**37**), homoeriodictyol (**38**), quercetin (**39**), quercitrin (**40**), luteolin (**41**), (-) catechin (**42**), (-) epicatechin (**43**), cinchonain Ia (**44**), cinchonain Ib (**45**), rhinchoin Ia (**46**), cinchonain Id (**93**),	Wungsintaweekul *et al*., 2011

Table 2.4 (continued)

***Smilax* species**	**Plant part**	**Chemical constituents**	**References**
S. corbularia Kunth	Rhizome	(4*R*,8*R*,9*S*)-4,8-bis(3,4-dihydroxyphenyl)-3,4,9,10-tetrahydro-5,9-dihydroxy-2H,8H-benzo[1,2-b:3,4-b']dipyran-2-one (**47**), phyllocoumarin (**48**), epiphyllocoumarin (**49**), trans-resveratrol (**50**), piceatannol (**52**), isorhapontigenin (**53**), eucryphin (**54**), (-) syringaresinol (**55**), 5-*O*-caffeoylshikimic acid (**56**), caffeic acid (**57**), protocatechuic acid (**58**), (4*S*,8*R*,9*S*)-4,8-bis(3,4-dihydroxyphenyl)-3,4,9,10-tetrahydro- 5,9-dihydroxy-2H,8H-benzo[1,2-b:3,4-b']dipyran-2-one (**92**)	Wungsintaweekul *et al*., 2011
S. glabra	Rhizome	**Flavonoid**: quercetin (**39**), **Steroids**: sitosterol-3-*O*-β-D-glucopyranoside (**9**) stigmasterol (**7**), β-sitosterol (**6**) **Chromone**: tufulingoside (**59**)	Yiqing *et al*.,1996

Table 2.4 (continued)

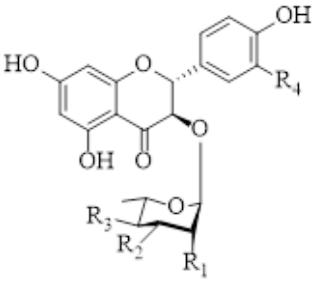
***Smilax* species**	**Plant part**	**Chemical constituents**	**References**
S. glabra	Rhizome	**Phenylpropanoids**: smiglasides A (**60**), smiglasides B (**61**), smiglasides C (**62**), smiglasides D (**63**), smiglasides E (**64**), helonioswide (**65**)	Chen *et al*., 2000
	Rhizome	**Phenylpropanoids**: smiglabrone A (**66**), smiglabrone B (**67**), smilachromanone (**68**), smiglastilbene (**69**), smiglactone (**70**), smiglabrol (**71**) smiglycerol (**72**), taxifolin (**24**) naringenin (**25**), dihydrokaempferol (**26**), sakuranetin (**27**), isoastilbin (**28**), astilbin (**29**), neoastilbin (**30**), neoisoastilbin (**31**), engeletin (**32**), arthromerin B (**33**), sinensin (**34**), (2*R*, 3*R*)-taxifolin 3-*O*-β-D-glucopyranoside (**35**), (2*S*,3*S*)-glucodistylin (**36**)	Xu *et al*., 2013

Table 2.4 (continued)

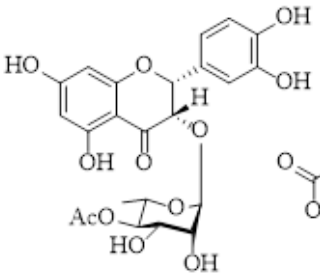
***Smilax* species**	**Plant part**	**Chemical constituents**	**References**
S. glabra	Rhizome	**Flavanes**: (-)-epicatechin (**43**), (+)-catechin (**73**), cinchonain Ia (**44**), cinchonain Ib (**45**) **Flavonoids**: apigenin (**74**), quercetin (**39**), luteolin (**41**), myricetin (**75**), **Chalcone**: kukulkanin B (**76**) **Aurones**: 4,4,6-trihydroxyaurone (**77**), aureusidin (**7**8), **Lignans**: (-)-secoisolariciresinol (**79**), 4-ketopinoresinol (**80**), smiglabranol (**81**), (+)-lyoniresinol (**82**), kompasinol A (**83**), aiphanol (**84**), **Stilbenes**: *trans*-resveratrol (**50**), *trans*-piceid (**51**), piceatannol (**52**) **Phenylpropanoids**: *trans*-caffeic acid (**57**), 5-*O*-caffeoylshikimic acid (**56**), 3-*O*-*p*-coumaroylshikimic acid (**85**), smiglycerol (**72**), juncusyl ester B (**86**),1-*O*-*p*-coumaroylglycerol (**87**),	Xu *et al*., 2013

Table 2.4 (continued)

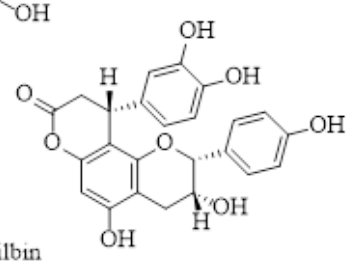
***Smilax* species**	**Plant part**	**Chemical constituents**	**References**
S. glabra	Rhizome	**Phenolics**: vanillin (**94**), *p*-hydroxy-benzaldehyde (**96**), acetovanillone (**95**), (+)-scytalone (**88**), glucosyringic acid (**89**), protocatechuic acid (**90**), 3-methoxygallic acid (**91**), , hydroxytyrosol (**97**), **Triterpene**: acetyl-11-keto-*β*-boswellic acid (**98**), **Steroids**: stigmasterol (**7**), *β*-sitosterol (**6**), daucosterol (**9**), smilagenin (**99**), 5-hydroxymaltol(**100**), 5-hydroxyuridine (**101**), 2-methylbutanedioic acid-4-ethyl ester (**102**)	Xu *et al*., 2013



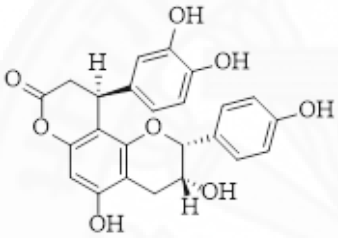
	R_1	R_2	R_3	R_4
2"-acetyl astilbin (**13**)	OAc	OH	OH	OH
3"-acetyl astilbin (**14**)	OH	OAc	OH	OH
4"-acetyl astilbin (**15**)	OH	OH	OAc	OH
3"-acetyl engeletin (**16**)	OH	OAc	OH	H



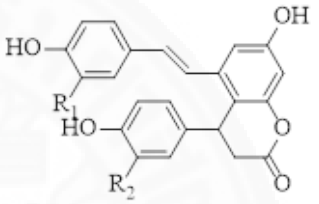
(2*R*,3*S*)-4"-acetyl isoastilbin (**17**)



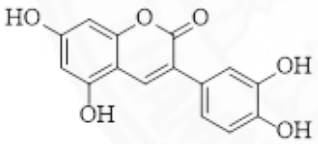
corbulain Ia (**18**)



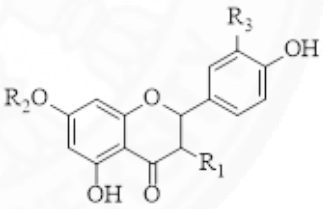
corbulain Ib (**19**)



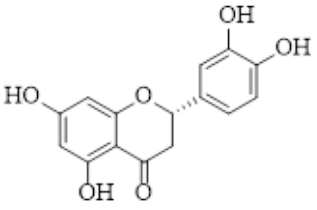
	R_1	R_2
gnetumontanin E (**20**)	H	OH
gnetumontanin F (**21**)	OH	OH
gnetumontanin G (**22**)	H	OMe



5,7,3',4'-tetrahydroxy-3-phenylcoumarin (**23**)



taxifolin (**24**)	$R_1 = R_3 = OH$, $R_2 = H$ 2*R*, 3*R*
naringenin (**25**)	$R_1 = R_2 = R_3 = H$, 2*S*
dihydrokaempferol (**26**)	$R_1 = OH, R_2 = R_3 = H$ 2*R*, 3*R*
sakuranetin (**27**)	$R_1 = R_3 = H, R_2 = CH_3$ 2*S*
isoastilbin (**28**)	R_1 = ORha, $R_2 = H, R_3 = OH$ 2*R*, 3*S*
astilbin (**29**)	R_1 = ORha, $R_2 = H$, $R_3 = OH$ 2*R*, 3*R*
neoastilbin (**30**)	R_1 = ORha, $R_2 = H, R_3 = OH$ 2*S*, 3*S*
neoisoastilbin (**31**)	R_1 = ORha, $R_2 = H, R_3 = OH$ 2*S*, 3*R*
engeletin (**32**)	R_1 = ORha, $R_2 = R_3 = H$ 2*R*, 3*R*
arthromerin B (**33**)	R_1 = OGlc, $R_2 = R_3 = H$ 2*R*, 3*R*
sinensin (**34**)	$R_1 = OH, R_2 = Glc, R_3 = H$ 2*R*, 3*R*
(2*R*, 3*R*)-taxifolin 3'-*O*-β-D-glucopyranoside (**35**)	$R_1 = OH$, $R_2 = H$, R_3 = OGlc 2*R*, 3*R*
(2*S*,3*S*)-glucodistylin (**36**)	R_1 = OGlc, $R_2 = H, R_3 = OH$ 2*S*, 3*S*



eriodictyol (**37**)

Figure 2.12 Chemical structure of compounds found in *Smilax* spp

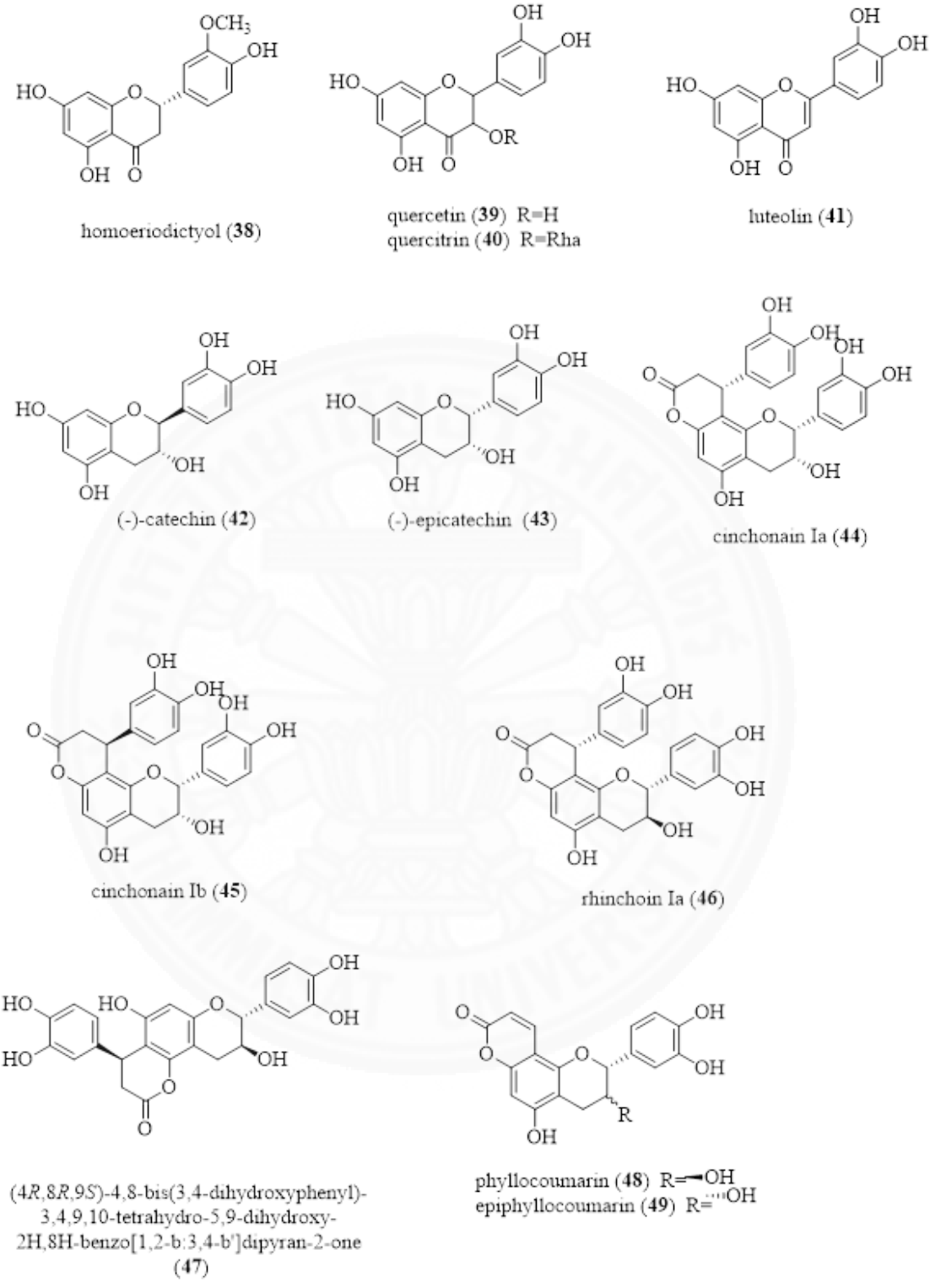
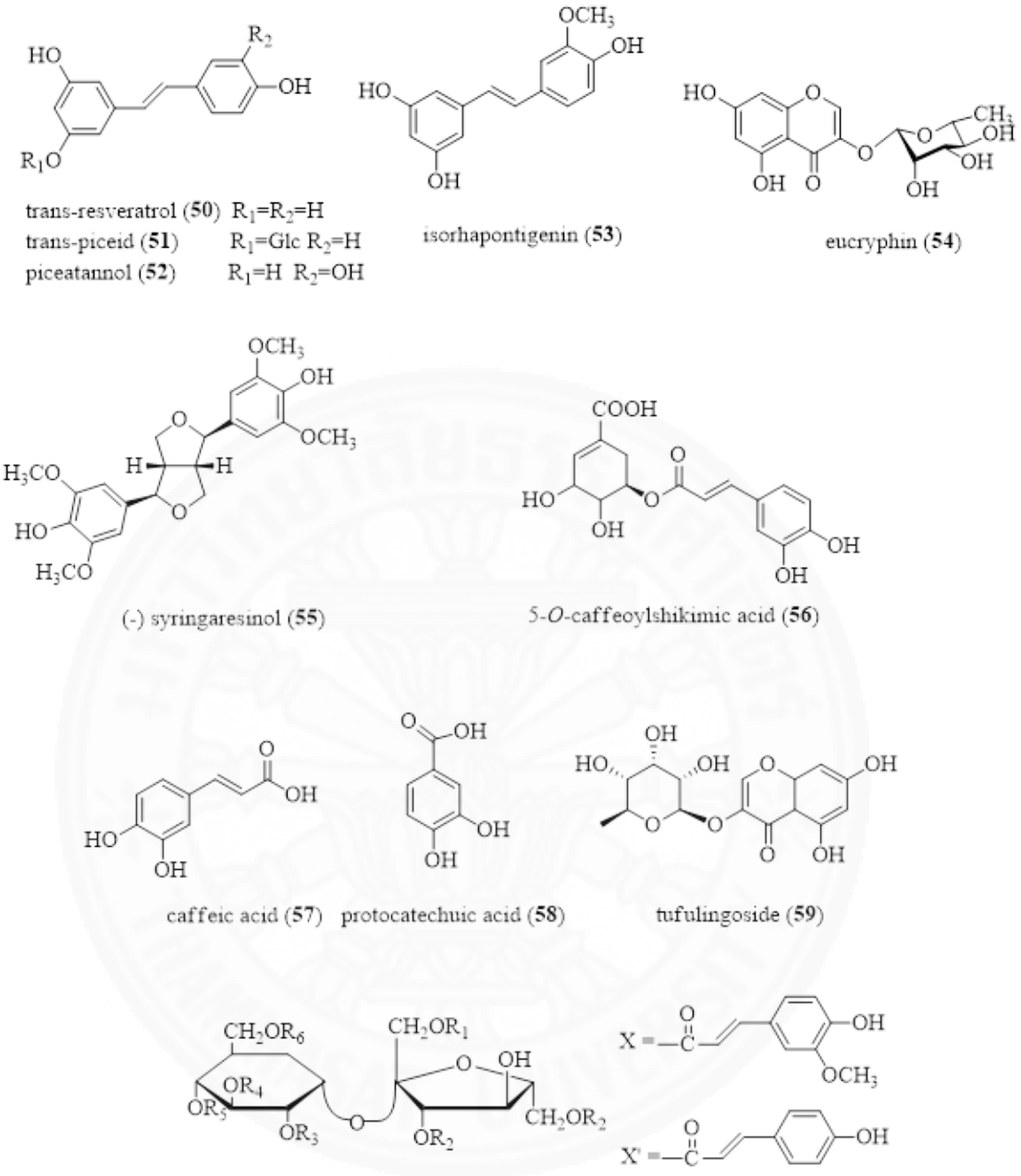


Figure 2.12 (continued). Chemical structure of compounds found in *Smilax* spp



	R_1	R_2	R_3	R_4	R_5	R_6
smiglaside A (**60**)	X	X	Ac	H	Ac	Ac
smiglaside B (**61**)	X	X	Ac	H	H	Ac
smiglaside C (**62**)	H	X	Ac	H	Ac	Ac
smiglaside D (**63**)	X'	X	Ac	H	Ac	Ac
smiglaside E (**64**)	X'	X	Ac	H	H	Ac
helonioswide (**65**)	H	X	H	H	H	H

Figure 2.12 (continued). Chemical structure of compounds found in *Smilax* spp

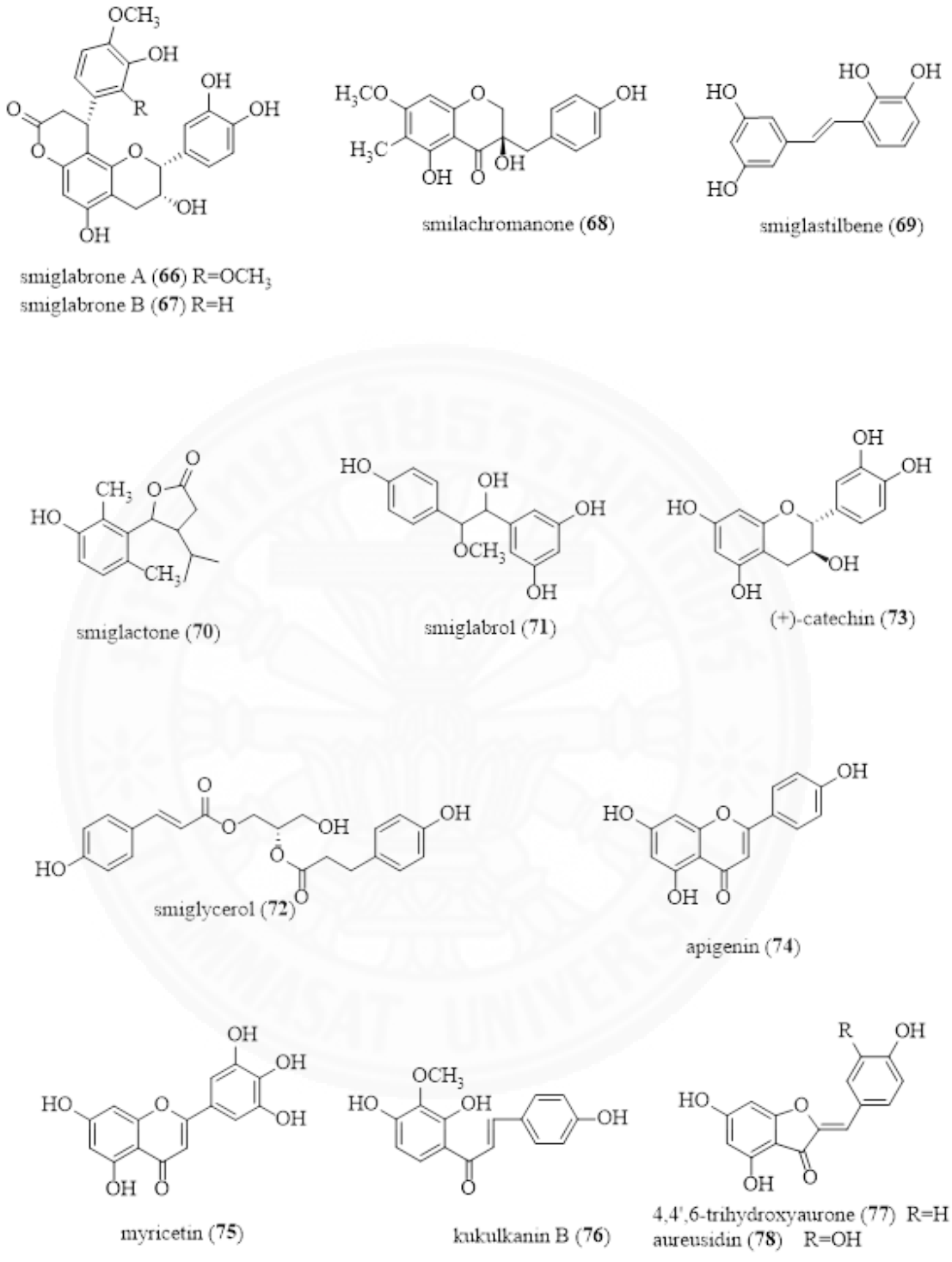


Figure 2.12 (continued). Chemical structure of compounds found in *Smilax* spp

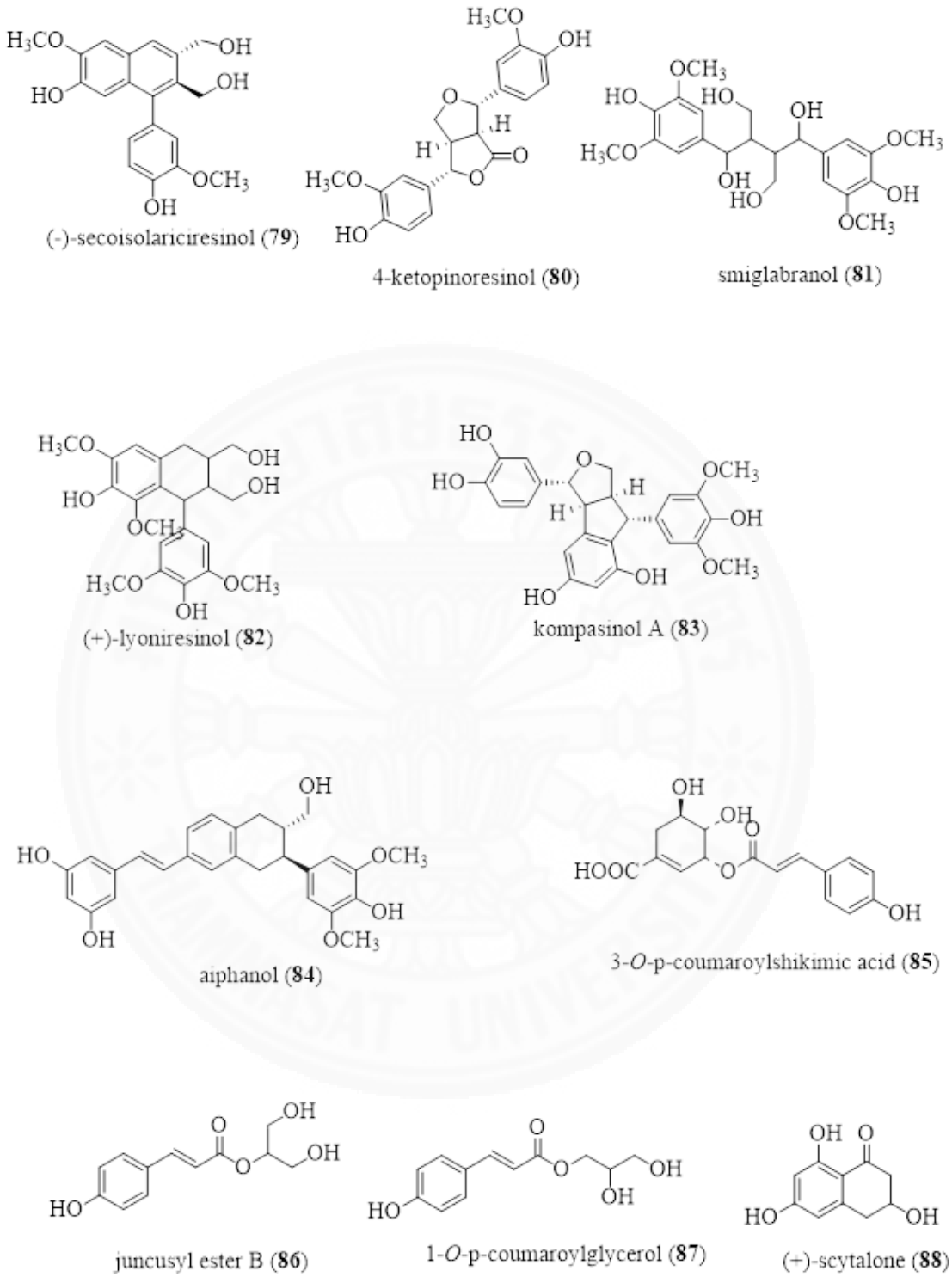
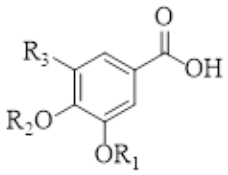
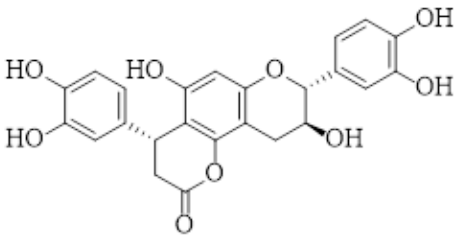


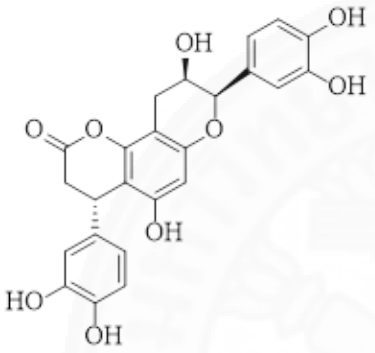
Figure 2.12 (continued). Chemical structure of compounds found in *Smilax* spp



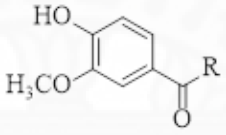
glucosyringic acid (**89**) R_1=CH_3, R_2=Glc, R_3=OCH_3
protocatechuic acid (**90**) R_1=R_2=R_3=H
3-methoxygallic acid (**91**) R_1=CH_3, R_2=H, R_3=OH



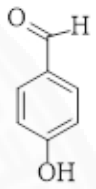
(4*S*,8*R*,9*S*)-4,8-bis(3,4-dihydroxyphenyl)-3,4,9,10- tetrahydro-5,9-dihydroxy-2H, 8H-benzo[1,2-b:3,4-b']dipyran-2-one (**92**)



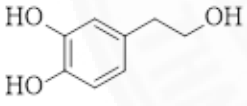
cinchonain Id (**93**)



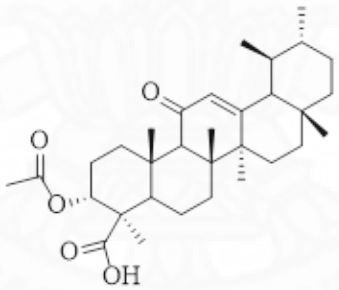
vanillin (**94**) R = H
acetovanillone (**95**) R = CH_3



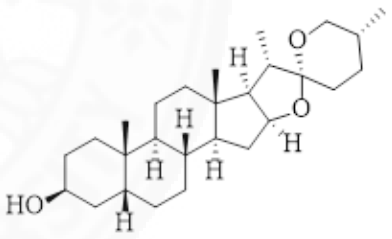
p-hydroxy-benzaldehyde (**96**)



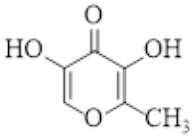
hydroxytyrosol (**97**)



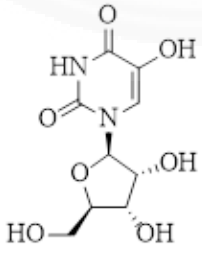
acetyl-11-keto-b-boswellic acid (**98**)



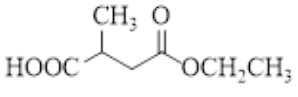
smilagenin (**99**)



5-hydroxymaltol (**100**)



5-hydroxyuridine (**101**)



2-methylbutanedioic acid-4-ethyl ester (**102**)

Figure 2.12 (continued). Chemical structure of compounds found in *Smilax* spp

2.4 *Pygmaeopremna herbacea* (Roxb.) Mold (Verbenaceae)

Figure 2.13 *Pygmaeopremna herbacea* (Roxb.) Mold (Itharat, 2002)

Scientific name: *Pygmaeopremna herbacea* (Roxb.) Mold

Synonym: *Premna herbacea* Roxb., *Premna gandaria* Hamilton, *Clerodendron humile* Hamilton ex wall, *Tatea subaculis* F. Muell., *Pygmaeopremna obovata* Merr., *Pygmaeopremna herbacea* (Roxb.) Mold. Ex Saxena

Family name: Verbenaceae

Local name: Khaang Hua lek (ข่างหัวเล็ก), Phaen din yen (แผ่นดินเย็น), Som Kor (ส้มก้อ), Hua-Khao-Yen-Tai (หัวข้าวเย็นใต้)

It is widely distributed from Burma, Southern China, Thailand, and Indochina to the Philippines, Indonesia and New Guinea to Australia. It is said to be cultivated in India and Ceylon. It is often found in hard, stiff, dry soils in open barren places. The root and rhizomatous stem have been used as medicine in India and Ceylon. The juice is described as hot, bitter, pungent and digestive and has been used in the treatment of cough, asthma, fever and rheumatism (Dassanayake *et al.*, 1983).

2.4.1 General description of *Pygmaeopremna herbacea* (Roxb.) Mold

A description of *Pygmaeopremna herbacea* (Roxb.) Mold, which is shown in **Figure 2.13** and **2.14** is dwarf or very small subshrub or undershrub, often forming extensive colonies in open ground, with a long woody taproot and ligneous

perennial root, stems rhizomatous, annual, aerial, 20-30 cm tall, glabrous; leaves few, decussate-opposite, bright green above, 1-3 pairs (usually only 4) or crowed in a rosette-like whorl at the much abbreviated branch apex, sessile or subsessile, all subequal in size or the uppermost smaller, obovate, 2.5-15 cm long, 1.5-7.7 cm wide, apically obtuse, basically cuneate, glabrous on both surfaces, microscopically dotted above, pubescent. Inflorescence, cymose, corymbiform, flower small, purple in bud, fragrant; calyx pale green, somewhat 2-lipped, pubescent externally; corolla varying from yellow or pale yellow to yellowish-purple, cream, white, greenish-white, or greenish, 2.5-4 mm across, the tube 1.5-3 mm long, externally glabrous and pubescent above, the lower lip often white, yellow at the base, stamen 4, didynamous; filaments very short, less than 1 mm long; anthers about 0.5 mm long; style short; fruit drupaceous, globose, 5-10 mm in diameter, at first green, finally black and persistant fruiting-calyx, 1-4 chambered, 1-4 seeded (Dassanayake *et al*., 1983).

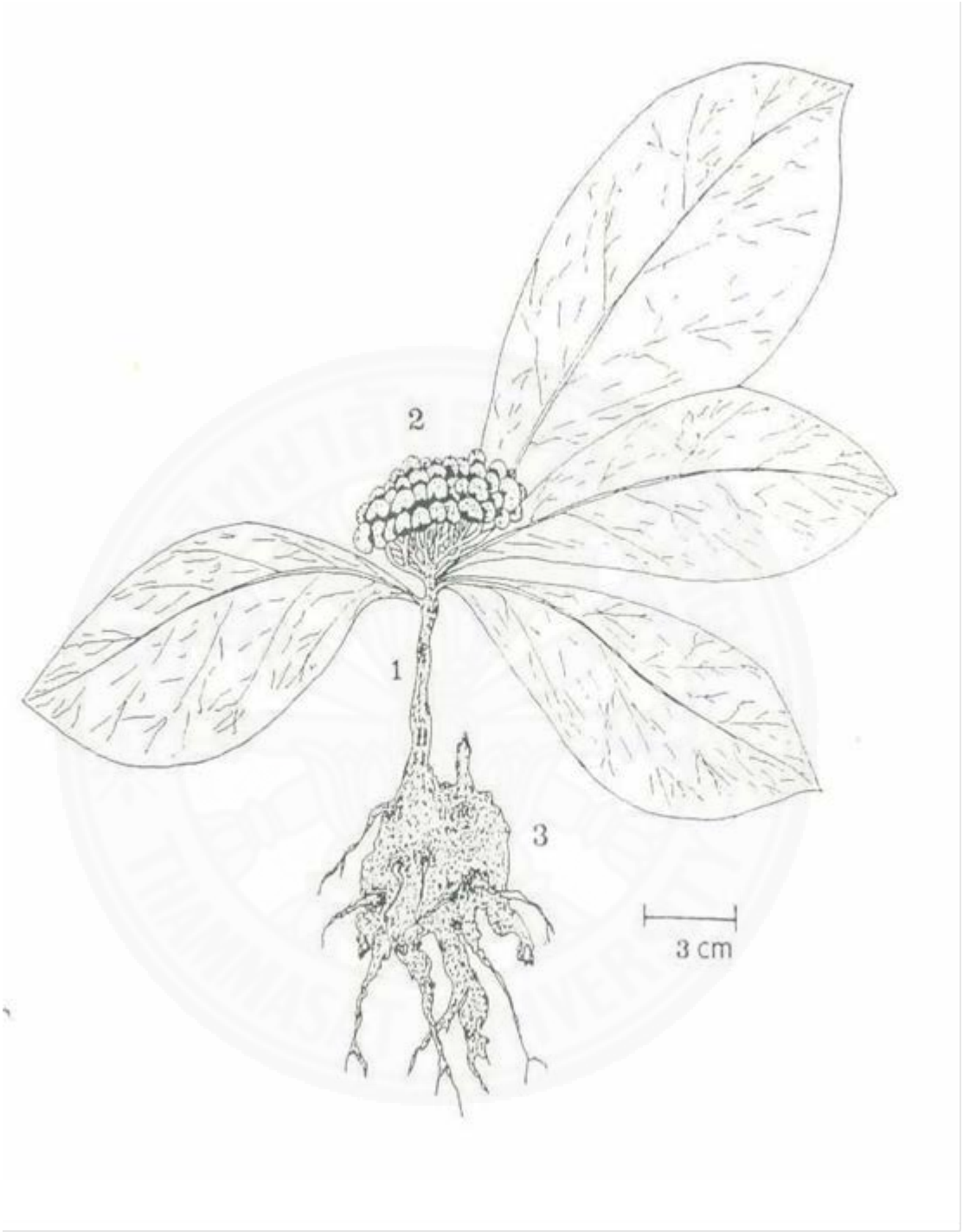


Figure 2.14 *Pygmaeopremna herbacea* (Roxb.) Mold; 1whole plant; 2 fruits; 3 rhizome (Itharat, 2002; Boonyarattanakornkit & Chantaptavan, 1993)

2.4.2 Biological activity of *Pygmaeopremna herbacea* (Roxb.) Mold

The investigations on the biological activity of *Pygmaeopremna herbacea* (Roxb.) Mold wich found in Thailand are shown in **Table 2.5**.

Table 2.5 Biological activity of *Pygmaeopremna herbacea*

Scientific name	Plant part	Activity	Result of biological activity	References
Pygmaeopremna herbacea	Root	Analgesic	Ethanolic extract injected intragastric in both sexes of mouse showed active at dose 100 mg/kg.	Narayanan *et al*., 2000
	Rhizome	Anti-HIV	The EtOH and water extracts of *P. herbacea* were apparently inactive (IC_{50} > 100µg/mL) against HIV-1 IN and HIV-1 PR.	Tewtrakul *et al*., 2006
	Root	Anti-inflammatory	Ethanolic extract injected intragastic in both sexes of rat showed active at dose 200 mg/kg with cotton pellet granuloma but inactive at dose 400 mg/kg with carrageenan-induced pedal edema.	Narayanan *et al*., 2000
	Rhizome	Anti-inflammatory	Ethanolic extract of *P. herbacea* exhibited less potent inhibitory activities against LPS induced NO production in RAW 264.7 cell lines (IC_{50} = 54.2 µg/mL), while the water extracts had no inhibitory activity (IC_{50} >100 µg/mL).	Tewtrakul & Itharat, 2007

Table 2.5 (continued)

Scientific name	Plant part	Activity	Result of biological activity	References
Pygmaeopremna herbacea	Root nodules	Antimicrobial	Bharangin and its derivative bharangin monoacetate showed good in vitro antimicrobial activity, the last one displaying a better activity against gram (+) and gram (−) bacteria, and both showing the same potency against *Candida albicans* and *Saccharomyces cerevisiae* (MIC values (10–12.5 µg/mL).	Murthy *et al.*, 2006
	Root	Antipyretic	Ethanolic extract injected intragastric in both sexes of rabbit showed activity at dose 100 mg/kg with tab vaccine-induced pyrexia.	Narayanan *et al*., 2000
	Root	Cytotoxic	Ethanol-water (1:1) extract inactive against CA-KB (IC_{50} >20 µg/mL).	Dhar *et al*., 1973
	Rhizome	Cytotoxic	Ethanol and water extract were test cytotoxic activity using SRB assay exhibited less cytotoxic activity against lung, breast and colon cancer (IC_{50} >50 µg/mL).	Itharat *et al.*, 2004

Table 2.5 (continued)

Scientific name	Plant part	Activity	Result of biological activity	References
Pygmaeopremna herbacea	Root nodules	Cytotoxic	The cytotoxic activities of isolated compounds showed very good cytotoxic activity using the MTT assay with doxorubicin as positive control. Bharangin displayed selective cytotoxicity against MCF-7 cells (IC_{50} 0.006 µg/mL), A549 (IC_{50} 2.03 µg/mL) and PC-3 cells (IC_{50} 2.95 µg/mL) and neobharangin showed promising cytotoxicity against on MCF-7 (0.39 µg/mL) and A549 (2.61µg/mL). 11-hydroxy-5,7,9,13-abietatetraene-2,12-dione showed cytotoxicity against PC-3 (1.7 µg/mL)	Satish *et al*., 2011

2.4.3 Chemical constituents of *Pygmaeopremna herbacea* (Roxb.) Mold

Chemical constituents isolated from *Pygmaeopremna herbacea* are summarized in **Table 2.6** and **Figure 2.15**. Information from SciFinder Scholar copyright in 2014 will be presented and classified into groups: diterpenoids and flavonoids.

Table 2.6 Chemical constituents of *Pygmaeopremna herbacea*

Scientific name	Plant part	Chemical constituents	References
Pygmaeopremna herbacea	root nodules	**Diterpenoid quinonemethides**: bharangin (**106**), bharangin monoacetate (**107**)	Murthy *et al*., 2006
		isobharangin (**105**), 11-hydroxy-5,7,9,13-abietatetraene-2,12-dione (**103**), bharangin fuerstione (**108**), 15-deoxyfuerstione (**109**) 3β-acetoxyfuerstione (**110**) neobharangin (**104**)	Satish *et al*., 2011
	Rhizomes	**Flavone**: scutellarein (**111**)	Verma *et al*., 2012

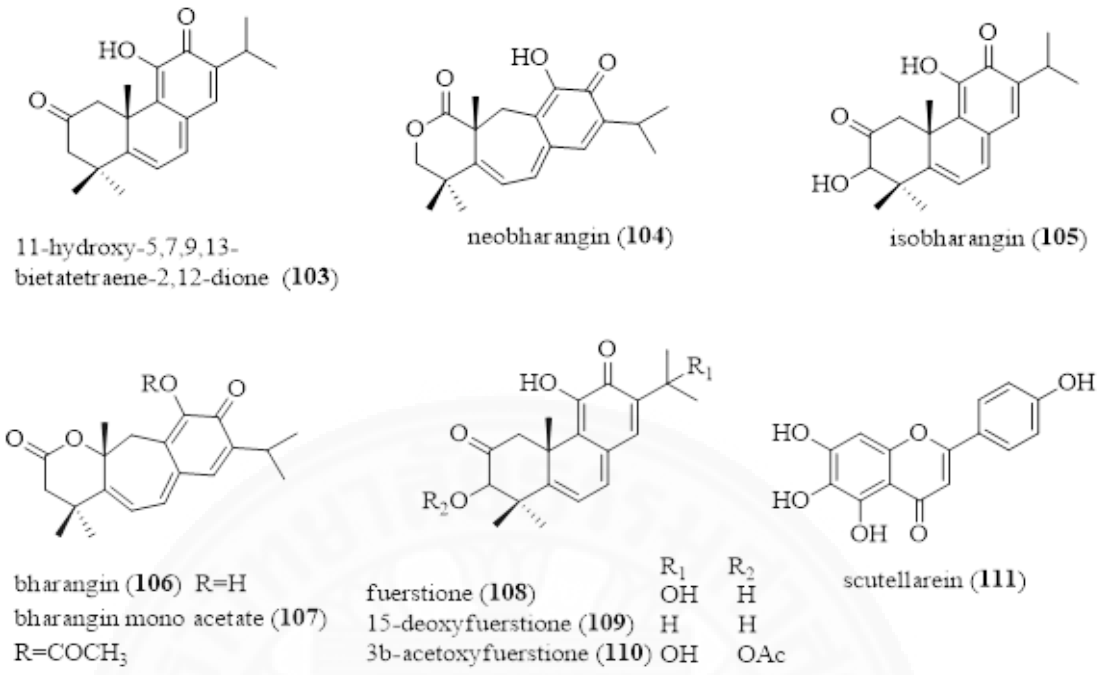


Figure 2.15 Chemical structure of compounds found in *Pygmaeopremna herbacea*

2.5 Natural product and anticancer drug

In the literature, several anti-cancer drugs from plants have a long history of use in the treatment of cancer (Cragg & Snader, 2003). These are podophyllotoxin (**112**), a compound obtained from *Podophyllum peltatum*. It acts as an anticancer agent by binding irreversibly to tubulin (Srivastava *et al*., 2005). Etoposide (**113**) and teniposide (**114**), the modified analogs of podophyllotoxin are used in the treatment of varioustype of cancers.

Vincristine (**115**) and vinblastine (**116**), anticancer alkaloids from *Catharanthus roseus* (Madagascar periwinkle) are now known to prevent cell division by inhibiting mitosis in the cell cycle. They irreversibly bind to tubulin, thereby blocking cell multiplication and eventually causing cell death (Noble, 1990).

Paclitaxel (Taxol®) (**117**) from *Taxus brevifolia*, the Pacific yew tree. Paclitaxel was the first compound to be discovered to promote microtubule formation. It has been used in the treatment of several types of cancer, but most commonly for ovarian and breast cancers as well as non-small cell lung tumors (Kinghorn & Seo, 1996). Camptothecin (**118**), discovered from the deciduous tree *Camptotheca*

acuminata, is an anticancer agent which has a unique mechanism of action. Camptothecin and its derivatives are topoisomerase-I inhibitors, and cause cell death by DNA damage (Hsiang *et al.*, 1985). The camptothecin water-soluble analogs, namely, topotecan (**119**) and irinotecan (**120**) have been developed as effective drugs.

These substances embrace some of the most exciting new chemotherapeutic agents currently available for use in a clinical setting.

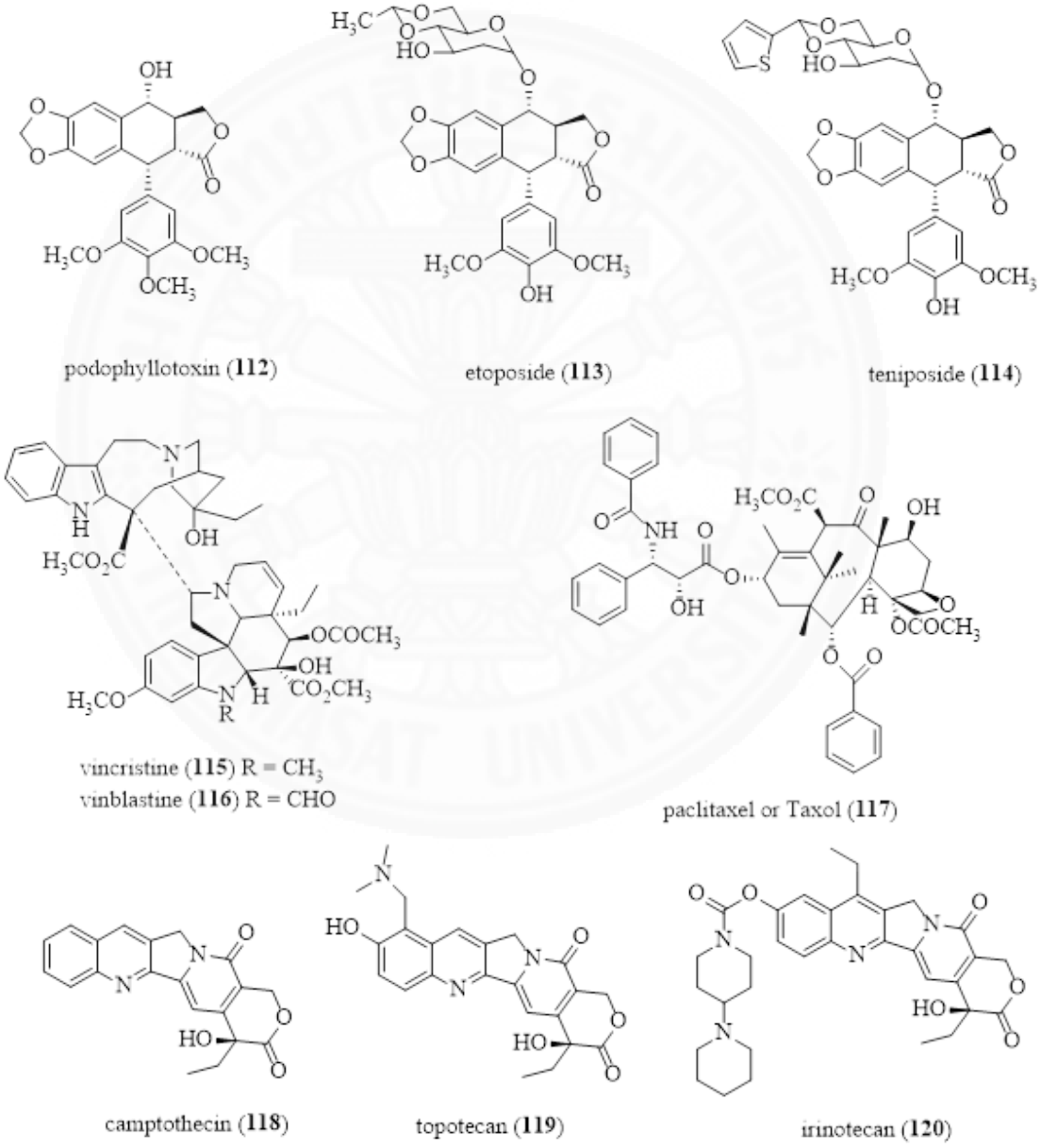
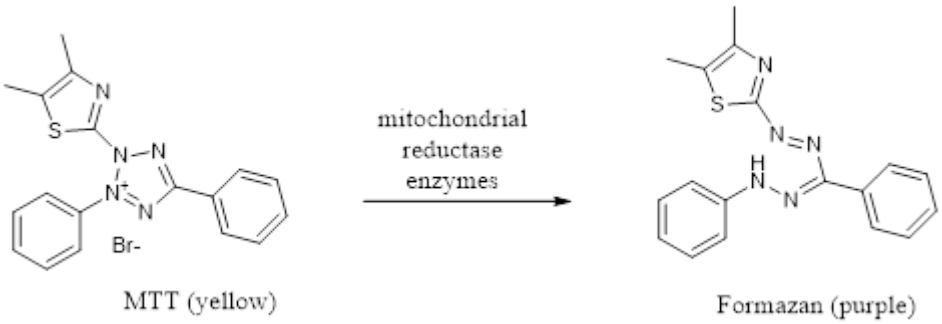


Figure 2.16 Chemical structures of anticancer compounds from plants

2.6 Cytotoxic activity test

Over the last twenty-five years, a very large number of plant extracts have been screened for cytotoxic effects against cancer cell lines and have resulted in some significant drugs being introduced such as paclitaxel. In addition, the traditional use of a considerable number of plants for cancer has been justified to some extent by the findings that the plant extracts are cytotoxic, especially if selectivity is demonstrated, either between different cancer cell lines or between cancer and non-cancer cell lines (Houghton *et al*., 2007).

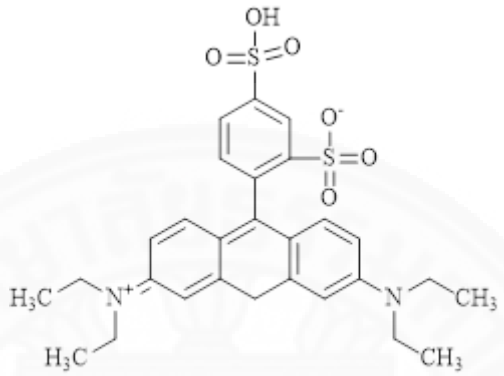
Cytotoxicity testing is based on one or more mammalian cell lines being grown under conditions where they are actively growing and undergoing mitotic division. Cells are cultured in a microtitre well plate and the rate of multiplication and growth is measured indirectly by formation of a colour, the intensity of which is directly proportional to the number of cells present. A variety of experiments can be used and the most basic is to compare the rate of proliferation of a cancer cell lines in the presence and absence of the test substance, usually after a specified time. Ideally, several different cancer cell lines can be used so that selectivity can be assessed and the addition of normal cell lines to the battery enables selectivity between cancer cell lines and normal cell lines to be determined. This gives an indication of potential usefulness in a clinical setting, for which a selectivity of at least two orders of magnitude for the cancer cell lines being the more susceptible is required (Houghton *et al*., 2007). Such tests can also be used to determine whether the cytotoxic effect is merely cytostatic i.e. it stops cells growing or dividing, or cytocidal, where the cells are killed. For such a determination, two sets of identical cells are both exposed to the test agent under identical conditions and for the same period of time. At the end of the exposure period, one set of cells is assayed whilst, for the other set, the medium containing the test substance is discarded and replaced with fresh medium alone. The cells are then incubated for a fixed time before the assay for cell growth is conducted. If the agent has only a cytostatic effect, the cells will grow and undergo mitosis in the fresh medium but, if they have been killed during the initial exposure time, no such increase in number of cells will be observed (Houghton *et al*., 2007).



Two major techniques are used to assess the cell growth. The first major technique, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) and XTT (2,3-bis(2-methoxy- 4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide sodium salt) were developed and were introduced in 1986 and 1988, respectively. Both of these reagents are metabolically reduced by the mitochondria in viable cells to a coloured formazan product, the intensity of which can be measured spectrophotometrically in a plate reader. The use of XTT is preferred since the formazan produced is soluble in water and the solubilisation step required if MTT is used is eliminated. However many cell lines were not so efficient at reducing XTT compared with MTT but the addition of phenazine methosulphate (PMS) showed that reduction was much better (Houghton *et al*., 2007).With both of these reagents the formation of colour relies on the activity of the mitochondria, so, if the function of these is inhibited by variations in cellular levels of NADH, glucose and other factors, variable results are obtained and a similar result may be given as if the cells were not alive or not proliferating (Houghton *et al.*, 2007). Because of these limitations, the second major technique i.e. the sulphorhodamine B (**121**) (SRB) assay is more preferred for testing cytotoxicity. This assay, which was developed in 1990 (Skehan *et al*., 1990), remains one of the most widely used methods for *in vitro* cytotoxic screening. The assay relies on the ability of SRB to bind to protein components of cells that have been fixed to tissue-culture plates by trichloroacetic acid (TCA).

The SRB (2-(3-diethylamino-6-diethylazaniumylidene-xanthen-9-yl)-5-sulfo-enzenesulfonate) is a bright-pink aminoxanthene dye with two sulfonic groups that bind to basic amino acid residues under mild acidic conditions, and dissociate under basic conditions. As the binding of SRB is stoichiometric, the amount of dye extracted from stained cells is directly proportional to the cell mass.

The advantage of SRB assay is sensitive, simple, reproducible and more rapid than the formazan- based assays and gives better linearity, a good signal-to-noise ratio and has a stable end-point that does not require a time-sensitive measurement, as do the MTT or XTT assays (Houghton *et al*., 2007).

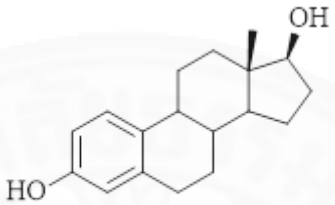


SRB (121)

2.7 Cell proliferation assay

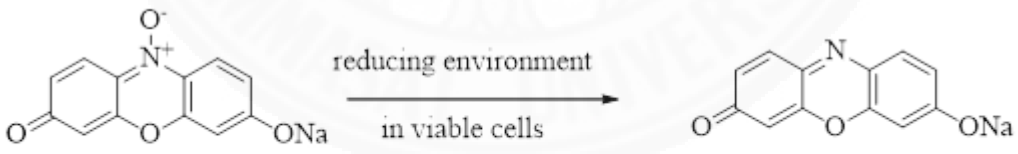
The proliferation of estrogen-dependent cancer cells has been used for detect estrogenic activity test. Cell proliferation assay, the E-Screen test has been used (Sato *et al*., 1995). For this assay, breast cancer cells (MCF-7) were chosen because they are genuine human estrogen-sensitive cells. They remain quiescent when inoculated into ovariectomized hosts. They require the presence of estrogen to growing as tumors in hosts. The MCF-7 cell assay is based on the ability of this polyclonal human breast cancer cell line to proliferate in response to estrogen. This cell line has been used for many years in the search for estrogen antagonists for breast cancer therapies (Odum *et al*., 1998). Umehara *et al*., 2004, have introduced *in vitro* cell proliferation assay; estrogenic activity, which was developed from E-screen test, to determine estrogenic potency by measuring the proliferation of cells and stimulate potency of substance in an estrogen-dependent of breast cancer cells (MCF-7 and T47D). These cell lines are grown in culture medium supplemented with a nonestrogen charcoal-stripped serum that proliferation is prevented. When estrogen is added, the cells proliferate. This assay compare to the cell yield achieved after 4 days

of culture in medium supplemented with 5-10% charcoal-dextran stripped serum in the presence of 17β-estradiol (**122**) (positive control) or absence (negative control) and with diverse concentration of xenobiotics suspected of being estrogenic. When cell yield is examined over a wide range of test compound concentrations, it is possible to distinguish agonists, partial agonist and inactive compounds from one another (Korach *et al*., 1995).



17β-estradiol (**122**)

The alamar blue assay is based on the principle of the native, oxidized form of alamar blue reagent is reduced intracellularly by oxidoreductases and the mitochondrial electron transport chain, with a corresponding shift in its absorbance and fluorescence (Goegan *et al*., 1995). The dye contains resazurin (**123**), which is blue and non-fluorescent. In a reducing environment, resazurin is converted to its reduced form, resorufin (**124**), which is pink and fluorescent (Bonnier *et al*., 2015).



resazurin (**123**) resorufin (**124**)

CHAPTER 3
RESEARCH METHODOLOGY

3.1 General information

1D and 2D NMR spectra were measured on a JEOL ECX-500 instrument operating at 500 and 125 MHz for ^{1}H and ^{13}C, respectively. Chemical shifts are expressed in a δ (ppm) scale with tetramethylsilane as an internal standard, and coupling constants (*J*) are in hertz. The UV spectra were measured with a UV-2550 spectrophotometer (Shimadzu, Tokyo, Japan). The IR spectra were recorded on Vertex 70 FT-IR Bruker spectrometer (Bruker, Karlsruhe, Germany). The ESI-TOF mass spectra, both HR- and LR-, were performed using a micrOTOF Bruker spectrometer (Bruker) for measurements. Vacuum liquid chromatography (VLC) and column chromatography (CC) were carried out on silica gel (Merck, Darmstadt, Germany) type 60 (230-400 mesh for vacuum liquid chromatography and 70-230 mesh for column chromatography, respectively). Precoated plates of silica gel (Kieselgel 60 F_{254}, 0.25 mm, Merck, Darmstadt, Germany) and RP-18 (F254S, Merck KGaA) were used for analytical TLC. The spots on TLC were detected under UV light at 254 nm and by heating at 120 ˚C after spraying with acidic anisaldehyde or dilute H_2SO_4 reagent (Sigma-Aldrich, St Galen, Switzerland). Repeated HPLC was carried out mainly with a JASCO model 887-PU pump, and isolates were detected by an 875-UV variable wavelength detector. Reversed-phase columns for preparative separations; Tosoh TSK gel ODS-80Ts, 5 μm, 6 × 60 × 2 cm ;Tosoh Chemicals Co. Ltd., Tokyo, Japan; flow rate 45 mL/min with detection at 205 nm) and Inertsil® ODS, 3×50 cm (GL Sciences, Inc., Tokyo, Japan; flow rate 10 mL/min with detection at 205 nm) and semipreparative separations (Cosmosil Cholester, 5 μm, 2 × 25 cm, Nacalai Co. Ltd., Kyoto, Japan; YMCPack R&D ODS, 5 μm, 2 × 25 cm, YMC Co. Ltd., Kyoto, Japan; flow rate 9 mL/min with detection at 205 nm) were used.

3.2 Reagents and chemicals

RPMI Medium 1640 (RPMI 1640), Minimum Essential Medium (MEM), Dulbecco's Modified Eagle Medium (DMEM), Fetal bovine serum (FBS), Penicillin Streptomycin (PS) and 0.5% trypsin-EDTA were purchased from Gibco BRL Life Technologies (Grand Island, NY, USA). Phosphate buffer saline (PBS) was purchased from from Amresco (Ohio, USA). Nutrient Mixture F-12 Ham, Sulforhodamine B (SRB), MEM non-essential amino acid solution (NEAA), HEPES buffer solution, vincristine sulphate, Trisma base, Trichloroacetic acids (TCA), paclitaxel, vincristine sulfate and tamoxifen were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was purchased from Fluka (Munich, Germany). Acetic acid and sulfuric acid were purchased from Merck (Darmstadt, Germany). Analytical grade reagent; hexane, chloroform ($CHCl_3$), ethyl acetate (EtOAc), and methanol (MeOH) were purchased from RCI Labscan (Thailand). Anisaldehyde reagent was purchased from Sigma-Aldrich (St Galen, Switzerland). CO_2 humidified incubator was purchased from Shellab (OR, USA). Laminar air flow cabinet was purchased from Boss tech (Bangkok, Thailand). Microplate reader was purchased from Bio Tek instrument, Inc. (VT, USA).

3.3 Plant materials

The rhizomes of five species of Hua-Khao-Yen were collected. The rhizomes of *D. membranacea* were collected from Amphor Pa-tue, Chumporn province, Thailand in August 2011 (voucher number, SKP A062041305), *S. corbularia* were collected from Amphor Mae Taeng, Chiang Mai province, Thailand in March 2010 (voucher number, SKP A179190315), *S. glabra* were collected from Amphor Dan Sai, Loei province, Thailand in October 2010 (voucher number, SKP 179190701), *P. herbacea* were collected from Kanchanaburi province, Thailand in October 2010 (voucher number, SKP 202160801), and *D. birmanica* were collected from department of Medical Sciences, Chanthaburi province, Thailand in 2008 (voucher number, SKP A062001002). Authentication of plant materials was carried out at the herbarium of the Department of Forestry, Bangkok, Thailand where plant

specimen samples have been lodged. The specimens were kept in the herbarium of the Southern Centre of Thai Medicinal Plants, at the Faculty of Pharmaceutical Science, Prince of Songkhla University, Songkhla. Macroscopic and Microscopic description were also used to identify plant powder according to the THP (Thai Herbal Pharmacopoeia, 2000) and compare these characteristics to the previous report (Itharat, 2002).

3.4 Preparation of plant extracts

The rhizomes of five species of Hua-Khao-Yen were washed, chopped, dried (50 °C) in an oven, and milled to obtain powder. The powders were extracted by procedures used by Thai traditional doctors with ethanol or water extracts. Dried powder was macerated with 95% ethanol for 3 days at room temperature, filtered. Filtrate was dried in evaporator, and the residue was further macerated twice. The three extracts were combined and concentrated to dryness under reduced pressure to yield crude ethanolic extracts. For the water extract, powder was boiled for 30 minutes in water, filtering and drying using lyophilizer. All of the crude extracts were dried to constant weight in a vacuum desiccator and stored in glass container at -20 ^{o}C until required for experimentation.

3.5 Extraction and isolation

3.5.1 Extraction and isolation of ethnolic extract of *Dioscorea membranacea*

The rhizomes of *D. membranacea* were washed, chopped, dried (50 °C) in an oven, and milled to obtain powder. Dried powdered (2 kg) were extracted three times (3×8 L, for 3 days each) with 95% ethanol at room temperature. The residue from filtering was continuously extracted twice. The extracts from 3 times were pooled and concentrated to dryness under reduced pressure to yield crude ethanolic extract (70 g) as a dark brown residue.

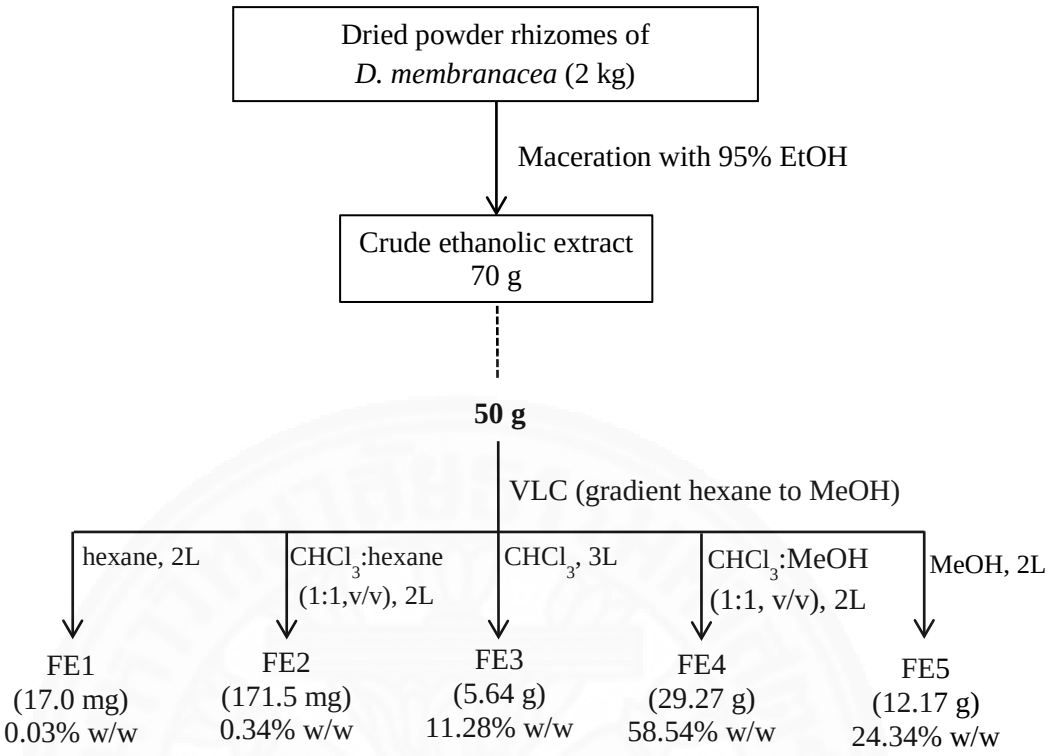


Figure 3.1 Extraction and isolation of dried powder rhizomes of *Dioscorea membranacea*

The crude ethanolic extract (50 g) was separated by vacuum liquid chromatography (VLC) on silica gel 60 using hexane as eluent and increasing polarity with chloroform and methanol, successively to give five fractions as followed: hexane (2000 mL), $CHCl_3$: hexane (1:1, 2000 mL), $CHCl_3$ (3000 mL), MeOH: $CHCl_3$ (1:1, 2000 mL) and MeOH (2000 mL), respectively. Each fraction was dried by evaporator to yield FE1 (17.0 mg), FE2 (171.5 mg), FE3 (5.64 g), FE4 (29.27 g) and FE5 (12.17 g) and the percentage of yield as % w/w of starting weight of crude extract are shown in **Figure 3.1**.

According to the cytotoxic activity against four cell lines; hepatocellular carcinoma (HepG2), cholangiocarcinoma (KKU-M156) and breast cancer (MCF-7 and T47D) cell lines by using the SRB assay (see **Table 4.2**), fraction FE3 was selected for the bioassay-guided isolation as it showed highest cytotoxicity against all types of cancer cell lines (HepG2, KKU-M156, MCF-7 and T47D) with the IC_{50} value of IC_{50} value of 18.30 ± 0.05, 14.31 ± 3.08, 5.62 ± 0.77 and 9.49 ± 1.38 µg/mL,

respectively, while FE1, FE2, FE4 and FE5 showed no cytotoxic activity (IC_{50} >50 µg/mL) (**Table 4.2**).

Fraction FE3 (5.64 g) was subjected to column chromatography (CC) on silica gel 60 (150 g) using EtOAc: hexane (1:9) gradient solvent system with increasing polarity each fraction was collected for 40 mL and pooled for TLC analysis to give nine fractions (fr.1-fr.9).

Subfraction fr. 3 (120.0 mg) was further purified by CC on silica gel 60 (30 g) using $CHCl_3$: hexane (3:2, 500 mL) as eluent to yield DME1 (37.6 mg, 0.08% w/w) as a pale yellow solid (R_f = 0.57, EtOAc: hexane, 3:7).

Subfraction fr. 4 (1.94 g) and 5 (1.67 g) were chromatographed using CC on silica gel 60 (100 g) and eluting with $CHCl_3$: hexane (3:2), to afford DME4 (72.5 mg, 0.15% w/w) as a white solid (R_f = 0.50, EtOAc: hexane, 3:7) and DME5 (17.1 mg, 0.03% w/w) as a pale yellow solid (R_f = 0.30, EtOAc: hexane, 3:7), respectively.

Subfraction fr. 6 (1.46 g) was further purified by CC on silica gel 60 (100 g) using EtOAc: hexane (3:7) as eluent to afford DME2 (45.8 mg, 0.09% w/w) as a pale yellow solid (R_f = 0.33, EtOAc: hexane, 3:7).

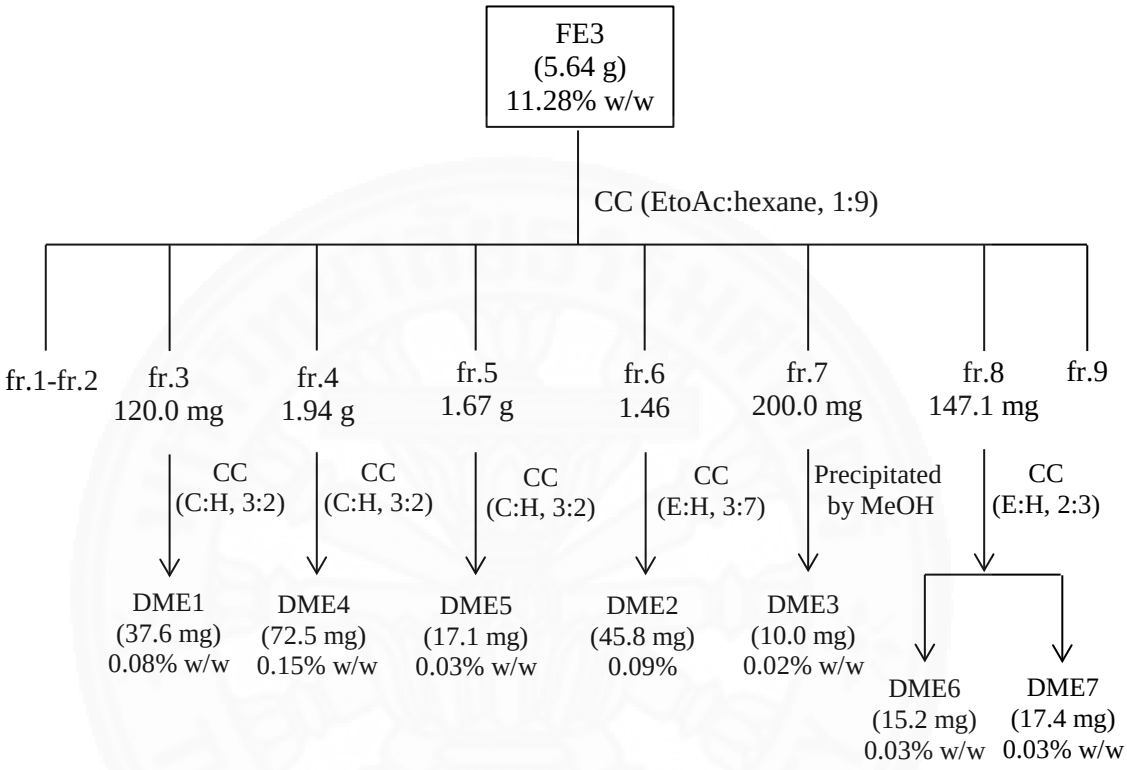
Subfraction fr. 7 (200.0 mg) was filtered and washed by MeOH to yield DME3 (10.0 mg, 0.02% w/w) as a yellow solid (R_f = 0.21, EtOAc: hexane, 3:7).

Subfraction fr. 8 (147.1 mg) was also purified by CC on silica gel 60 (100 g) and eluting with EtOAc: hexane (2:3) to afford DME6 (15.2 mg, 0.03% w/w) as a white amorphous solid (R_f = 0.33, EtOAc: hexane, 1:1) and DME7 (17.4 mg, 0.03% w/w) as a white amorphous powder (R_f = 0.26, EtOAc: hexane, 1:1).

In addition, each compound was also demonstrated to be pure by using TLC, comparison with authentic pure compounds using three different solvent systems of varying polarity and detection with UV and acidic anisaldehyde spraying reagent.

Compound DME6: white amorphous solid; UV (MeOH) λ_{max} (log ε) 224 (3.69) nm; IR (neat) ν_{max} 3432, 2927, 1687 cm^{-1}; HR-ESI-TOF-MS *m/z* $[M+Na]^+$ 481.2561 (calcd for $C_{27}H_{38}O_6Na$, 481.2566); ^1H NMR (400 MHz in $CDCl_3$) and ^{13}C NMR (100 MHz in $CDCl_3$) see **Table 4.9**.

Compound DME7: white amorphous powder; UV (MeOH) λ_{max} (log ε) 224 (4.23) nm; IR (neat) ν_{max} 3488, 2928, 1686 cm^{-1}; HR-ESI-TOF-MS *m/z* $[M+Na]^+$ 481.2561 (calcd for $C_{27}H_{38}O_6Na$, 481.2566); ^1H NMR (400 MHz in $CDCl_3$) and ^{13}C NMR (100 MHz in $CDCl_3$) see **Table 4.11**.



Notes: C = $CHCl_3$, E = EtOAc, H = hexane and %w/w = %w/w of crude extract

Figure 3.2 Isolation of DME1-DME7 from chloroform fraction (FE3) of ethanolic extract of *Dioscorea membranacea*

3.5.2 Extraction and isolation of water extract of *Dioscorea membranacea*

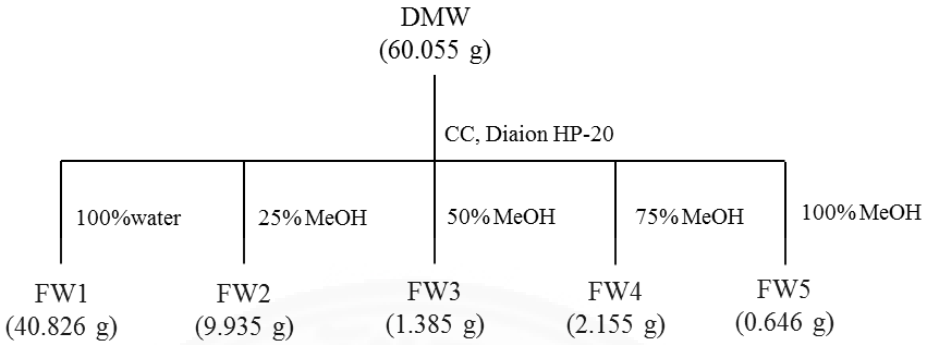


Figure 3.3 Isolation of water extract of *Dioscorea membranacea* by Diaion HP-20

The water extract (60.05 g) of *D. membranacea* was chromatographed using diaion HP-20 and eluted with 100% water to get rid of sugar amino acid and then eluted with 25% MeOH, 50% MeOH, 75% MeOH and 100% MeOH, respectively to give five fractions (FW1, FW2, FW3, FW4 and FW5, respectively). Each fraction was detected by TLC fingerprint normal phase and reverse phase and compared with DMW were shown in **Figure 3.4.**

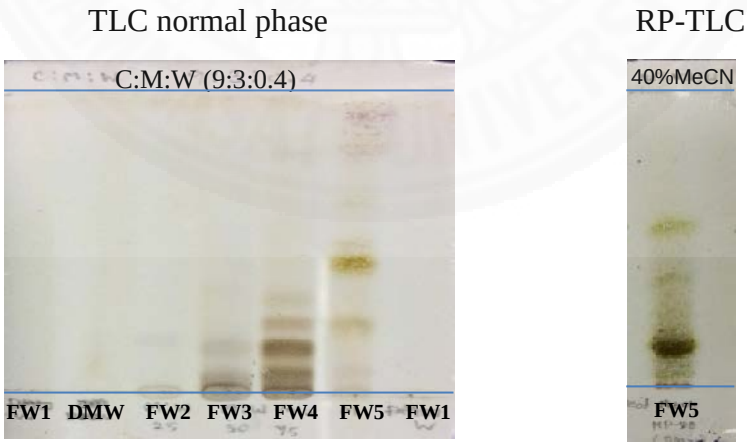


Figure 3.4 TLC fingerprint of fractions (FW1-FW5) from DMW detected by TLC normal phase and reverse phase

From these fractions, Fraction FW5 was tried the solvent system by analytical HPLC (C_{18}- InertSustain ODS (4.6×250 mm) using isocratic MeCN-water (45:55) as eluent (λ 205 nm, flow rate 1 mL/min). The HPLC chromatogram was shown in **Figure 3.5**.

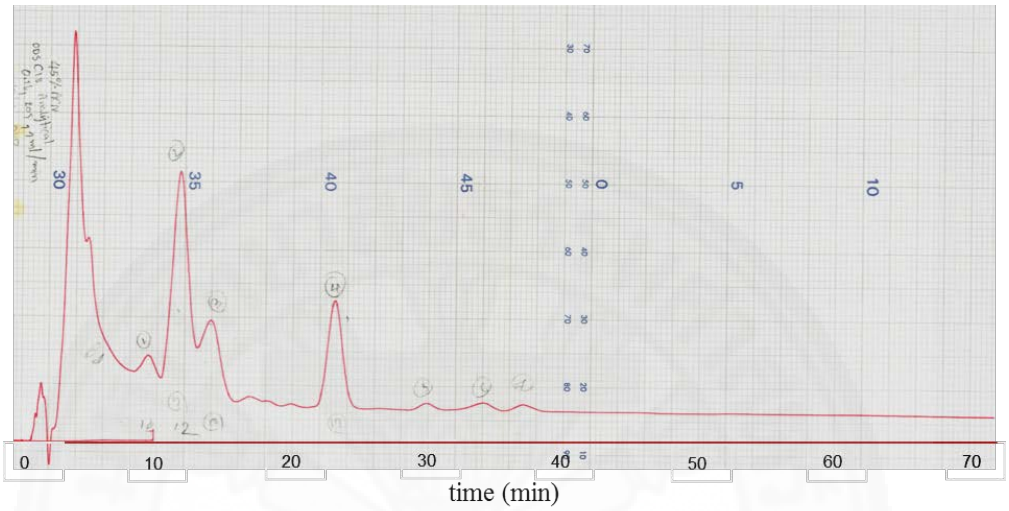
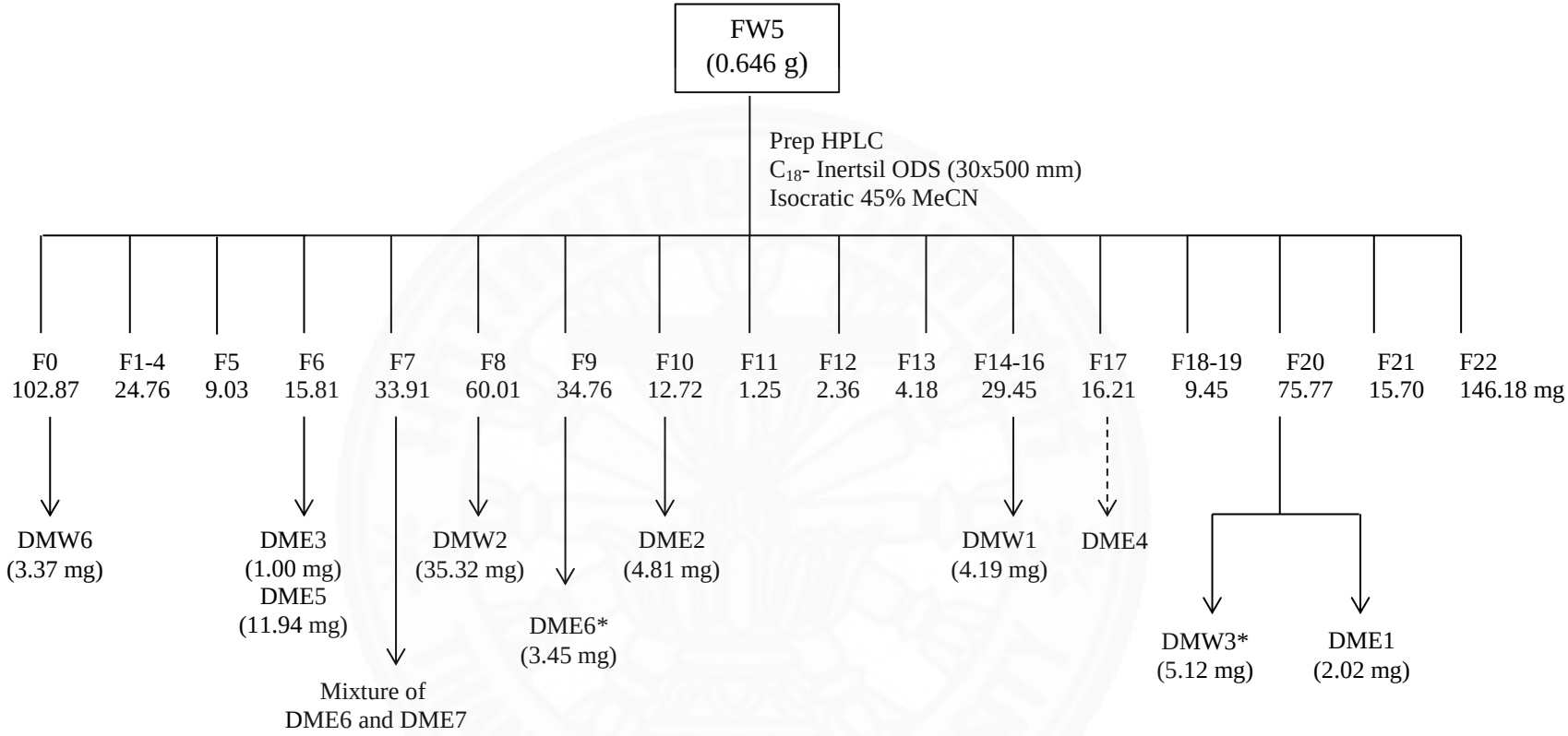


Figure 3.5 HPLC chromatogram of fraction FW5 separated by analytical HPLC using 45% MeCN as eluent

Then, fraction FW5 (0.646 g) was purified by preparative HPLC (column C_{18}- Inertsil ODS (30×500 mm) using isocratic MeCN-water (45:55) as eluent (flow rate 10 mL/min). Each fraction was collected and pooled by their characteristic of chromatogram to afford seventeen combined fractions (see **Figure 3.6**).



Note: *compound was precipitated by MeOH

Figure 3.6 Isolation of DME1-DME7, DMW1-DMW3 and DMW6 from fraction FW5 (100% MeOH) of water extract of *Dioscorea membranacea*

Subfraction F6 (15.81 mg, t_R 98 min) was precipitated by MeOH to give two pure compounds; F6(S) (1.00 mg) and F6 (L) (11.94 mg). By comparison of ^1H and ^{13}C NMR and characteristic of spot on TLC with authentic sample of dioscreanone and 2,5,6-trihydroxy-3,4-dimethoxy-9.10-dihydrophenanthrene, thus F6(S) and F6(L) was identified as dioscreanone (DME3) and 2,5,6-trihydroxy-3,4-dimethoxy-9.10-dihydrophenanthrene (DME5), respectively.

Subfraction F17 (16.21 mg, t_R 220 min) was showed characteristic of ^1H and ^{13}C NMR and TLC same with previously reported data of 5,6-dihydroxy-2,4-dimethoxy-9,10-dihydrophenanthrene. F17 was identified as 5,6-dihydroxy-2,4-dimethoxy-9,10-dihydrophenanthrene (DME4).

Subfraction F0 (102.87 mg) was precipitated in MeOH. The mother solution (75.40 mg) was subjected to preparative HPLC (column C_{18}- Inertsil ODS (30×500 mm), flow rate 10.0 mL/min) using gradient 25% MeCN to 50% MeCN as eluent to give DMW6 (white powder, 3.37 mg, t_R 193 min). By comparison of their ^1H and ^{13}C NMR data with those previously reported data, DMW6 was identified as 3*β*, 26-dihydroxy-25 (*R*)-furosta-$\Delta^{5,20(22)}$-diene-3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-β-D-glucopyranoside.

Subfraction F7 (33.91 mg) was subjected to semi-preparative HPLC using 35% MeCN as eluent (column C_{18}- Inertsil ODS-3 (20 × 250 mm), flow rate 9.0 mL/min) to give mixture of DME6 and DME7 (compared the ^1H NMR data with panthogenin B (DME6) and epipanthogenin B (DME7) which found in ethanolic extract of *D. membranacea*.

Subfraction F8 (47.42 mg) was separated by semi-preparative HPLC using 35% MeCN (column C_{18}- Inertsil Ph-3 (20 × 250 mm), flow rate 9.0 mL/min) to afford DMW2 (35.32 mg, t_R 32 min).

Subfraction F9 (34.76 mg) was precipitated by MeOH to give F9s (3.45 mg, R_f = 0.2) same with DME6.

Subfraction F10 (12.72 mg) was precipitated by MeOH to give F10s (4.81 mg, R_f = 0.3, 40% EtOAc; hexane). By comparison of their ^1H NMR data with authentic dioscorealide B, F10s was identified as dioscorealide B (DME2).

Subfraction F14-16 (29.45 mg) was subjected to semi-preparative HPLC using 45% MeCN (column C_{18}- Inertsil ODS-EP (20 × 250 mm), flow rate 9.0 mL/min) to give DMW1 (white powder, 4.19 mg, t_R 28 min).

Subfraction F20 (brown solid, 75.77 mg) was precipitated by MeOH to give DMW3 (5.12 mg, R_f =0.4, $CHCl_3$: MeOH: Water, 8:2:0.2). By comparison of their ^1H and ^{13}C NMR data, DMW3 was identified as Prosapogenin A of Dioscin. Then the mother solution (40.99 mg) was purified by semi-preparative HPLC using 25% MeCN (HPLC (column C_{18}- Inertsil Ph-3 (20 × 250 mm), flow rate 9.0 mL/min) as the mobile phase, followed by semi-preparative HPLC using 55% MeCN (HPLC (column C_{18}- Inertsil ODS (20 × 250 mm), flow rate 9.0 mL/min) as eluent to give DMW3 (2.81 mg) and FWL11 (2.02 mg, t_R 39 min). By comparison of their ^1H and ^{13}C NMR data, FWL11 was identified as dioscorealide A = DME1.

Fraction FW4 (2.10 g, eluted with 75% MeOH) was subjected to Reversed-phase column for preparative separation (Tosoh TSK gel ODS-80Ts, 5 μm, 60 × 600 mm × 2 columns; Tosoh Chemicals Co. Ltd., Tokyo, Japan; flow rate 30 mL/min using gradient of 40-55% MeOH as the mobile phase, to collect thirteen fractions (Fm1-Fm13) (**Figure 3.7**).

Subfraction Fm6 (425.42 mg) was subjected to preparative HPLC (column C_{18}- Inertsil ODS (30 × 500 mm) using isocratic 25% MeCN, flow rate 6.4 mL/min) as eluent, followed by semi-preparative HPLC to afford DMW4 (white powder, 5.72 mg, t_R 14 min, chiral column CD-Ph 10 × 250 mm, using 23% MeCN as eluent).

Subfraction Fm12 (262.82 mg) was subjected to preparative HPLC (column C_{18}- Inertsil ODS (30×500 mm) using isocratic 25% MeCN (flow rate 7.2 mL/min) as eluent, followed by semi-preparative HPLC (YMC ODS column) using 25% MeCN to afford DMW5 (white powder, 1.08 mg, t_R 22 min).

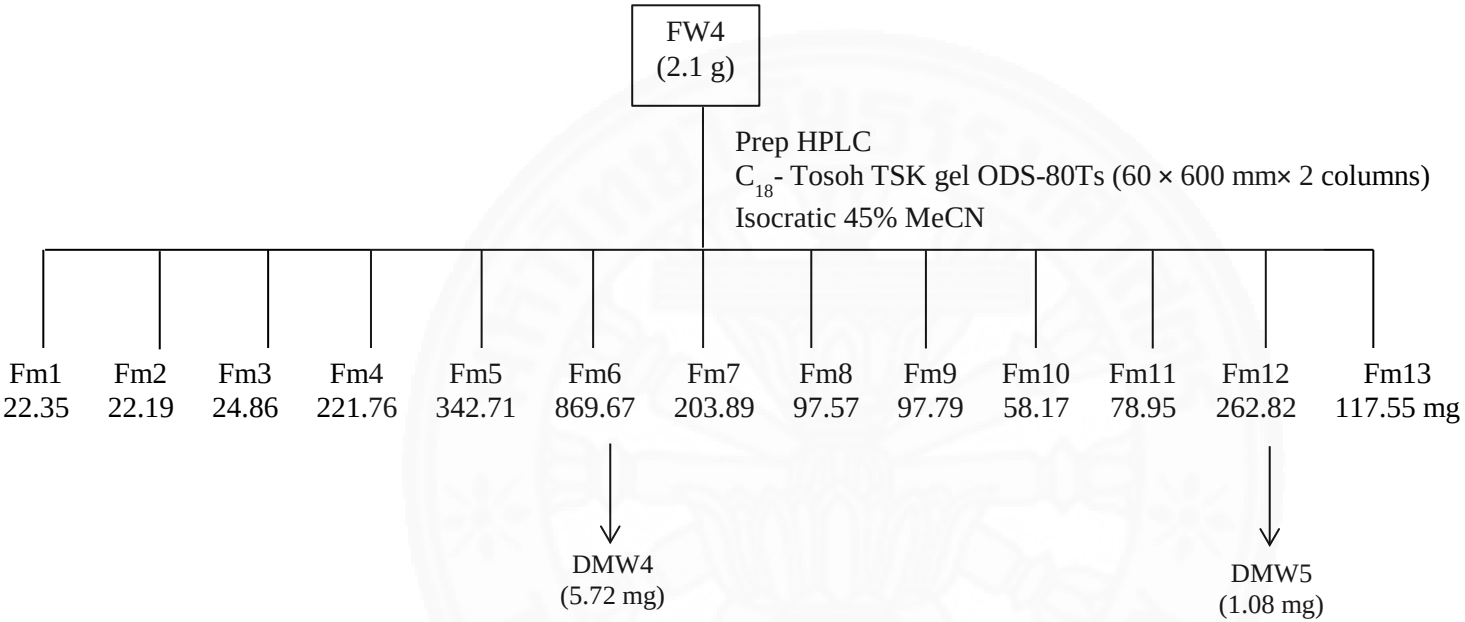


Figure 3.7 Isolation of DMW4 and DMW5 from fraction FW4 (75% MeOH) of water extract of *Dioscorea membranacea*

3.6 *In vitro* assay for cytotoxic activity

The human cancer cell lines: hepatocellular carcinoma (HepG2) cell line and cholangiocarcinoma cell line (KKU-M156) were used for cytotoxicity test of crude extract and its pure compounds. HepG2 (ATTC No. HB-8065) cells were cultured in Minimum Essential Media (MEM) (Gibco BRL Life Technologies, Grand Island, NY, USA) supplemented with 10% heated-inactivated fetal bovine serum (FBS) (Gibco BRL Life Technologies, Grand Island, NY, USA), and 1% Penicillin Streptomycin (PS) (Gibco BRL Life Technologies, Grand Island, NY, USA). KKU-M156 was established and kindly provided by Dr. Piti Aungareewithaya and Prof. Dr. Veeraphol Kukongviriyapan, Faculty of Medicine, Khon Kaen University, Khon Kaen province and cells were cultured in HAM's F12 (Sigma-Aldrich Inc., St. Louis, MO, USA) supplemented with 10% heated-inactivated FBS, 1% PS, and 12.5 mM hepes (Sigma-Aldrich Inc. (St. Louis, MO, USA). One normal cell line was human keratinocyte immortal cells (HaCaT), purchased from CLS cell lines service (No. 300493-SF) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL Life Technologies, Grand Island, NY, USA) supplemented with 10% heated-inactivated FBS and 1% PS. All cell lines were maintained at 37 °C in a 5% CO_2 atmosphere with 95% humidity (Itharat *et al*., 2014). For breast cancer cell lines both MCF-7 and T47D were mentioned in *in vitro* assay for estrogenic activity.

According to their growth profiles, the optimal plating densities of each cell line were determined (2×10^3, 3×10^3, 3×10^3, 7×10^3 and 8×10^3 cells/well for KKU-M156, HepG2, MCF-7, T47D and HaCaT, respectively). The extracts were diluted in medium to produce 4 concentrations and 100 µL/well of each concentration was add to the plates in four replicates to obtain final concentration ranges of 1-100 µg/mL for extract and 0.1-50 µg/mL for pure compounds. The plates were incubated for 72 hours, then incubated for a recovery period of 6 days and cell numbers were analyzed by SRB assay (Skehan *et al*., 1990; Itharat *et al*., 2004, Houghton *et al*., 2007). Paclitaxel (Sigma-Aldrich Inc. St. Louis, MO, USA) and vincristine sulfate (Sigma-Aldrich Inc. St. Louis, MO, USA) were used as the positive control.

The absorbance (OD) of each well was determined by a Power Wave X plate reader (Bio-Tek instrument, Inc. VT, USA) at 492 nm). The percentage of

cytotoxicity was measured as the percentage absorbance compared the control (non-treated cells). Percentage of cytotoxicity was calculated by using equation below.

$$\% \text{ Cytotoxicity} = \frac{(OD_{control} - OD_{sample})}{OD_{control}} \times 100$$

The IC_{50} values were calculated using GraphPad Prism version 6.01 (GraphPad Software, San Diego, CA, USA). According to National Cancer Institute guideline (Boyed, 1997) extract with an IC_{50} values < 20 µg/mL and compounds IC_{50} values < 4 µg /mL were considered active.

3.7 *In vitro* assay for estrogenic and anti-estrogenic activity

Human cell lines

MCF-7 and T47D human breast cancer cells were purchased from ATCC. The MCF-7 (ATCC No. 59681580) cells were grown in MEM supplemented with 6 ng/mL insulin, 1 mM sodium pyruvate, 1 mM nonessential amino acids, 2 mM glutamine, 10% FBS, and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin), under a 5% CO_2 humidified atmosphere at 37 °C. The T47D (ATCC No. 59681609) cells were grown in RPMI-1640 supplemented with 1mM sodium pyruvate, 1 mM nonessential amino acids, 2 mM glutamine, 10% FBS, and antibiotics (100 U/mL penicillin, 100 µg/ mL streptomycin), under a 5% CO_2 humidified atmosphere at 37 °C.

Cell proliferation assay

Cells were seeded into 96-well tissue culture plates in 5% DCC treated-FBS supplemented RPMI phenol red-free medium at a density of 10^4 cells/well. Each test sample was dissolved in 70% EtOH solution at concentrations 100-fold higher than desired final concentrations in unit of µg/ml (prepare stock solution at concentration of 2 mg/mL). Then samples in liquid condition were diluted by serial dilution with 70% EtOH at 1, 10 and 100 times diluted solutions. Then all samples solutions were diluted for 10 times with culture medium, and 10 µLof each sample

solution was added to 90 µL of the cell suspension seeded in 96 well platesand incubated at 37 °C with 5% CO_2 for 96 h. In all experiments, serial dilutions of estradiol were added as a positive control. Alamar Blue reagent was used to evaluate relative cell concentrations. After 3 h, fluorescence was measured at 590 nm with excitation at 550 nm using a Wallac 1420 ARVOsx multilabel counter (Perkin-Elmer Inc., Wellesley.MA) (Umehara *et al*., 2009).

Estrogenic activity

Estrogenic assay will be conducted according to the procedure for the cell proliferation assay with minor modifications. MCF-7 or T47D cells were seeded (1×10^4 cells/well) in 96-well plates in 90 µL of 5% DCC-treated, FBS-supplemented RPMI phenol red-free medium. A 5 µL portion of each test compound was added to each well with concentrations ranging from 0.01 to 10 µM and incubated at 37 °C in a CO_2 incubator for 96 h. In all experiments, serial dilution of estradiol were added as a positive control at concentrations ranging from 1 to 100 pM. Add 10 µL Alamar Blue reagent in the wells and incubate for 3 h., the plate will be measured by fluorescence 590 nm with excitation 550 nm. The result was calculated from the cell populations and the EqE values of each sample (EqE100, EqE10, and EqE1) were determined for the concentration required to enhance the E2 effect to the equivalent level of 100, 10, and 1 pM, respectively.

Anti-estrogenic activity

All of the extracts and isolated compounds were tested for antiestrogenic activity following the procedure described in a previous report (Umehara *et al*., 2009). Breast cancer cells MCF-7 and T47D cells were seeded at a density of 1.0×10^4 cells/well in 96-well plates in 90 µL of 5% DCC-treated, FBS-supplemented RPMI phenol red-free medium. After 3 h incubation, 5 µL of each test compound at four different concentrations ranging from 0.01 to 10 µM was added to each well along with 5 µL of estradiol (E2) at a concentration of 20 nM to make a final volume 100 µL in each well. Finally, the plates were incubated at 37 °C in a CO_2 incubator for 96 h. 5 µL of serially diluted tamoxifen at concentrations ranging from 0.01 to 10 µM was used as a positive control. The result was calculated from the cell populations,

and the iEqE values of each sample (iEqE50, iEqE10, and iEqE1) were determined for the concentration required to inhibit the E2 effect (iEqE50, iEqE10, and iEqE1, with a concentration suppressing the E2 effect to the equivalent level of 50, 10, and 1 pM, respectively). From the results of the concentrations tested, samples were categorized as strong (S) if they suppressed E2 activity to a level of 10 pM, and mild (M) if they suppressed the activity level to between 10 and 50 pM.

Data and statistical analysis

Statistical differences will be determined by analysis of variance followed by Dunnett's multiple comparison tests using GraphPad Prism version 6.01. Statistical significance is established at the $p < 0.05$ level.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Identification of plant materials called 'Hua-Khao-Yen'

Five plants materials namely *Dioscorea birmanica* Prain & Burkill, *Dioscorea membranacea* Pierre, *Smilax corbularia* Kunth, *Smilax glabra* Roxb and *Pygmaeopremna herbacea* (Roxb.) Mold, were identified by comparison with authentic voucher specimens (SKP A062001002, SKP A062041305, SKP A179190315, SKP 179190701 and SKP 202160801, respectively), which have been kept in the herbarium of Southern Center of Thai Medicinal Plants at Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand. The characteristic of this plant were identified by comparison tissues and plant powder of their rhizomes using macroscopic and microscopic method and compared its characteristic with the previous report (Itharat, 2002).

***Dioscorea birmanica* Prain & Burkill**

Description: Odour, no odour; taste, slightly bitter; texture, brittle and light

Macroscopical Outside colour rhizomes as brown but inside colour as red white (**Figure 4.1**).

Microscopical Powder drug of rhizomes of *Dioscorea birmanica* Prain & Burkill were found the characteristics of the 4-16 μm round shape starch grains (**Figure 4.2**), the rhaphide crystal of calcium oxalate in groups (**Figure 4.3**), and the pale brown cell walls of cork cells found in fair amount. Based on its identical characteristics to those of the previous report (Itharat, 2002), this plant was identified as *Dioscorea birmanica*.

***Dioscorea membranacea* Pierre**

Description: Odour, no odour; taste, no taste; texture, brittle and light

Macroscopical Outside colour rhizomes as black brown but inside colour as yellow white (**Figure 4.1**).

Microscopical Powder drug of rhizomes of *Dioscorea membranacea* Pierre were found the characteristics of the 28-48 μm × 32-100 μm (width × length) elliptical shape starch grains (**Figure 4.2**), the rhaphide crystal of calcium oxalate in the parenchyma cells (**Figure 4.3**), and the dark brown cell wall of cork cells found in large quantity. Based on its identical characteristics to those of the previous report (Itharat, 2002), this plant was identified as *Dioscorea membranacea*.

***Smilax corbularia* Kunth**

Description: Odour, indistinct; taste, indistinct; texture, hard and strong

Macroscopical Outside colour rhizomes as deeply red brown but inside colour as red brown (**Figure 4.1**).

Microscopical Powder drug of rhizomes of *Smilax corbularia* Kunth were found the characteristics of the 10-60 μm (large quantity of the 32-40 μm) round shape starch grains (**Figure 4.2**), the rhaphide crystal of calcium oxalate in groups of cells shaped like a woven-ball (**Figure 4.3**), and the dark brown cell wall of cork cells found in small quantity. Based on its identical characteristics to those of the previous report (Itharat, 2002), this plant was identified as *Smilax corbularia*.

***Smilax glabra* Roxb**

Description: Odour, indistinct; taste, bitter; texture, hard and strong

Macroscopical Outside colour rhizomes as red brown but inside colour as pale white (**Figure 4.1**).

Microscopical Powder drug of rhizomes of *Smilax glabra* Roxb were found the characteristics of the 8-56 μm (large quantity of the 20-28 μm) round shape starch grains (**Figure 4.2**), the rhaphide crystal of calcium oxalate in a single cell shaped like a bowl (**Figure 4.3**), and the yellow cell walls of cork cells found in small quantity. Based on its identical characteristics to those of the previous report (Itharat, 2002), this plant was identified as *Smilax glabra*.

***Pygmaeopremna herbacea* (Roxb.) Mold**

Description: Odour, indistinct; taste, indistinct; texture, hard and strong

Macroscopical Outside colour rhizomes as gray brown but inside colour as yellow white (**Figure 4.1**).

Microscopical Powder drug of rhizomes of *Pygmaeopremna herbacea* (Roxb.) Mold were found the characteristics of the 6-32 μm (large quantity) round shape starch grains (**Figure 4.2**), the rectangular prism of calcium oxalate found in bast fibre (**Figure 4.3**), and the brown cell walls of cork cells found in fair quantity. Based on its identical characteristics to those of the previous report (Itharat, 2002), this plant was identified as *Pygmaeopremna herbacea*.

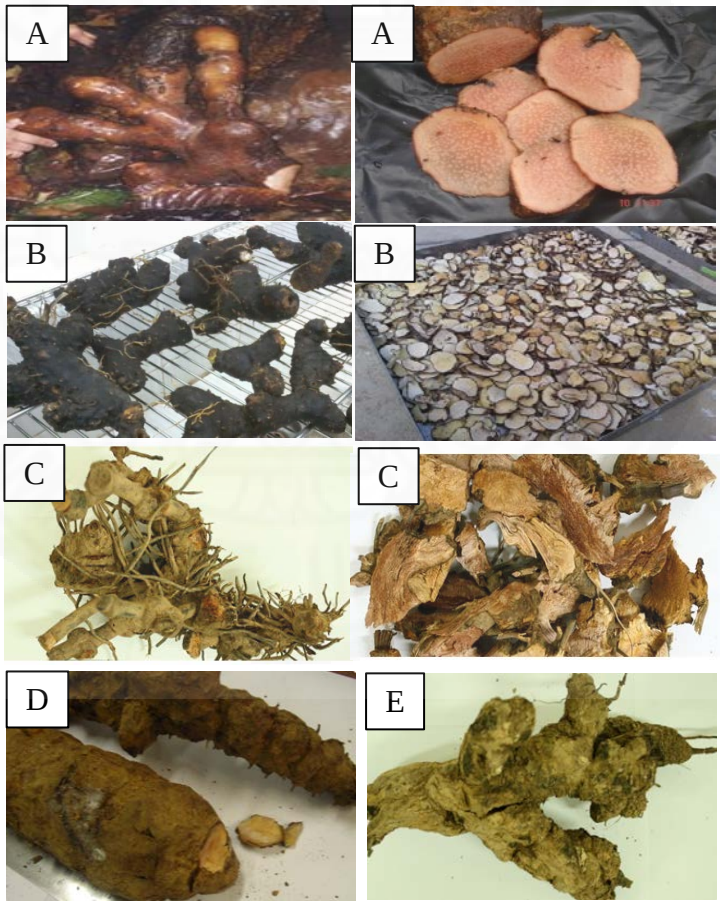


Figure 4.1 The fresh rhizomes of *Dioscorea birmanica* Prain et Burkill (A), *Dioscorea membranacea* Pierre (B), *Smilax corbularia* Kunth. (C), *Smilax glabra* Roxb. (D) and *Pygmaeopremna herbacea* Roxb (E) (Ruangnoo, 2012; Itharat, 2002)

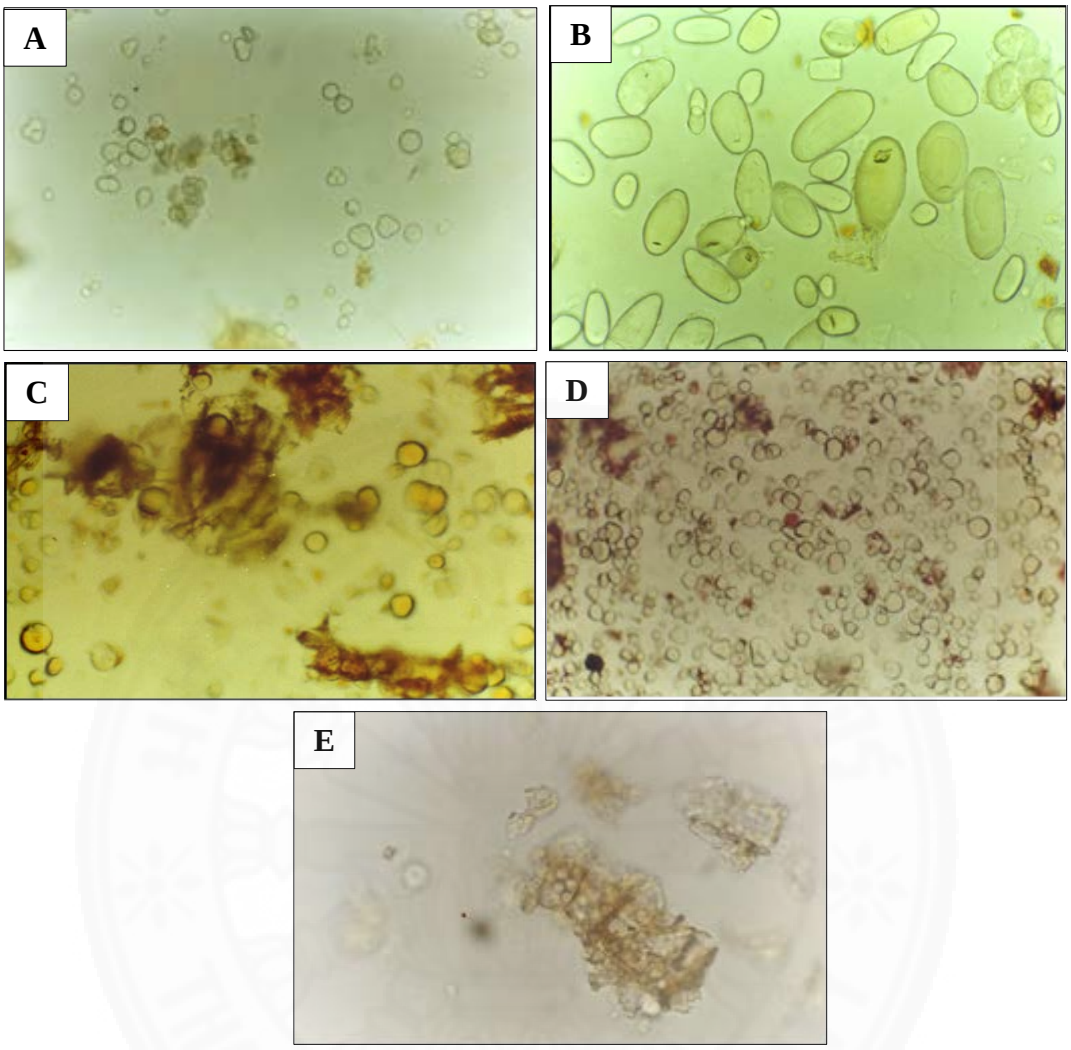


Figure 4.2 Starch grains from different types of Hua-Khao-Yen under microscope (x375); *Dioscorea birmanica* Prain et Burkill (A), *Dioscorea membranacea* Pierre (B), *Smilax corbularia* Kunth. (C), *Smilax glabra* Roxb. (D) and *Pygmaeopremna herbacea* Roxb (E) (Itharat, 2002)

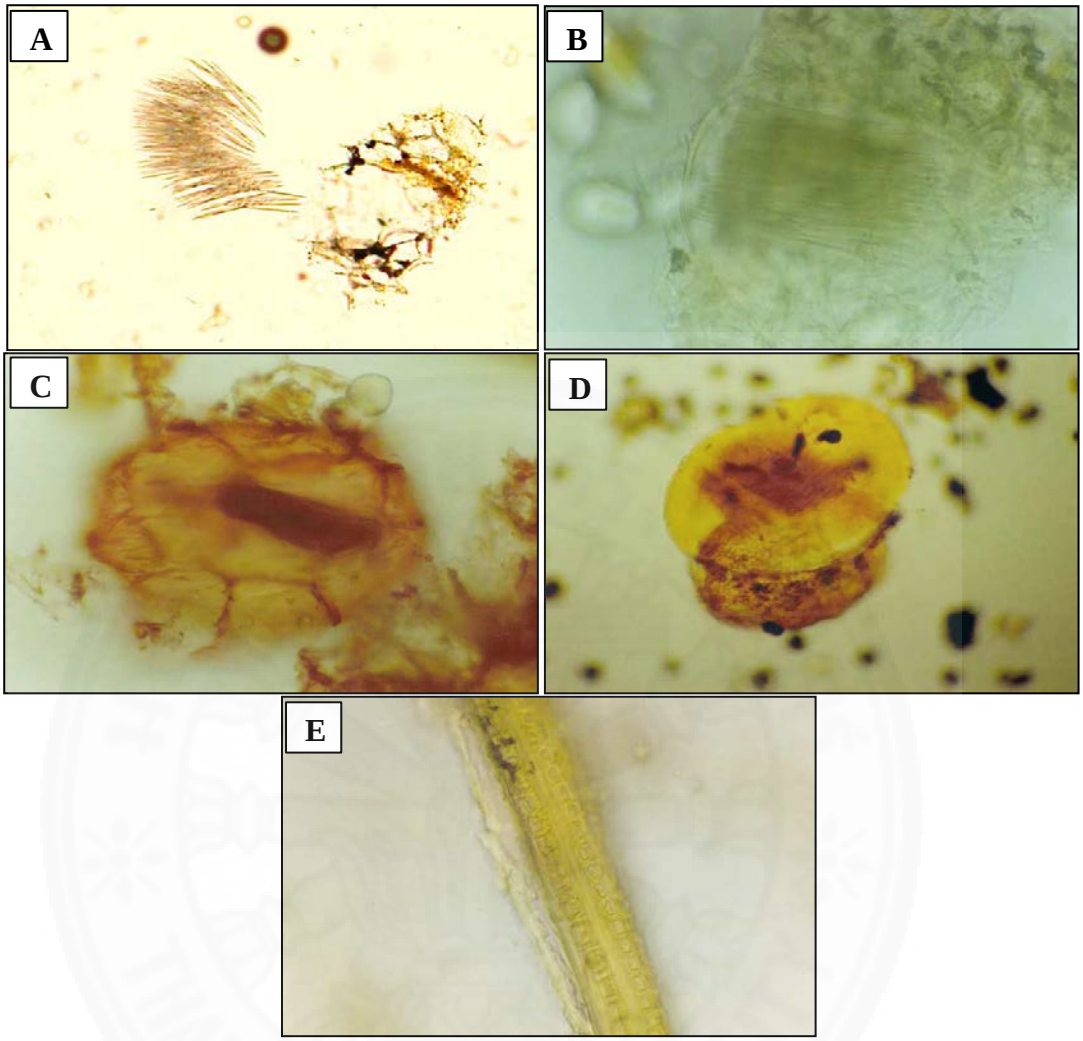


Figure 4.3 Calcium oxalate crystals from different types of Hua-Khao-Yen under microscope (x375); *Dioscorea birmanica* Prain et Burkill (A), *Dioscorea membranacea* Pierre (B), *Smilax corbularia* Kunth. (C), *Smilax glabra* Roxb. (D) and *Pygmaeopremna herbacea* Roxb (E) (Itharat, 2002)

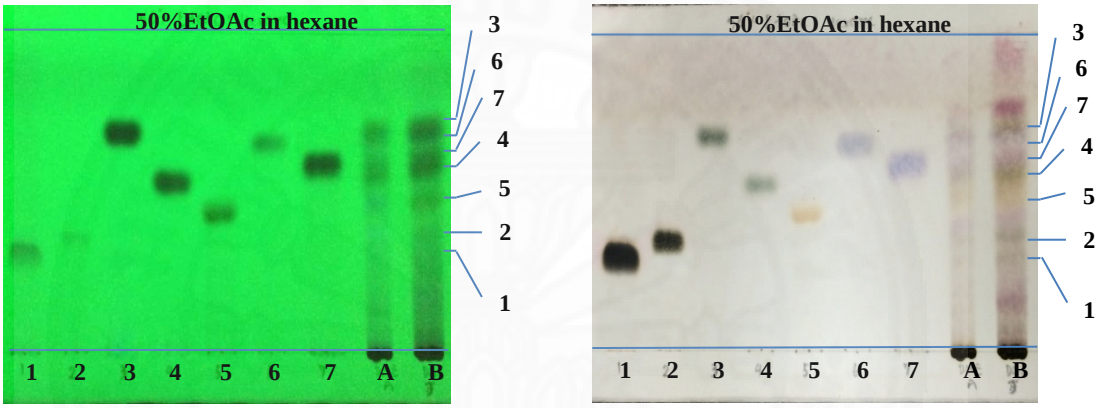
4.2 Plant extraction

The percentage yield of the ethanolic and water extracts of dried powder rhizomes from five species of Hua-Khao-Yen including *Dioscorea birmanica* Prain & Burkill *Dioscorea membranacea* Pierre, *Pygmaeopremna herbacea* (Roxb.) Mold, *Smilax corbularia* Kunth and *Smilax glabra* Roxb. were shown in **Table 4.1** in which the ethanolic extract of *D.birmanica* and *S. corbularia* had highest yield (15.16% of both extract). For the water extract, the % yields (w/w) of *S. glaba* (34.22%) > *D. birmanica* (31.12%) > *D. membranacea* (24.90%) > *P. herbacea* (13.92%) > *S. corbularia* (8.25%).

4.3 *In vitro* assay for cytotoxic activity of five species of Hua-Khao-Yen extracts

The cytotoxic activity of the ethanolic and water extracts of five species of Hua-Khao-Yen against KKU-M156, HepG2, MCF-7 and T47D cell lines were summarized in **Table 4.2**. This data showed that the water extract of *D. membranacea* exhibited the highest activity against four cancer cell lines with IC_{50} <0.3 µg/mL, while the water extracts of the other species had no cytotoxicity (IC_{50} >100 µg/mL). The ethanolic extract of *D. membranacea* exhibited high potency against breast cancer cell (MCF-7) with IC_{50} = 13.19 ± 2.86 µg/mL and weak activity for T47D, KKU-M156 and HepG2, respectively. For the other species showed weak or no cytotoxic activity, *S. corbularia* showed less activity than *P. herbacea*, while the ethanolic extracts of *D. birmanica* and *S. glabra* showed no activity (IC_{50} > 100 µg/mL). Although, the ethanolic of *D. membranacea* exhibited the cytotoxicity against liver and breast cancer cells in the present study but the previous study of this plant showed the difference results (Itharat *et al*., 2014). By the reason of different results, the rhizome of *D. membranacea* of the previous described was harvested in February as summer season (Itharat *et al*., 2014) and this work was collected this plant in August as rainy season. In the previous work (Itharat *et al*., 2014), active compound dioscorealide B showed high quantity with the percentage of yield more than 5 % but in this work it can produced this compound less than 1% .

Regarding the collection of rhizomes in different seasons, the active compounds of crude ethanolic extract of *D. membranacea* has reduced in rainy season i.e. dioscorealide B (DME2) and dioscoeanone (DME3) when compare with crude ethanolic extract in summer season which were detected by TLC fingerprint (**Figure 4.4**). Therefore, the results support the principle of harvesting in Thai traditional medicine which the rhizome and root part had the highest active ingredients should be collected on summer season but collecting rhizome or root part in the rainy season make less quality or weak activity (Itharat *et al*., 1998).



a **b**

Note: TLC fingerprints (50% EtOAc in hexane) of isolated compounds (1 = DME7, 2 = DME6, 3 = DME1, 4 = DME2, 5 = DME3, 6 = DME4 and 7 = DME5) and crude ethanolic extract of *Dioscorea membranacea* Pierre rhizomes in rainy (**A**) and summer season (**B**) were detected under UV light at 254 nm (**a**) and by heating at 120°C after spraying with acidic anisaldehyde reagent (**b**).

Figure 4.4 TLC fingerprints of isolated compounds (DME1-DME7) and crude ethanolic extract of *Dioscorea membranacea* Pierre rhizomes in rainy and summer season

Table 4.1 The percentage of yield and cytotoxic activity against four types of cancer cell lines of each crude extract (n=3)

Plant species	**extract**	**code**	**% yield**	**Cytotoxic activity (IC_{50} µg/mL ±SEM)**			
				KKU-M156	**HepG2**	**MCF-7**	**T47D**
Dioscorea birmanica Prain &	EtOH	DBE	15.16	>100	>100	>100	>100
Burkill	water	DBW	31.12	>100	>100	>100	>100
Dioscorea membranacea Pierre	EtOH	DME	4.25	30.49±0.82	38.97±2.04	13.19±2.86	25.09±4.90
	water	DMW	24.90	0.001±0.00	0.25±0.16	0.07±0.01	0.03±0.01
Pygmaeopremna herbacea	EtOH	PHE	3.50	38.48±3.36	40.05±0.39	46.62±4.07	51.04±4.72
(Roxb.) Mold	water	PHW	13.92	>100	>100	>100	>100
Smilax corbularia Kunth	EtOH	SCE	15.16	84.53±1.62	40.45±1.46	87.92±3.50	>100
	water	SCW	8.25	>100	>100	>100	>100
Smilax glabra Roxb.	EtOH	SGE	10.65	>100	>100	>100	>100
	water	SGW	34.22	>100	>100	>100	>100

The 50 g of crude ethanolic extract was separated by VLC to obtain five fractions (FE1-FE5). According to the cytotoxic activity against KKU-M156, HepG2, MCF-7 and T47D cell lines by the SRB assay, fraction FE3 or chloroform fraction was selected for the bioassay-guided isolation as it showed highest cytotoxicity against four types of cancer cell lines (HepG2, KKU-M156, MCF-7 and T47D) with the IC_{50} value of 18.30 ± 0.05, 14.31 ± 3.08, 5.62 ± 0.77 and 9.49 ± 1.38 µg/mL, respectively, while FE1, FE2, FE4 and FE5 showed no cytotoxic activity (IC_{50} >50 µg/mL) (**Table 4.2**). The % yield of these fractions as F5 >F4 >F3 >F2 >F1 (**Table 4.2**).Therefore, fraction FE3 should be purified the active constituents for supporting Thai traditional use of this plant for cancer treatment.

Table 4.2 The percentage of yield and cytotoxic activity of VLC fractions from ethanolic extract of *Dioscorea membranacea* (n=3)

fraction	**%yield**	**Cell lines (IC_{50} µg/mL ±SEM)**			
	(w/w)	**KKU-M156**	**HepG2**	**MCF-7**	**T47D**
FE1	0.03	>100	>100	>100	>100
FE2	0.34	96.80±3.20	63.81±4.32	40.49±1.44	66.24±3.31
FE3	11.28	18.30±0.05	14.31±3.08	5.62±0.77	9.49±1.38
FE4	58.54	60.55±1.75	50.15±5.80	35.50±4.02	44.72±5.95
FE5	24.34	>100	77.90±5.03	88.18±3.79	>100

For the crude water extract of *D. membranacea* was also tested the cytotoxicity against four human cancer cell lines (KKU-M156, HepG2, MCF-7 and T47D). The result showed that fraction FW1 (100% water) exhibited the highest cytotoxicity against cholangiocarcinoma cell (KKU-M156) with the IC_{50} = 1.68 ± 0.26 µg/mL followed by breast cancer cells (MCF-7 and T47D) and hepatocellular carcinoma cell (HepG2), successively (**Table 4.3**). Fraction FW5 (100% MeOH) showed specific cytotoxicity against breast cancer cell only one type (MCF-7) with the IC_{50} = 10.04 ± 0.03 µg/mL, but it showed weak activity against T47D, KKU-M156 and HepG2. Fractions FW2-FW4 showed no cytotoxicity aginst these four cell lines (IC_{50} >50 µg/mL). In this result, FW1 specific effect for bile duct cancer or

cholangiocarcima cells (KKU-M156) and FW5 exhibited specific cytotoxic activity for breast cancer cell (MCF-7).

Table 4.3 The percentage of yield and cytotoxic activity of HP-20 diaion fractions of water extract of *Dioscorea membranacea* (n=3)

fraction	**%yield**	**Cell lines (IC_{50} µg/mL ±SEM)**			
	(w/w)	**KKU-M156**	**HepG2**	**MCF-7**	**T47D**
FW1	67.98	1.68±0.26	11.58±5.16	8.86±3.92	12.41±1.19
FW2	16.54	>100	>50	>50	>50
FW3	2.26	>100	>50	>50	>50
FW4	3.59	>100	>50	>50	>50
FW5	1.08	37.56±5.09	35.42±5.07	10.04±0.03	46.53±1.60

4.4 *In vitro* assay for estrogenic and anti-estrogenic activities of all crude extracts and fractions from water extract of *Dioscorea membranacea*

All extracts of five species of Hua-Khoa-Yen and fractions from water extract of *D. membranacea* were tested estrogenic and anti-estrogenic effect using estrogen-responsive breast cancer cell lines MCF-7 and T47D with increasing concentrations ranging from 0.01 µg/mL to 10 µg/mL, and their stimulatory activity was determined by comparing their effects with a positive control, estradiol (E2) at concentrations ranging from 1 to 100 pM. The results revealed that there is no extracts which showed estrogenic activitity against T47D cell through concentration of 0.01-10 µg/mL. The crude extracts SGE and PHW showed no effect at concentration of 0.1-10 µg/mL against E2-enhance T47D cell proliferation when treated with 100 pM of estradiol (E2) by comparison with a positive control at100 pM of E2 (**Table 4.4**).

The crude extracts SCE, SCW and SGW exhibited high anti-estrogenic activity at concentration lower than 0.1 µg/mL that they inhibited cell proliferation enhanced by 100 pM of E2 to equivalent of those induced by 50 pM ($iEqE_{10}$) or 50% suppressive activity. However, they showed weak anti-estrogenic activity.

Table 4.4 Inhibitory activities (μg/mL) of all crude extracts of Hua-Khao-Yen and fractions of water extract of *Dioscorea membranacea* against E2-enhance cell proliferation

Crude extract and fraction	**T47D**		
	$iEqE_{50}$ (μg/mL)[a]	**$iEqE_{10}$(μg/mL)[a]**	**$iEqE_1$ (μg/mL)[a]**
DME	<0.1	<0.1	2.43
PHE	>10	>10	0.10
DBE	>10	6.24	3.19
SCE	<0.1	5.62	7.20
SGE	ND	ND	ND
DMW	<0.01	<0.01	0.17
PHW	ND	ND	ND
DBW	>10	1.67	<0.1
SCW	<0.1	8.31	>10
SGW	<0.1	>10	>10
FW1 (100% water)	<1.0	1.00	1.60
FW2 (25% MeOH)	<1.0	1.00	9.62
FW3 (50% MeOH)	<1.0	1.00	5.34
FW4 (75% MeOH)	<1.0	1.00	>10
FW5 (100% MeOH)	>10	1.00	<1.0
Tamoxifen[b]	0.1 μM	0.8 μM	9 μM

[a] $iEqE_{50}$, $iEqE_{10}$, and $iEqE_1$ represent the concentrations of the crude extracts/fractions (μg/mL) that inhibited cell proliferation enhanced by 100 pM of E2 to equivalent levels of those induced by 50 pM, 10 pM, and 1 pM E2 treatment, respectively. These values were determined by linear regression analysis using three different concentrations. Mild inhibition (M), more than 50% inhibition through the concentrations tested. Strong inhibition (S), more than 90% inhibition through the concentrations tested.

[b] positive control

ND = not determined

The crude extracts PHE, DBE and DBW showed 99% suppressive activity at concentration of 0.1, 3.19 and <0.1 μg/mL, respectively against E2-induced T47D cell proliferation. The crude ethanolic extract (DME) showed strong activity at concentration lower than 0.1 μg/mL that inhibited cell proliferation enhanced by 100 pM of E2 to equivalent of those induced by 10 pM ($iEqE_{10}$) or 90% suppressive activity. Crude water extract (DMW) was recognized the anti-estrogenic activity at 100 times less concentration, in which DMW showed 90% suppression ($iEqE_{10}$) at concentration of lower than 0.01 μg/mL and showed 99% suppression ($iEqE_1$) at concentration of 0.17 μg/mL against E2-induced T47D cell proliferation.

An order of potency of these crude extracts were DBE, DME, PHE, DBW, DMW > SCE, SCW, SGW > SGE, PHW (no anti-estrogenic activity), respectively.

All fraction displayed anti-estrogenic activity, in which fraction FW5 (100% MeOH) showed 99% suppressive activity at concentration lower than 1.0 μg/mL against E2-induced T47D cell proliferation. For other fractions showed 90% suppressive activity at concentration of 1.0 μg/mL.

According to the anti-estrogenic activity against E2-enhance cell proliferation, crude ethanolic and water extracts of *D. membranacea* were selected to study its active constituents.

4.5 Structure elucidation of compounds from ethanolic extract of *Dioscorea membranacea*

The fraction FE3 from crude ethanolic extract of *Dioscorea membranacea* was purified by column chromatography to give seven compounds (DME1-DME7). Their structures were elucidated by 1D (^1H, ^{13}C and ^1H-^1H COSY) and 2D (HMQC and HMBC) spectroscopic data. The bioassay-guided fractionation of the rhizome of ethanolic extract of *D. membranacea* led to the isolation of two napthofuranoxepins [dioscorealide A (DME1) and dioscorealide B (DME2)], phenanthraquinone [dioscoreanone (DME3)], two phenanthrenes [5,6-dihydroxy-2,4-dimethoxy-9,10-dihydrophenanthrene (DME4) and 2,5,6-trihydroxy-3,4-dimethoxy, 9, 10-

dihydrophenanthrene (DME5)] and two steroids [panthogenin B (DME6) and epipanthogenin B (DME7)] (**Figure 4.16**). Compounds DME1-DME5 gave identical spectra to authentic sample which were given from Dr. Arunporn Itharat (Itharat *et al*., 2004; Itharat *et al*., 2014)

4.5.1 Compound DME1

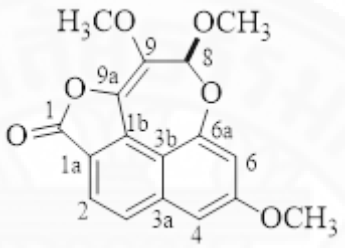


Figure 4.5 Chemical structure of DME1

Compound DME1 was obtained as a white solid. It gave a green acidic anisaldehyde test. The ^{1}H NMR data of DME1 (**Table 4.5**, **Figure 4.5**) displayed four aromatic protons in which two *ortho*-coupled protons at δ 7.75 and 7.69 (each 1H, d, J = 8.5, H-2 and H-3, respectively) and two *meta*-coupled aromatic protons at δ 7.05 and 6.96 (each 1H, d, J = 2.0, H-4 and H-6, respectively) including three methoxy groups at δ 4.27 (3H, s, 9-OMe), 3.96 (3H, s, 5-OMe), 3.58 (3H, s, 8-OMe) and a oxymethine proton at δ 5.59 (1H, s, H-8). The ^{1}H NMR data of DME1 was corresponded to previous reported data of dioscorealide A which gave from Arunporn Itharat (Itharat *et al*., 2003). Thus, DME1 was identified as dioscorealide A.

Table 4.5 Comparison of ^{1}H NMR spectral data between DME1 and dioscorealide A

position	δ_H (ppm)	
	DME1 (in $CDCl_3$)	**dioscorealide A (in $CDCl_3$)**
2	7.75 (d, 8.5)	7.72 (d, 8.5)
3	7.69 (d, 8.5)	7.66 (d, 8.5)
4	7.05 (d, 2.0)	7.03 (d, 2.0)
6	6.96 (d, 2.0)	6.94 (d, 2.0)
8	5.59 (s)	5.57 (s)
5-OCH_3	3.96 (s)	3.96 (s)
8-OCH_3	3.58 (s)	3.56 (s)
9-OCH_3	4.27 (s)	4.24 (s)

4.5.2 Compound DME2

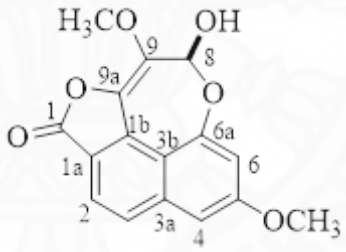


Figure 4.6 Chemical structure of DME2

Compound DME2 was obtained as a pale yellow solid. It gave green acidic anisaldehyde test. By comparison of ^{1}H NMR spectral data of compound DME2 (**Table 4.6, Figure 4.6**) and DME1 revealed close structural similarity. The difference was shown the hydroxyl group located at C-8 instead of the methoxyl group. Therefore, DME2 was established as dioscorealide B by comparison with the previously reported data (Itharat *et al*., 2003).

Table 4.6 Comparison of ^{1}H NMR spectral data between DME2 and dioscorealide B

position	δ_H (ppm)	
	DME2 (in $CDCl_3$+CD_3OD)	**dioscorealide B (in DMSO-d_6)**
2	7.68 (d, 8.5)	7.76 (d, 8.5)
3	7.74 (d, 8.5)	7.88 (d, 8.5)
4	7.05 (d, 2.0)	7.36 (d, 2.5)
6	6.91 (d, 2.0)	6.88 (d, 2.5)
8	5.58 (s)	6.03 (s)
5-OMe	3.97 (s)	3.96 (s)
9-OMe	4.28 (s)	4.13 (s)

4.5.3 Compound DME3

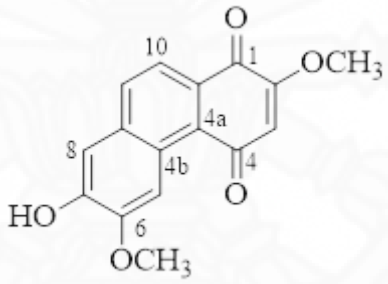


Figure 4.7 Chemical structure of DME3

Compound DME3 was obtained as a yellow solid. It gave yellow acidic anisaldehyde test. The ^{1}H NMR spectral data of DME3 (**Table 4.7**, **Figure 4.7**) was corresponded to dioscoreanone which was reported previously (Itharat *et al*., 2003).

Table 4.7 Comparison of ^{1}H NMR spectral data between DME3 and dioscoreanone

position	δ_H (ppm)	
	DME3 (in $CDCl_3$)	**dioscoreanone (in $CDCl_3$+CD_3OD**
3	6.09 (s)	6.11 (s)
5	9.16 (s)	9.13 (s)
8	7.24 (s)	7.24 (s)
9	7.93 (d, 8.5)	7.92 (d, 8.5)
10	8.08 (d, 8.5)	8.06 (d, 8.5)
2-OCH_3	3.91 (s)	3.93 (s)
6- OCH_3	4.13 (s)	4.12(s)

4.5.4 Compound DME4

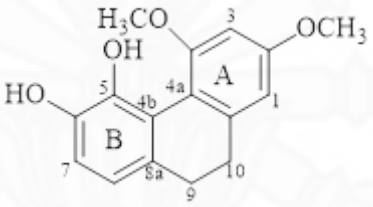


Figure 4.8 Chemical structure of DME4

Compound DME4 was obtained as a white solid. Its HREIMS *m/z* $[M]^+$ 272.1043 (calcd for $C_{16}H_{16}O_4$, 272.1049). Its IR spectrum showed an absorption band at 3468 (hydroxyl) cm^{-1}, and UV absorption bands at λ_{max} 216, 275 and 297 nm supporting the presence of 9,10-dihydrophenanthrene chromophore (Majumder *et al*., 2001).

The ^{13}C NMR and DEPT spectral data of DME4 (**Table 4.8**, **Figure 4.8**) indicated the presence of 16 carbons as two methylenes ($2\times CH_2$), twelve aromatic ($4\times CH$, $4\times C$ and $4\times C$-O) and two methoxy carbons ($2\times OCH_3$). The ^{1}H NMR spectral data (**Table 4.7**) displayed the presence of *ortho*-coupled protons in ring-B at δ 6.84 and 6.77 (each 1H, d, J = 8.0 Hz, H-7 and H-8, respectively) and *meta*-coupled protons in ring-A at δ 6.64 and 6.57 (each 1H, d, J = 2.5, H-1 and H-2). The

appearance of proton signals at δ 3.87 (3H, s) and 3.98 (3H, s) were assigned to 2-OMe and 4-OMe, respectively. Additionally, two benzylic methylene protons at δ 2.64 (2H, m, H-9) and 2.70 (2H, m, H-10) indicated that the signal pattern of DME4 similar to those of 9, 10-dihydrophenanthrene in the literature (Majumder *et al*., 2001). Two proton signals at δ 8.26 (s) and 6.02 (s) suggested the presence of hydroxy groups at 5-OH and 6-OH, respectively. In the HMBC experiment (**Figure 4.9**) showed that the methylene protons at δ 2.70 (2H-10) correlated with carbons at δ 143.7 (C-10a), 130.9 (C-8a), 115.5 (C-4a), 107.4 (C-1) and 30.0 (C-9), another methylene protons at δ 2.64 (2H-9) correlated with the carbons at δ 143.7 (C-10a), 130.9 (C-8a), 119.6 (C-8), and 31.9 (C-10), the aromatic proton at δ 6.64 (H-1) correlated with the carbons at δ 159.6 (C-2), 115.5 (C-4a), 98.8 (C-3) and 31.9 (C-10), and the aromatic proton at δ 6.77 (H-8) correlated with the carbons at δ 145.7 (C-6), 130.9 (C-8a), 120.4 (C-4b), 115.5 (C-4a) and 30.0 (C-9). From these data, compound DME4 was identified as 5,6-dihydroxy-2,4 dimethoxy-9,10-dihydrophenanthrene, which has been reported from *Dioscorea prazeri* (Rajaraman & Rangaswami, 1975) and also reported from *Dioscorea membranacea* by Itharat *et al*., 2014.

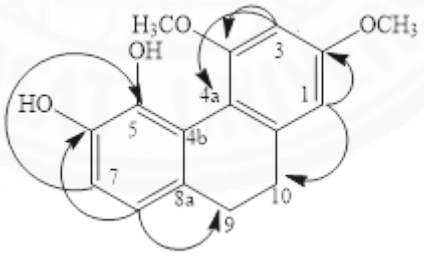


Figure 4.9 Selected HMBC correlations of DME4

4.5.5 Compound DME5

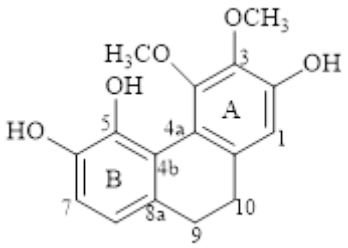


Figure 4.10 Chemical structure of DME5

Compound DME5 was obtained as a yellow solid. Its HREIMS *m/z* $[M]^+$ 288.1030 (calcd for $C_{16}H_{16}O_5$, 288.0998). Its IR spectrum showed an absorption band at 3375 (hydroxyl) cm^{-1}, and UV absorption bands at λ_{max} 216, 275 and 297 nm supporting the presence of 9,10-dihydrophenanthrene chromophore (Majumder *et al*., 2001).

The ^1H and ^{13}C NMR spectral data of DME5 (**Table 4.8**, **Figure 4.10**) were similar to those of DME4 (**Table 4.8**), except aromatic proton in ring-A instead of one singlet signal of aromatic proton at δ 6.79 (1H, s, H-1). The appearance of proton signals at δ 4.01 (3H, s) and 3.78 (3H, s) were assigned to 3-OMe and 4-OMe, respectively. Three proton signals at δ 8.88 (s), 6.08 (s) and 5.83 (d, J = 2.0 Hz) suggested the presence of hydroxy groups at 5-OH, 6-OH and 2-OH, respectively. The location of methoxyl and hydroxy groups in ring-A were confirmed by HMBC and NOE experiments. The locations of two methoxy groups were confirmed by the NOE analysis (**Figure 4.11**), in which the results of strong enhancements of 3-OMe and 5-OH upon irradiation 4-OMe and of 3-OMe and H-1 upon irradiation of 2-OH revealed that the methoxy groups located at C-4 and C-3. From these data, DME5 was identified as 2, 5, 6-trihydroxy-3, 4-dimethoxy, 9, 10- dihydrophenanthrene, which has been described in the literature (Itharat *et al*., 2014).

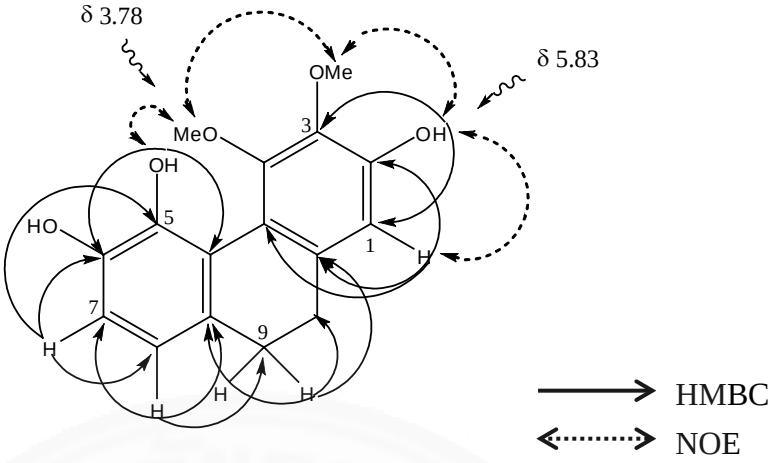


Figure 4.11 Selected HMBC and NOE experiments of DME5

Table 4.8 ^{1}H (500 MHz), ^{13}C (125 MHz) NMR and HMBC spectral data of DME4 and DME5 in $CDCl_3$

position	DME4			DME5		
	δ_C	δ_H (*J* in Hz)	HMBC ($\delta_H \rightarrow \delta_C$)	δ_C	δ_H (*J* in Hz)	HMBC ($\delta_H \rightarrow \delta_C$)
1	107.4	6.64 (d, 2.5)	C-2, C-3, C-4a, C-10	111.9	6.79 (s)	C-2, C-3, C-4a, C-10
2	159.6			148.3		
3	98.8	6.57 (d, 2.5)	C-1, C-2, C-4, C-4a	138.5		
4	154.9			147.8		
4a	115.5			118.8		
4b	120.4			119.9		
5	140.2			140.0		
6	145.7			145.7		
7	112.1	6.84 (d, 8.0)	C-5, C-6, C-8a	112.4	6.87 (d, 8.0)	C-5, C-6, C-8, C-8a
8	119.6	6.77 (d, 8.0)	C-4a, C-4b, C-6, C-8a, C-9	119.8	6.77 (d, 8.0)	C-4a, C-6, C-9
8a	130.9			130.8		
9	30.0	2.64 (m)	C-8, C-8a, C-10, C-10a	30.2	2.66 (m)	C-8, C-8a, C-10, C-10a
10	31.9	2.70 (m)	C-1, C-4a, C-8a, C-9, C-10a	31.0	2.63 (m)	C-1, C-4a, C-8a, C-9, C-10a
10a	143.7			137.5		
2-OH					5.83 (s)	C-1, C-2, C-3
5-OH		8.26 (s)	C-4b, C-5, C-6		8.88 (s)	C-4b, C-5, C-6
6-OH		6.02 (s)	C-5, C-6, C-7		6.08 (s)	C-5, C-6, C-7
2-OMe	55.5	3.87 (s)	C-2			
3-OMe				61.7	4.01 (s)	C-3
4-OMe	57.5	3.98 (s)	C-4	62.4	3.78 (s)	C-4

4.5.6 Compound DME6

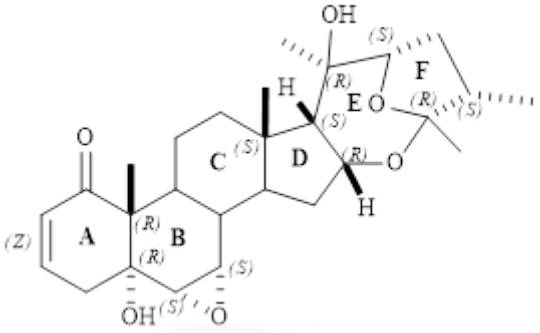


Figure 4.12 Chemical structure of DME6

Compound DME6 was obtained as a white amorphous solid. Its HR-ESI-TOF-MS showed the $[M+Na]^+$ ion at *m/z* 481.2561, corresponding to the molecular formula $C_{27}H_{38}O_6Na$. The IR spectrum showed the absorption bands at ν_{max} 3432 and 1687 cm^{-1} suggesting the presence of hydroxyl group and α, β-unsaturated ketone, respectively. It gave a green acidic anisaldehyde reagent test indicating a steroid.

The ^{13}C NMR and DEPT spectra suggested the presence of 27 carbons as five methyls, five methylenes, eleven methines and six quaternary carbons (**Table 4.9**, **Figure 4.12**). The ^1H NMR spectra showed characteristic of 27-norergostane type steroid (Shan *et al*., 2014) as four singlet methyl signals at δ_H 0.99 (H-18), 1.14 (H-21), 1.17 (H-19) and 1.32 (H-26) and a doublet methyl at δ_H 1.03 (1H, d, *J* = 7.0 Hz, H-27). The appearance of α, β-unsaturated ketone moiety at δ_H 5.84 (1H, dd, *J* = 10.0, 2.5 Hz, H-2) and 6.59 (1H, ddd, *J* = 10.0, 5.0, 2.5 Hz, H-3) and one epoxy group locating at δ_H 3.04 (1H, d, *J* = 4.0 Hz, H-6) and 3.27 (1H, brd, *J* = 4.0 Hz, H-7). On the basis of heteronuclear multiple bond correlation (HMBC), the carbonyl group was located at C-1 by long-range correlations of both H-3 (δ_H 6.59) and H-19 (δ_H 1.17) with C-1 (δ_C 203.0), the methylene proton at δ_H 2.70 and 2.52 (2H-4) showed correlations with C-2 (δ_C 129.1), C-3 (δ_C 139.5), C-5 (δ_C 73.3) and C-10 (δ_C 51.0), indicating the presence of hydroxyl group at C-5 and double bond between C-2 and C-3. From these data and ^1H-^1H COSY correlation, compound DME6 showed the presence of A-, B-, C-, and D- rings unit similar to those of (20*S*,22*R*,24*S*,25*R*)-5*R*,24*R*-dihydroxy-6*R*,7*R*-epoxy-1- oxowitha-2-en-26,22-olide isolated from *Dioscorea japonica* (Kim *et al*., 2011), except for the appearance of seven- and five-

membered ring in E and F unit, respectively. The connectivity of D-, E- and F-rings were confirmed by HMBC correlation (**Figure 4.13**), in which the methine proton at δ_H 4.50 (H-16) showed correlations with C-14 (δ_C 49.7), C-15 (δ_C 30.7), C-20 (δ_C 74.2) and C-25 (δ_C 106.4) suggesting the presence of oxygen atom between C-17 and C-25 and the methine proton at δ_H 3.87 (H-22) showed correlations with C-20 (δ_C 74.2), C-23 (δ_C 33.4), C-24 (δ_C 44.7) and C-25 (δ_C 106.4) indicated that the five-membered ring attached to E-ring at C-22 and C-25. These data confirmed the structure of DME6 as a 27-norergostane. The relative stereochemistry of DME6 was supported by difference nuclear Overhauser effect spectroscopy (NOESY), in which Me-18 showed cross peak with H-16, H-11b, H-12a and 20-OH were in β-orientation, Me-19 showed correlation with H-4 and H-8 were in β-orientation, Me-21 showed cross peak with H-17 and H-22 were in the same direction and Me-26 showed cross peak with H-16 and H-24, indicating the Me-26 were in β-orientation. From these data, compound DME6 was identified as panthogenin B (Shan *et al*., 2014) (**Table 4.10**).

Table 4.9 ^{1}H (400 MHz), ^{13}C (100 MHz) NMR and HMBC spectral data of DME6 in $CDCl_3$

position	DME6		
	δ_C	δ_H (*J* in Hz)	HMBC ($\delta_H \rightarrow \delta_C$)
1	203.0		
2	129.1	5.84 (dd, 10.0, 2.5)	C-4, C-10
3	139.5	6.59 (ddd, 10.0, 5.0, 2.5)	C-1, C-5
4	36.7	2.70 (m), 2.52 (dd, 18.5, 5.0)	C-2, C-3, C-5, C-10
5	73.3		
6	56.1	3.04 (d, 4.0)	C-4, C-5, C-10
7	57.1	3.27 (brd, 4.0)	C-8, C-9
8	34.6	1.76 (m)	C-14
9	35.7	1.63 (m)	
10	51.0		
11	21.2	2.74 (m), 1.36 (m)[a]	C-12
12	39.7	1.99 (m), 1.46 (m)	C-9, C-11, C-14
13	44.7		
14	49.7	1.76 (m)	
15	30.7	1.92 (m), 1.75 (m)	C-14
16	70.0	4.50 (td, 8.5, 3.5)	C-14, C-15, C-20, C-25
17	62.1	1.41 (d, 8.5)	C-12, C-13, C-16, C-18
18	15.1	0.99 (s)	C-12, C-13, C-14, C-17
19	14.8	1.17 (s)	C-1, C-5, C-9, C-10
20	74.2		
21	22.2	1.14 (s)	C-17, C-20, C-22
22	88.8	3.87 (dd, 9.0, 6.0)	C-17, C-20, C-21, C-23, C-24, C-25
23	33.4	2.16 (m), 1.26 (m)	C-20, C-22, C-24, C-25
24	44.7	1.98 (m)	C-25
25	106.4		
26	22.3	1.32 (s)	C-24, C-25
27	13.1	1.03 (d, 7.0)	C-23, C-24, C-25
5-OH		3.17 (brd, 1.0)	C-4, C-5, C-6
20-OH		2.52 (brs)	C-21

[a] Deduced from ^{1}H-^{1}H COSY experiment

Table 4.10 Comparison of ^{1}H and ^{13}C NMR data between DME6 (recorded in $CDCl_3$) and panthogenin B (recorded in pyridine-d_5)

Position	DME6		panthogenin B	
	δ_C (100 MHz)	δ_H (*J* in Hz, 400 MHz)	δ_C (150 MHz)	δ_H (*J* in Hz, 600 MHz)
1	203.0		204.8	
2	129.1	5.84 (dd, 10.0, 2.5)	130.1	6.03 (dd, 10.0, 2.4)
3	139.5	6.59 (ddd, 10.0, 5.0, 2.5)	141.5	6.56 (m)
4	36.7	2.70 (m), 2.52 (dd, 18.5, 5.0)	38.7	2.66 (d, 19.0), 2.55 (dd, 19.0, 5.0)
5	73.3		74.9	
6	56.1	3.04 (d, 4.0)	56.9	3.09 (d, 3.2)
7	57.1	3.27 (brd, 4.0)	57.2	3.23 (d, 3.2)
8	34.6	1.76 (m)	36.1	1.77 (m)
9	35.7	1.63 (m)	37.1	2.12 (m)
10	51.0		52.5	
11	21.2	2.74 (m), 1.36 (m)[a]	22.6	3.06 (d, 3.8), 1.44 (d, 2.8)
12	39.7	1.99 (m), 1.46 (m)	41.2	2.00 (m), 1.30 (m)
13	44.7		45.9	
14	49.7	1.76 (m)	51.3	1.77 (m)
15	30.7	1.92 (m), 1.75 (m)	32.1	2.12 (m), 1.87 (m)
16	70.0	4.50 (td, 8.5, 3.5)	71.6	5.03 (m)
17	62.1	1.41 (d, 8.5)	63.9	1.42 (d, 8.0)
18	15.1	0.99 (s)	16.4	1.25 (s)
19	14.8	1.17 (s)	15.7	1.20 (s)
20	74.2		74.9	
21	22.2	1.14 (s)	24.3	1.23 (s)
22	88.8	3.87 (dd, 9.0, 6.0)	90.6	4.13 (dd, 9.0, 6.2)
23	33.4	2.16 (m), 1.26 (m)	35.0	2.12 (m), 1.30 (m)
24	44.7	1.98 (m)	45.9	2.00 (m)
25	106.4		107.3	
26	22.3	1.32 (s)	23.9	1.46 (s)
27	13.1	1.03 (d, 7.0)	14.6	1.11 (d, 6.8)
5-OH		3.17 (brd, 1.0)		
20-OH		2.52 (brs)		

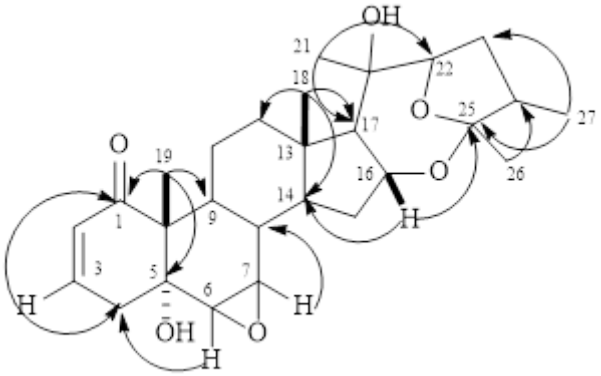


Figure 4.13 Selected HMBC correlations of DME6

4.5.7 Compound DME7

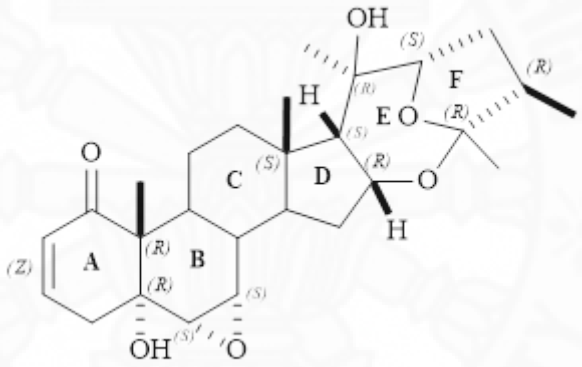


Figure 4.14 Chemical structure of DME7

Compound DME7 was isolated as a white amorphous powder. Its HR-ESI-TOF-MS showed the $[M+Na]^+$ ion at *m/z* 481.2561, corresponding to the molecular formula $C_{27}H_{38}O_6Na$. The IR spectrum (ν_{max} 1686 cm^{-1}) and UV absorption (λ_{max} 224 nm) suggesting the presence of α,β-unsaturated ketone. It gave a green acidic anisaldehyde reagent test indicating a steroid.

The ^1H and ^{13}C NMR spectroscopic features of the compound DME7 (**Figure 4.14**) were closely related with DME6 which has been reported in *Dioscorea panthica* (Shan *et al*., 2014) (**Table 4.11** and **4.12**), except for some signals consisting F-ring were slightly shifted. The coupling constant of H-22 (δ_H 3.98 (dd, *J* = 9.0, 6.0)], an oxymethine attaching the E/F ring juncture, showed the same value with that

of DME6 [δ_H 3.87 (dd, *J* = 9.0, 6.0, H-22)], and Me-26 attaching another E/F ring juncture commonly showed NOESY correlation with H-16 [δ_H 4.50 (td, *J* = 8.5, 3.5)] in these compounds. Moreover, Me-27 [δ_H 0.93 (d, *J* = 7.0)] displayed NOE correlations with Me-26 [δ_H 1.31 (3H, s)] and H-22 [δ_H 3.98 (dd, *J* = 9.0, 6.0)] in their NOESY spectra of DME7, while Me-27 [δ_H 1.03 (d, *J* = 7.0)] did not exhibit any NOE correlation in the NOESY spectra of DME6 (**Figure 4.15**). According to their 1D and 2D NMR spectral data, DME7 (epipanthogenin B) was identified as (5*R*, 6*S*, 7*S*, 10*R*, 13*S*, 16*R*, 17*S*, 22*S*, 24*R*, 25*R*) 6, 7:16, 25:22, 25-triepoxy-5, 20-dihydroxyergost-2-en-1-one, an epimer at C-24 of panthogenin B (Thongdeeying *et al*., 2016).

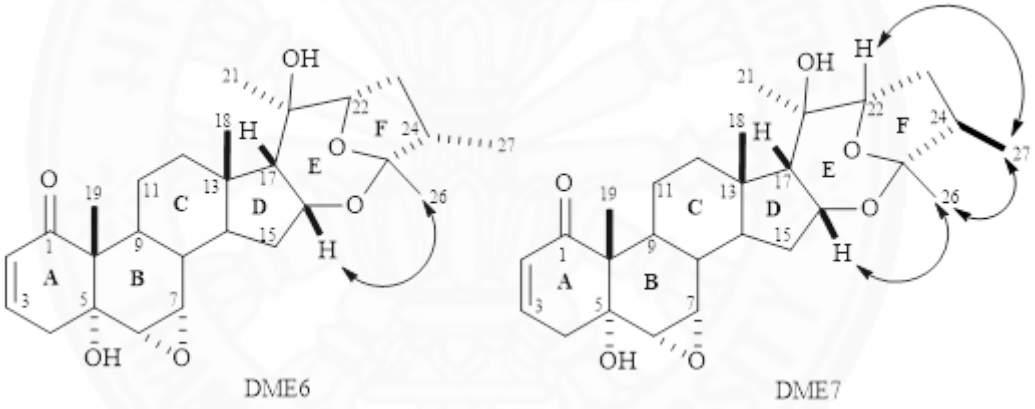


Figure 4.15 Selected NOESY correlations of DME6 and DME7

Table 4.11 ^{1}H (400 MHz), ^{13}C (100 MHz) NMR and HMBC spectral data of DME7 in $CDCl_3$

position	DME7		
	δ_C	δ_H (*J* in Hz)	HMBC ($\delta_H \rightarrow \delta_C$)
1	203.3		
2	129.3	5.84 (dd, 10.0, 2.5)	C-4, C-10
3	139.8	6.58 (ddd, 10.0, 5.0. 2.5)	C-1, C-4, C-10
4	37.0	2.67 (m), 2.51 (dd, 18.5, 5.0)	C-2, C-3
5	73.5		
6	56.3	3.02 (d, 4.0)	C-4, C-5, C-7, C-10
7	57.3	3.26 (brd, 4.0)	C-6, C-8, C-9
8	34.8	1.75 (m)	
9	35.9	1.63 (m)	C-8
10	51.2		
11	21.4	2.73 (m), 1.36 (m)	C-12
12	39.8	2.00 (m), 1.43 (m)	C-9
13	44.8		
14	50.0	1.73 (m)	C-8, C-15
15	31.0	1.87 (m), 1.79 (m)	
16	70.2	4.50 (td, 8.5, 3.5)	C-14, C-15, C-20, C-25
17	62.2	1.45 (d, 8.5)	C-12, C-13, C-16, C-18
18	15.4	0.99 (s)	C-12, C-13, C-14, C-17
19	15.0	1.17 (s)	C-1, C-5, C-9, C-10
20	74.4		
21	22.5	1.15 (s)	C-17, C-20, C-22
22	89.3	3.98 (dd, 9.0, 6.0)	C-17, C-20, C-21, C-23, C-24, C-25
23	33.9	1.98 (m), 1.63 (m)	C-22, C-25, C-27
24	44.7	2.20 (quint,7.0)	C-22, C-25, C-27
25	109.3		
26	20.6	1.31 (s)	C-24, C-25
27	18.3	0.93 (d, 7.0)	C-23, C-24, C-25
5-OH		3.16 (brd, 1.0)	C-4, C-5, C-6
20-OH		2.50 (brs)	C-21, C-22

Table 4.12 Comparison of ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectral data between DME6 and DME7 in $CDCl_3$

position	DME6		DME7	
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	203.0		203.3	
2	129.1	5.84 (dd, 10.0, 2.5)	129.3	5.84 (dd, 10.0, 2.5)
3	139.5	6.59 (ddd, 10.0, 5.0, 2.5)	139.8	6.58 (ddd, 10.0, 5.0. 2.5)
4	36.7	2.70 (m), 2.52 (dd, 18.5, 5.0)	37.0	2.67 (m), 2.51 (dd, 18.5, 5.0)
5	73.3		73.5	
6	56.1	3.04 (d, 4.0)	56.3	3.02 (d, 4.0)
7	57.1	3.27 (brd, 4.0)	57.3	3.26 (brd, 4.0)
8	34.6	1.76 (m)	34.8	1.75 (m)
9	35.7	1.63 (m)	35.9	1.63 (m)
10	51.0		51.2	
11	21.2	2.74 (m), 1.36 (m)[a]	21.4	2.73 (m), 1.36 (m)
12	39.7	1.99 (m), 1.46 (m)	39.8	2.00 (m), 1.43 (m)
13	44.7		44.8	
14	49.7	1.76 (m)	50.0	1.73 (m)
15	30.7	1.92 (m), 1.75 (m)	31.0	1.87 (m), 1.79 (m)
16	70.0	4.50 (td, 8.5, 3.5)	70.2	4.50 (td, 8.5, 3.5)
17	62.1	1.41 (d, 8.5)	62.2	1.45 (d, 8.5)
18	15.1	0.99 (s)	15.4	0.99 (s)
19	14.8	1.17 (s)	15.0	1.17 (s)
20	74.2		74.4	
21	22.2	1.14 (s)	22.5	1.15 (s)
22	88.8	3.87 (dd, 9.0, 6.0)	89.3	3.98 (dd, 9.0, 6.0)
23	33.4	2.16 (m), 1.26 (m)	33.9	1.98 (m), 1.63 (m)
24	44.7	1.98 (m)	44.7	2.20 (quint,7.0)
25	106.4		109.3	
26	22.3	1.32 (s)	20.6	1.31 (s)
27	13.1	1.03 (d, 7.0)	18.3	0.93 (d, 7.0)
5-OH		3.17 (brd, 1.0)		3.16 (brd, 1.0)
20-OH		2.52 (brs)		2.50 (brs)

[a] Deduced from ^{1}H-^{1}H COSY experiment

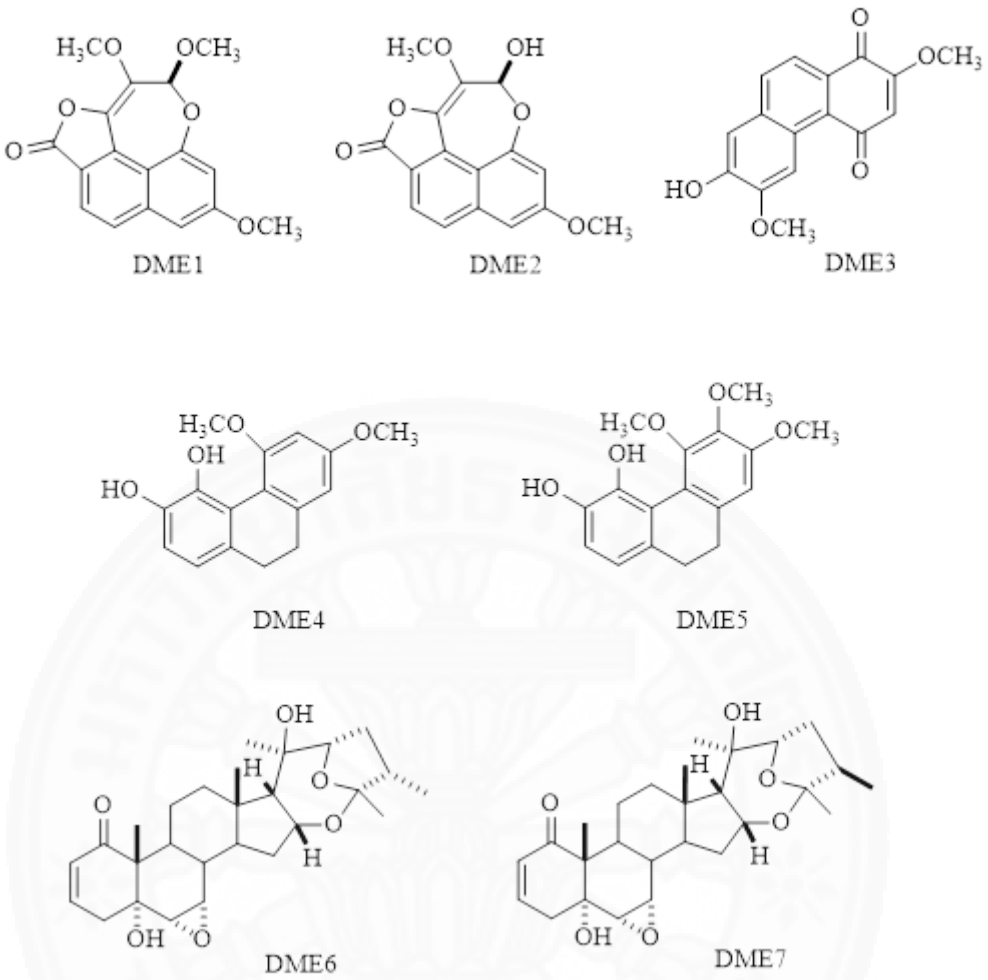


Figure 4.16 The structures of DME1-DME7 isolated from ethanolic extract of the rhizomes of *Dioscorea membranacea*

4.6 Structure elucidation of compounds from water extract of *Dioscorea membranacea*

The crude water extract of *Dioscorea membranacea* was purified by HPLC technique to give six steroid saponin compounds (DMW1-DMW6) (**Figure 4.27**) and seven compounds which were the same compounds isolated from ethanolic extract of *D. membranacea* (DME1-DME7) (**Figure 4.16**). Their structures were elucidated by 1D (^{1}H, ^{13}C and ^{1}H-^{1}H COSY) and 2D (HMQC and HMBC) spectroscopic data.

4.6.1 Compound DMW1

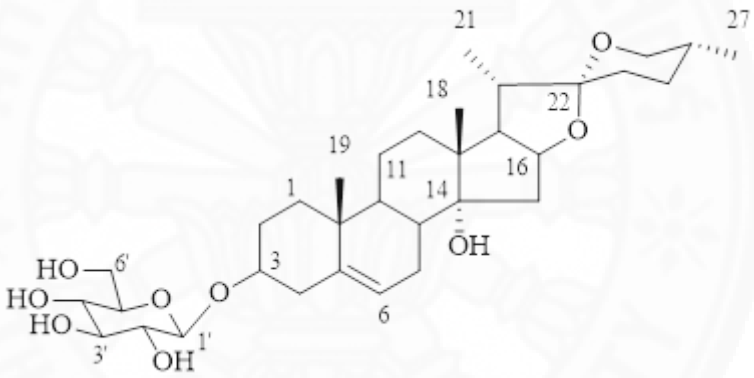


Figure 4.17 Chemical structure of DMW1

Compound DMW1 was obtained as a white solid. Its ESI TOF MS showed the $[M+Na]^{+}$ ion peak at *m/z* 615.3504, corresponding to the molecular formula $C_{33}H_{52}O_9Na$. From the molecular formula and ^{13}C NMR spectral data of DMW1 suggested the presence of a glucopyranosyl moiety.

The ^{1}H and ^{13}C NMR spectral data of compound DMW1 (**Table 4.13**, **Figure 4.17**) showed characteristic of spirostane steroid whereas two singlet methyls appeared at δ 1.08 (s, H-18)/20.3 and 1.00 (s, H-19)/19.6, two doublet methyls at δ 1.18 (d, J = 7.0 Hz, H-21)/15.6 and 0.68 (d, J = 5.0 Hz, H-27)/17.6, an olefinic proton at δ 5.32 (brd, J = 5.0, H-6)/122.3 and hydroxy group at δ 4.95 (s) which was located at C-14 (δ 82.2) in the steroid unit. For the sugar unit showed an anomeric proton of

glucopyranosyl at δ 5.01 (d, J = 7.5, H-1')/102.8 and oxymethylene proton AB system of H-6' were shown at δ 4.53 (dd, J = 12.0, 2.0) and 4.38 (dd, J = 12.0, 5.0). The linkage of sugar moiety and steroid unit was determined through heteronuclear multiple-bond correlations (HMBC) experiment in which anomeric proton at δ 5.01 showed correlation with C-3 (δ 102.8) (**Figure 4.18**). By comparison of the ^{1}H and ^{13}C NMR data with the previously reported data (Zhang *et al*., 2014), compound DMW1 was identified as 25 (*R*)-spirost-5-en-3β,14α-diol-3-*O*-β-D-glucopyranoside or polygodoside E (**Table 4.14**).

Table 4.13 ^{1}H (400 MHz), ^{13}C (100 MHz) NMR and HMBC spectral data of DMW1 in pyridine-d_5

position	δ_C	δ_H	**HMBC ($\delta_H \rightarrow \delta_C$)**
1	38.0	1.76(m), 1.03 (m)	C-10
2	30.6	2.10 (m), 1.75 (m)	C-1
3	78.4	3.91 (m)	
4	39.7	2.72 (m), 2.48 (m)	C-5, C-6
5	140.8		
6	122.3	5.39 (brd, 4.5)	C-10
7	27.0	2.48 (m), 1.86 (m)	C-5, C-6
8	35.9	2.04 (m)	C-9
9	43.9	1.82 (m)	
10	37.7		
11	20.7	1.60 (m), 1.56 (m) overlap	
12	32.3	2.25 (m), 1.46 (m)	C-11
13	45.4		
14	86.7		
15	40.2	2.32 (dd, 13.0, 7.5), 1.86 (dd, 13.0, 7.5)	C-13, C-14, C-16, C-17
16	82.2	5.07 (q, 7.5)	C-13

Table 4.13 (Continued)

position	δ_C	δ_H	HMBC ($\delta_H \rightarrow \delta_C$)
17	60.2	2.78 (t, 7.5)	C-12, C-13, C-16, C-18, C-20, C-21
18	20.3	1.08 (s)	C-12, C-13, C-14, C-17
19	19.6	1.00 (s)	C-5, C-9, C-10
20	42.4	2.09 (m)	C-13, C-17, C-21, C-22
21	15.6	1.18 (d, 7.0)	C-17, C-20, C-22
22	109.9		
23	32.3	1.72 (m), 1.46 (m)	C-24
24	29.6	1.60 (m), 1.58 (m) overlap	C-25
25	30.9	1.59 (m)	
26	67.1	3.51 (m)	C-24, C-25
27	17.6	0.68 (d, 5.0)	C-24, C-25, C-26
OH		4.95 (s)	C-13, C-14, C-15
Glucose			
1'	102.8	5.01 (d, 7.5)	C-3
2'	75.7	4.03 (m)	C-1', C-3'
3'	78.9	4.27(m)	C-4'
4'	72.1	4.24 (m)	C-3'
5'	78.7	3.94 (m)	
6'	63.2	4.53 (dd, 12.0, 2.0), 4.38 (dd, 12.0, 5.0)	

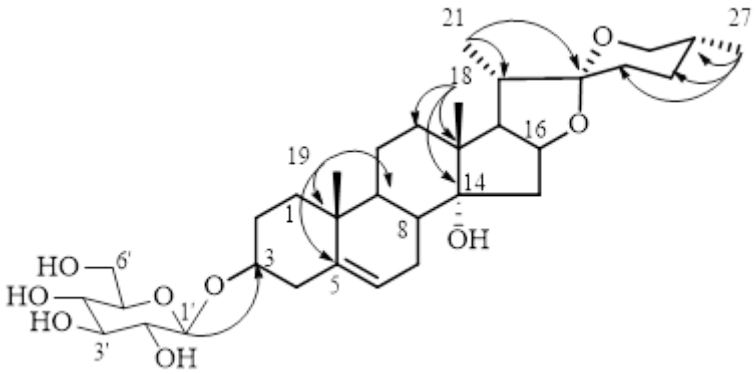


Figure 4.18 Selected HMBC correlations of DMW1

Table 4.14 Comparison of ^{13}C NMR spectral data of DMW1 and polygodoside E (recorded in pyridine-d_5)

position	δ_C (ppm)	
	DMW1	**polygodoside E**
1	38.0	37.7
2	30.6	30.3
3	78.4	78.0
4	39.7	39.9
5	140.8	140.5
6	122.3	122.2
7	27.0	26.3
8	35.9	35.6
9	43.9	43.6
10	37.7	37.4
11	20.7	20.4
12	32.3	32.0
13	45.4	45.0
14	86.7	86.4
15	40.2	39.4

Table 4.14 (Continued)

position	δ_C (ppm)	
	DMW1	**polygodoside E**
16	82.2	82.0
17	60.2	59.8
18	20.3	20.0
19	19.6	19.3
20	42.4	42.6
21	15.6	15.2
22	109.9	109.6
23	32.3	Not determined
24	29.6	29.4
25	30.9	30.6
26	67.1	66.9
27	17.6	17.3
OH		
Glucose		
1'	102.8	103.1
2'	75.7	72.7
3'	78.9	75.4
4'	72.1	70.3
5'	78.7	76.9
6'	63.2	62.6

4.6.2 Compound DMW2

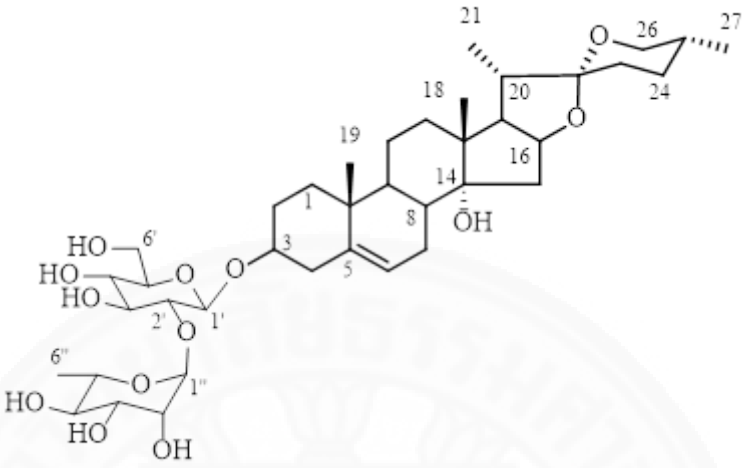


Figure 4.19 Chemical structure of DMW2

Compound DMW2 was obtained as a white powder. Its HR ESI TOF MS showed $[M+Na]^+$ ion peak at *m/z* 761.4083, corresponding to the molecular formula $C_{39}H_{62}O_{13}Na$.

The ^1H and ^{13}C spectral data of DMW2 (**Table 4.15**, **Figure 4.19**) and DMW1 (**Table 4.13**) exhibited the same pattern, except for the ramnopyranosyl moiety located at C-2" in which anomeric proton at δ 6.33 (brs) showed correlation with δ 78.0 (C-2'), 72.9 (C-3") and 69.5 (C-5") by HMBC experiment (**Table 4.16**, and **4.17**, **Figure 4.20**). Thus on the basis of 1D and 2D spectroscopic data and comparison with previous reported data (Zheng *et al*., 2004), compound DMW2 was determined as 25 (*R*)-spirost-5-en-3β, 14α-diol -3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-β-D-glucopyranoside or 25 (*R*,*S*)-dracaenoside F (**Table 4.17**).

Table 4.15 ^{1}H (400 MHz), ^{13}C (100 MHz) NMR and HMBC spectral data of DMW2 in pyridine-d_5

position	δ_C	δ_H	HMBC ($\delta_H \rightarrow \delta_C$)
1	37.8	1.81 (m), 1.02 (m)	C-3
2	30.3	2.10 (m), 1.90 (m)	C-1, C-3
3	78.0	3.91 (m)	C-1'
4	39.1	2.80 (m), 2.77 (m) overlap	C-2, C-3, C-5, C-6, C-10
5	140.5		
6	122.4	5.40 (brd, 5.0)	C-4, C-7, C-8, C-10
7	26.8	2.50 (m), 1.89 (m)	C-5, C-6, C-8, C-9, C-14
8	35.7	2.08 (m)	C-7, C-9, C-10
9	43.7	1.81 (m)	C-1, C-8, C-10, C-19
10	37.6		
11	20.4	1.60 (m), 1.40 (m)	C-8, C-9, C-12, C-13
12	32.0	2.24 (m), 1.44 (m)	C-9, C-11, C-13
13	45.2		
14	86.5		
15	40.0	2.34 (dd, 13.0, 7.5), 1.90 (dd, 13.0, 7.5)	C-13, C-14, C-16, C-17
16	81.9	5.07 (q, 7.5)	C-13
17	60.0	2.79 (t, 7.5)	C-12, C-13, C-16, C-18, C-20, C-21
18	20.0	1.07 (s)	C-12, C-13, C-14, C-17
19	19.4	1.13 (s)	C-5, C-9, C-10
20	42.1	2.10 (m)	C-13, C-17, C-21, C-22, C-23
21	15.3	1.18 (d, 7.0)	C-17, C-20, C-22
22	109.6		
23	32.0	1.72 (m), 142 (m)	C-24

Table 4.15 (Continued)

position	δ_C	δ_H	HMBC ($\delta_H \rightarrow \delta_C$)
24	29.4	1.60 (m), 1.58 (m) overlap	C-22, C-23, C-26
25	30.7	1.60 (m)	C-23, C-26
26	66.9	3.53 (m)	C-22, C-24, C-25
27	17.4	0.67 (d, 5.0)	C-24, C-25, C-26
14-OH		4.95 (s)	C-13, C-14, C-15
Glucose			
1'	100.4	5.00 (d, 7.0)	C-3, C-2", C-3"
2'	78.0	4.24 (m)	
3'	79.7	4.24 (m)	
4'	71.9	4.13 (m)	C-3'
5'	78.3	3.86 (m)	
6'	62.8	4.47 (dd, 12.5, 2.5), 4.32 (m)	
Rhamnose			
1"	102.1	6.33 (brs)	C-2', C-3", C-5"
2"	72.6	4.77 (m)	C-3", C-4"
3"	72.9	4.60 (dd, 9.0, 3.0)	C-4"
4"	74.3	4.32 (t, 9.0)	C-2", C-5"
5"	69.5	4.97 (m)	
6"	18.7	1.76 (d, 6.5)	C-4", C-5"

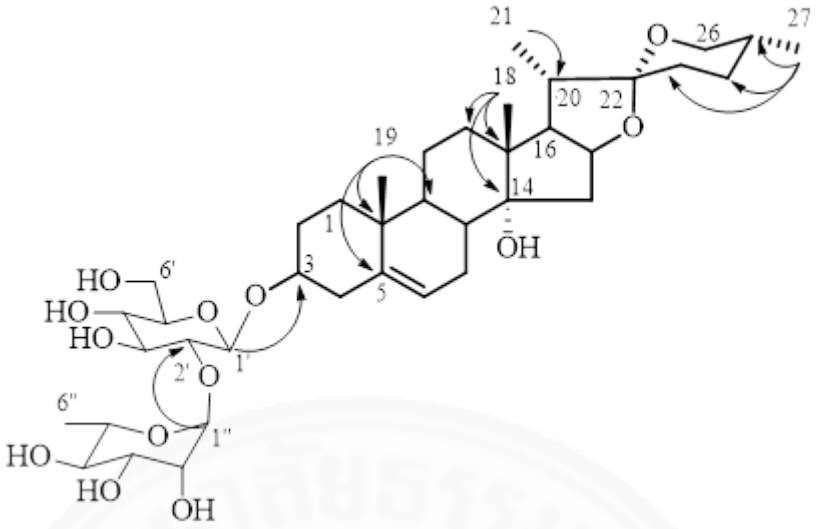


Figure 4.20 Selected HMBC correlations of DMW2

Table 4.16 Comparison of ^{1}H NMR spectral data between DMW1 and DMW2 (recorded in pyridine-d_5)

position	δ_H (ppm)	
	DMW1	**DMW2**
1	1.76(m), 1.03 (m)	1.81 (m), 1.02 (m)
2	2.10 (m), 1.75 (m)	2.10 (m), 1.90 (m)
3	3.91 (m)	3.91 (m)
4	2.72 (m), 2.48 (m)	2.80 (m), 2.77 (m) overlap
5		
6	5.39 (brd, 4.5)	5.40 (brd, 5.0)
7	2.48 (m), 1.86 (m)	2.50 (m), 1.89 (m)
8	2.04 (m)	2.08 (m)
9	1.82 (m)	1.81 (m)
10		
11	1.60 (m), 1.56 (m) overlap	1.60 (m), 1.40 (m)
12	2.25 (m), 1.46 (m)	2.24 (m), 1.44 (m)
13		
14		
15	2.32 (dd, 13.0, 7.5), 1.86 (dd, 13.0, 7.5)	2.34 (dd, 13.0, 7.5), 1.90 (dd, 13.0, 7.5)
16	5.07 (q, 7.5)	5.07 (q, 7.5)
17	2.78 (t, 7.5)	2.79 (t, 7.5)
18	1.08 (s)	1.07 (s)
19	1.00 (s)	1.13 (s)
20	2.09 (m)	2.10 (m)
21	1.18 (d, 7.0)	1.18 (d, 7.0)
22		
23	1.72 (m), 1.46 (m)	1.72 (m), 142 (m)
24	1.60 (m), 1.58 (m) overlap	1.60 (m), 1.58 (m) overlap

Table 4.16 (Continued)

position	δ_H (ppm)	
	DMW1	**DMW2**
25	1.59 (m)	1.60 (m)
26	3.51 (m)	3.53 (m)
27	0.68 (d, 5.0)	0.67 (d, 5.0)
14-OH	4.95 (s)	4.95 (s)
Glucose		
1'	5.01 (d, 7.5)	5.00 (d, 7.0)
2'	4.03 (m)	4.24 (m)
3'	4.27(m)	4.24 (m)
4'	4.24 (m)	4.13 (m)
5'	3.94 (m)	3.86 (m)
6'	4.53 (dd, 12.0, 2.0), 4.38 (dd, 12.0, 5.0)	4.47 (dd, 12.5, 2.5), 4.32 (m)
Rhamnose		
1"		6.33 (brs)
2"		4.77 (m)
3"		4.60 (dd, 9.0, 3.0)
4"		4.32 (t, 9.0)
5"		4.97 (m)
6"		1.76 (d, 6.5)

Table 4.17 Comparison of ^{13}C NMR spectral data between DMW1, DMW2 and 25(*R*, *S*)-dracaenoside F (recorded in pyridine-d_5)

position	δ_C (ppm)		
	DMW1	**DMW2**	**25 (*R*, *S*)-dracaenoside F**
1	38.0	37.8	37.5
2	30.6	30.3	30.1
3	78.4	78.0	78.3
4	39.7	39.1	39.1
5	140.8	140.5	140.4
6	122.3	122.4	122.5
7	27.0	26.8	26.8
8	35.9	35.7	35.7
9	43.9	43.7	43.7
10	37.7	37.6	37.8
11	20.7	20.4	20.5
12	32.3	32.0	32.0
13	45.4	45.2	45.3
14	86.7	86.5	86.6
15	40.2	40.0	40.0
16	82.2	81.9	82.1
17	60.2	60.0	60.0
18	20.3	20.0	20.1
19	19.6	19.4	19.5

Table 4.17 (Continued)

position	δ_C (ppm)		
	DMW1	**DMW2**	**(25*R*,*S*)-dracaenoside F**
20	42.4	42.1	42.6
21	15.6	15.3	15.5
22	109.9	109.6	109.7
23	32.3	32.0	32.1
24	29.6	29.4	29.4
25	30.9	30.7	30.7
26	67.1	66.9	66.9
27	17.6	17.4	17.4
14-OH Glucose			
1'	102.8	100.4	100.4
2'	75.7	78.0	78.3
3'	78.9	79.7	79.7
4'	72.1	71.9	71.9
5'	78.7	78.3	78.6
6'	63.2	62.8	62.8
Rhamnose			
1"		102.1	102.2
2"		72.6	72.7
3"		72.9	72.9
4"		74.3	74.2
5"		69.5	69.6
6"		18.7	18.8

4.6.3 Compound DMW3

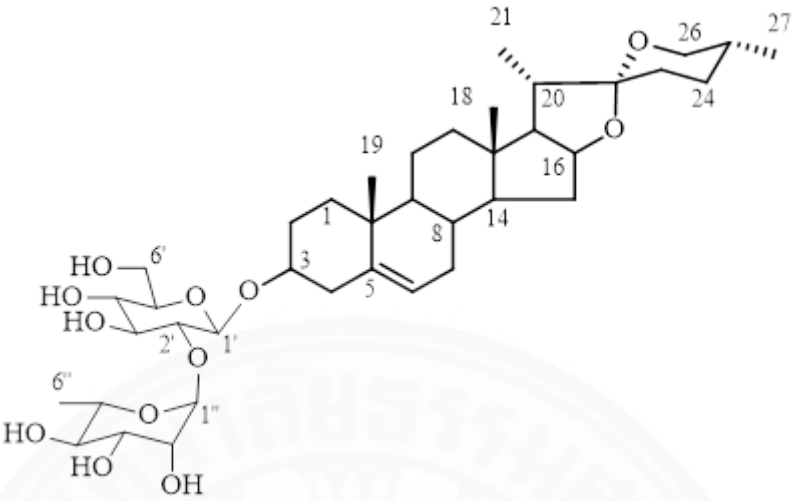


Figure 4.21 Chemical structure of DMW3

Compound DMW3 was obtained as a white solid. The molecular formula was assigned to be $C_{39}H_{62}O_{12}Na$ by ESITOFMS $[M+Na]^+$ at *m/z* 745.41. From the molecular formula and ^{13}C NMR spectral data of DMW3 suggested the presence of two glucopyranosyl moieties.

The ^{1}H and ^{13}C NMR of compound DMW3 (**Table 4.18**, **Figure 4.21**) were closely related to compound DMW2 (**Table 4.15**, **Figure 4.19**), except the hydroxyl group disappeared and the methyl proton (H-18) was shifted upfield to δ 0.86 (s) as compared to that of DMW2 at δ 1.07 (s). By comparison of the ^{1}H and ^{13}C NMR data with the previously reported data of (Ju & Jia, 1992), DMW3 was identified as diosgenin-3-*O*-α-L-rhamnosyl (1→2)-β-D-glucopyranoside or prosapogenin A of dioscin (**Table 4.18**, **Table 4.19**).

Table 4.18 Comparison of ^{1}H NMR spectral data between DME3 and prosapogenin A of dioscin (recorded in pyridine-d_5)

position	**δ_H (*J* in Hz)**	
	DMW3	**prosapogenin A of dioscin**
6	5.32 (brd, 5.0)	5.31 (m)
18	0.83 (s)	0.83 (s)
19	1.06 (s)	1.05 (s)
21	1.13 (d, 7.0)	1.13 (d, 7.0)
27	0.69 (d, 6.5)	0.68 (d, 5.0)

Table 4.19 Comparison of ^{13}C NMR spectral data between DMW2, DMW3 and prosapogenin A of dioscin (recorded in pyridine-d_5)

position		**δ_C (ppm)**	
	DMW2	**DMW3**	**prosapogenin A of dioscin**
1	37.8	37.6	37.6
2	30.3	30.3	30.2
3	78.0	78.1	78.2
4	39.1	39.1	39.1
5	140.5	141.0	141.0
6	122.4	121.8	121.8
7	26.8	32.4	32.4
8	35.7	31.8	31.8
9	43.7	50.4	50.5
10	37.6	37.2	37.2
11	20.4	21.2	21.2
12	32.0	39.9	39.3
13	45.2	40.5	40.0
14	86.5	56.7	56.8
15	40.0	32.3	32.3
16	81.9	81.2	81.6

Table 4.19 (Continued)

Position	δ_C (ppm)		
	DMW2	**DMW3**	**prosapogenin A of dioscin**
17	60.0	63.0	63.1
18	20.0	16.4	16.4
19	19.4	19.5	19.5
20	42.1	42.0	42.1
21	15.3	15.0	15.0
22	109.6	109.3	109.3
23	32.0	31.9	31.9
24	29.4	29.3	29.4
25	30.7	30.6	30.7
26	66.9	67.0	67.0
27	17.4	17.3	17.3
Glucose			
1'	100.4	100.5	100.5
2'	78.0	77.9	77.9
3'	79.7	79.7	79.7
4'	71.9	72.0	71.9
5'	78.3	78.3	78.3
6'	62.8	62.8	62.8
Rhamnose			
1"	102.1	102.1	102.1
2"	72.6	72.6	72.6
3"	72.9	72.9	72.9
4"	74.3	74.2	74.2
5"	69.5	69.5	69.5
6"	18.7	18.7	18.7

4.6.4 Compound DMW4

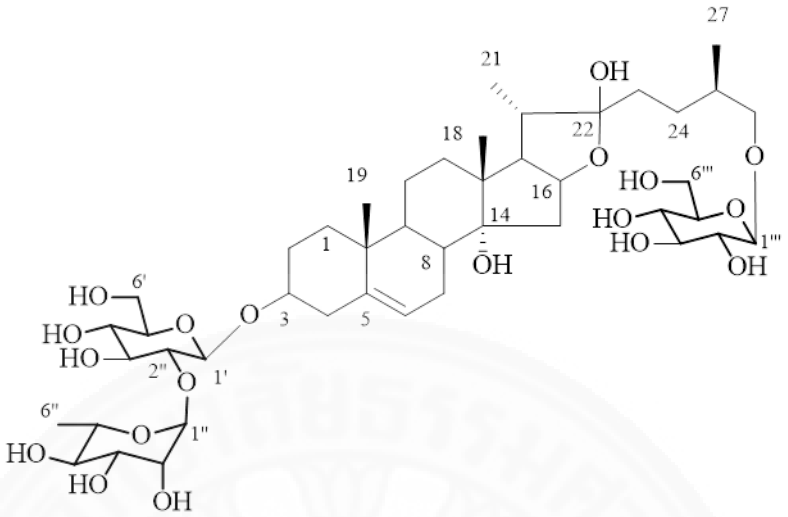


Figure 4.22 Chemical structure of DMW4

Compound DMW4 was obtained as a white powder. The molecular formula was assigned to be $C_{45}H_{74}O_{18}Na$ from the HRESITOFMS (*m/z* 941.4717 $[M+Na]^+$). From the molecular formula and ^{13}C NMR spectral data of compound DMW4 suggested the presence of two glucopyranosyl and one rhamnopyranosyl moieties.

The ^1H and ^{13}C NMR spectral data of DMW4 (**Table 4.20** and **4.21**, **Figure 4.22**) showed characteristic of furostane saponin as four methyl protons at δ 1.01 (d, *J* = 6.0, H-27), 1.12 (s, H-18), 1.14 (s, H-19) and 1.35 (d, *J* = 7.0 Hz, H-21) together with one methyl proton of rhamnopyranosyl at δ 1.76 (d, *J* = 6.0 Hz, H-6''). A vinylic proton at δ 5.40 (m) was located at C-6 (δ 122.4) by correlation with δ 26.8 (C-7) and 37.5 (C-10) in the HMBC experiment (**Figure 4.23**). The hydroxyl group was attached at C-14 (δ 86.4) from correlation of H-18 (δ 20.1) with C-12 (δ 32.0), C-13 (δ 45.5), C-14 (δ 86.4) and C-17 (δ 60.7). In addition, the location of glucopyranosyl at C-26 was confirmed from correlation of anomeric proton of H-1''' (δ 4.86) with C-26 (δ 75.3), C-2''' (δ 75.2) and C-3''' (δ 78.6). Compound DMW4 was identified as 26-*O*-β-D-glucopyranosyl- 25(*R*)-furost-5-en-3β,14α,22, 26-tetrol -3-O-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside or 25 (*R*,*S*)-dracaenoside N by comparison of its spectral data with the previous literature (Zheng *et al*., 2004).

Table 4.20 ^{1}H (400 MHz), ^{13}C (100 MHz) NMR and HMBC spectral data of DMW4 in pyridine-d_5

position	δ_C	δ_H	HMBC ($\delta_H \rightarrow \delta_C$)
1	37.8	1.80 (m), 1.05 (m)	C-3, C-10
2	30.3	2.12 (m), 1.90 (m)	
3	78.0	4.20 (m)	C-1'
4	39.1	2.81 (m), 2.75 (m)	C-2, C-3, C-5, C-6
5	140.5		
6	122.4	5.40 (m)	C-7, C-10
7	26.8	2.48 (m), 1.84 (m)	C-5, C-6
8	35.7	2.07 (m)	
9	43.7	1.80 (m)	C-5, C-8, C-10, C-19
10	37.5		
11	20.4	1.58 (m), 1.60 (m)	
12	32.0	2.29 (m), 1.49 (m)	
13	45.5		
14	86.4		
15	40.1	2.36 (dd, 13.0, 7.5), 1.90 (13.0, 5.5)	C-13, C-14, C-16, C-17
16	81.8	5.42 (m)	C-13
17	60.7	2.89 (brt, 7.0)	C-12, C-13, C-16, C-18, C-20
18	20.1	1.12 (s)	C-12, C-13, C-14, C-17
19	19.4	1.14 (s)	C-1, C-5, C-9, C-10
20	40.9	2.36 (m)	
21	16.7	1.35 (d, 7.0)	C-17, C-20, C-22
22	111.0		
23	37.3	2.07 (m), 2.00 (m)	C-20, C-22, C-24, C-25

Table 4.20 (Continued)

position	δ_C	δ_H	HMBC ($\delta_H \rightarrow \delta_C$)
24	28.4	2.07 (m), 1.73 (m)	
25	34.3	1.94 (m)	
26	75.3	3.93 (dd, 9.0, 7.0), 3.64 (dd, 9.0, 6.5)	C-24, C-25, C-27, C-1'''
27	17.5	1.01 (d, 6.0)	C-24, C-25, C-26
Glucose			
1'	100.4	5.00 (d, 7.0)	C-2', C-3
2'	77.9	4.24 (m)	
3'	79.7	4.24 (m)	
4'	71.9	4.12 (t, 7.0)	
5'	78.2	3.86 (m)	
6'	62.9	4.47 (brd, 11.0), 4.34 (m)	
Rhamnose			
1''	102.0	6.33 (brs)	C-2', C-3'', C-5''
2''	72.6	4.76 (m)	
3''	72.9	4.60 (dd, 9.0, 3.0)	
4''	74.2	4.32 (m)	
5''	69.5	4.97 (dd, 9.0,7.0)	
6''	18.7	1.76 (d, 6.0)	C-4'', C-5''
Glucose			
1'''	104.96	4.86 (brd, 7.0)[a]	C-26, C-2''', C-3'''
2'''	75.2	4.00 (t, 7.0)	
3'''	78.6	4.20 (m)	
4'''	71.8	4.20 (m)	
5'''	78.5	3.91 (m)	
6'''	62.8	4.52 (brd, 12.0) 4.34 (m)	

[a] It is interfered by solvent peak

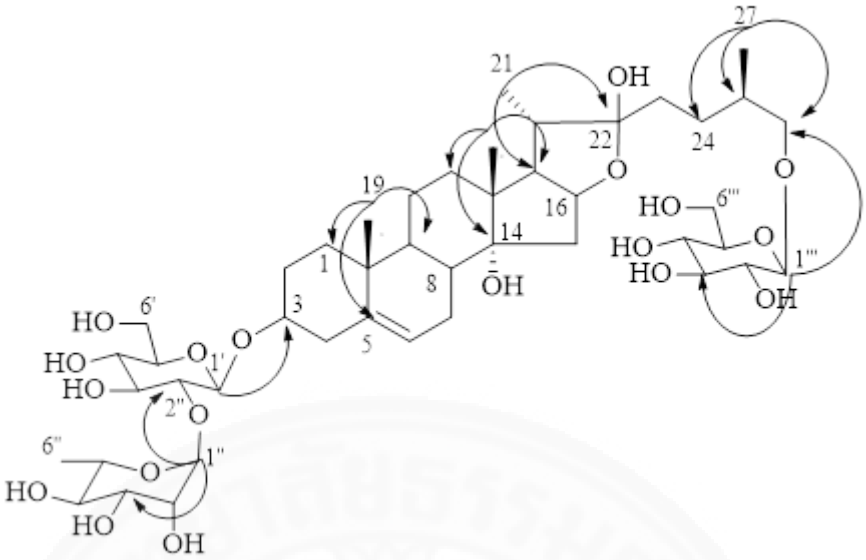


Figure 4.23 Selected HMBC correlations of DMW4

Table 4.21 Comparison of ^{13}C NMR spectral data between DMW4 and 25 (*R*,*S*)-dracaenoside N (recorded in pyridine-d_5)

position	δ_C (ppm)	
	DMW4	**25(*R*,*S*)-dracaenoside N**
1	37.8	37.9
2	30.3	30.2
3	78.0	78.3
4	39.1	40.0
5	140.5	140.3
6	122.4	122.4
7	26.8	26.7
8	35.7	35.5
9	43.7	43.7
10	37.5	37.9
11	20.4	20.4
12	32.0	31.8
13	45.5	45.2
14	86.4	86.2
15	40.1	39.0
16	81.8	82.2
17	60.7	60.1
18	20.1	18.7
19	19.4	19.4
20	40.9	40.1
21	16.7	16.7
22	111.0	110.3
23	37.3	31.0

Table 4.21 (Continued)

position	δ_C (ppm)	
	DMW4	**25(*R*,*S*)-dracaenoside N**
24	28.4	27.7
25	34.3	34.3
26	75.3	75.3
27	17.5	17.6
Glucose		
1'	100.4	100.3
2'	77.9	78.3
3'	79.7	79.7
4'	71.9	71.8
5'	78.2	78.7
6'	62.9	61.3
Rhamnose		
1"	102.0	102.2
2"	72.6	72.8
3"	72.9	72.9
4"	74.2	74.2
5"	69.5	69.6
6"	18.7	18.7
Glucose		
1"'	104.96	105.1
2"'	75.2	75.3
3"'	78.6	78.7
4"'	71.8	71.8
5"'	78.5	78.6
6"'	62.8	62.9

4.6.5 Compound DMW5

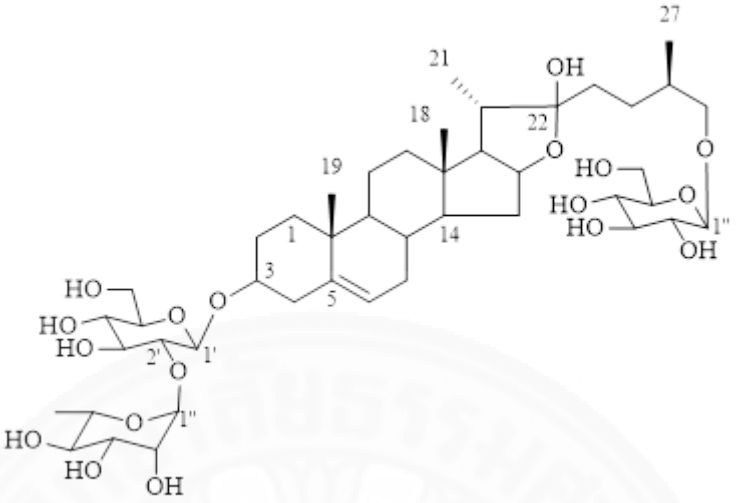


Figure 4.24 Chemical structure of DMW5

Compound DMW5 was obtained as a white powder. Its molecular formula showed $C_{45}H_{74}O_{18}Na$ from HRESITOFMS (*m/z* 925.4767 $[M+Na]^+$).

The ^1H and ^{13} C NMR spectral data of compound DMW5 (**Table 4.22**, **Figure 4.24**) were similar to those of DMW4 (**Table 4.20**), except for the hydroxy group at C-14 was replaced by methine proton at δ 1.08 (m, H-14). This structure was confirmed by HMBC experiment (**Figure 4.25**), in which methyl proton H-14 (δ 0.89) showed long-range correlation with C-12 (δ 39.1), C-13 (δ 40.9), C-14 (δ 56.7) and C-17 (δ 63.9). Thus on the basis of 1D and 2D spectral data by comparison with the previously reported data and DMW4, DMW5 was identified as 26-*O*-β-D-glucopyranosyl-22-hydroxyfurost-5-ene-3β,26-diol 3-*O*-α-L-rhamnopyranosyl (1 → 2)-β-D-glucopyranoside (Watanabe *et al*., 1983; Agrawal *et al*., 1985) (**Table 4.23** and **4.24**).

Table 4.22 ^{1}H (400 MHz), ^{13}C (100 MHz) NMR and HMBC spectral data of DMW5 in pyridine-d_5

position	δ_C	δ_H	HMBC ($\delta_H \rightarrow \delta_C$)
1	37.6	1.73 (m), 0.95 (m)	
2	30.3	2.13 (m), 1.88 (m)	
3	78.1	4.24 (m)	C-1'
4	40.0	1.73 (m), 1.13 (m)	
5	140.96		
6	121.8	5.30 (brd, 5.0)	C-8, C-10
7	32.4	1.88 (m), 1.43 (m)	
8	31.8	1.88 (m)	
9	50.4	0.89 (m)	
10	37.2		
11	21.2	1.45 (m), 1.40 (m)	
12	39.1	2.79 (m), 2.73 (m)	
13	40.9		
14	56.7	1.08 (m)	
15	32.5	2.04 (m), 1.43 (m)	
16	81.2	4.94 (m)	C-13, C-20
17	63.9	1.93 (dd, 8.5, 6.5)	C-12, C-13, C-16, C-18, C-20, C-21
18	16.5	0.89 (s)	C-12, C-13, C-14, C-17
19	19.4	1.06 (s)	C-1, C-5, C-9, C-10
20	40.7	2.36 (quint, 6.0)	
21	16.4	1.32 (d, 7.0)	C-17, C-20, C-22
22	110.7		
23	37.2	2.03 (m), 2.00 (m)	C-20, C-22, C-24, C-25
24	28.4	2.07 (m), 1.73 (m)	
25	34.3	1.93 (m)	

Table 4.22 (Continued)

position	δ_C	δ_H	HMBC ($\delta_H \rightarrow \delta_C$)
26	75.3	3.93 (dd, 10.0, 7.0), 3.61 (dd, 10.0, 6.0)	C-24, C-25, C-27, C-1'''
27	17.5	0.99 (d, 6.0)	C-24, C-25, C-26
Glucose			
1'	100.5	5.02 (d, 7.5)	C-2', C-3
2'	77.9	4.26 (m)	C-1'
3'	79.7	4.26 (m)	
4'	71.96	4.14 (t, 7.5)	
5'	78.25	3.88 (m)	
6'	62.9	4.48 (d, 12.0, 2.5), 4.35 (d, 12.0, 6.0)	
Rhamnose			
1''	102.0	6.35 (brd, 1.0)	C-2', C-3'', C-5''
2''	72.6	4.76 (m)	
3''	72.9	4.60 (dd, 9.0, 3.5)	
4''	74.2	4.31 (t, 9.0)	
5''	69.5	4.97 (m)	
6''	18.7	1.76 (d, 6.5)	C-4'', C-5''
Glucose			
1'''	104.97	4.80 (d, 7.5)	C-26
2'''	75.2	3.99 (t, 7.5)	
3'''	78.7	4.20 (m)	
4'''	71.8	4.20 (m)	
5'''	78.5	3.91 (m)	
6'''	62.8	4.53 (d 12.0, 2.5), 4.32 (d, 12.0, 6.0)	

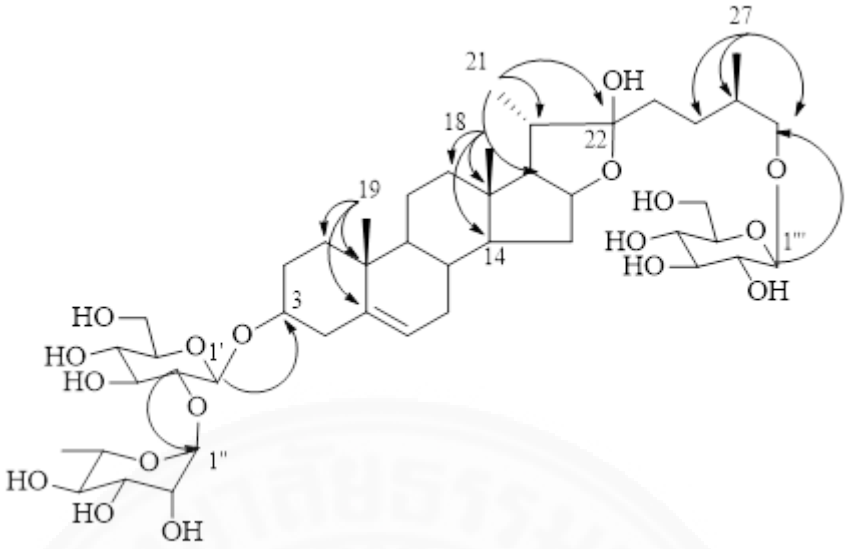


Figure 4.25 Selected HMBC correlations of DMW5

Table 4.23 Comparison of ^{1}H NMR and HMBC spectral data between DMW4 and DMW5 (recorded in pyridine-d_5)

position	δ_H (ppm)	
	DMW4	**DMW5**
1	1.80 (m), 1.05 (m)	1.73 (m), 0.95 (m)
2	2.12 (m), 1.90 (m)	2.13 (m), 1.88 (m)
3	4.20 (m)	4.24 (m)
4	2.81 (m), 2.75 (m)	1.73 (m), 1.13 (m)
5		
6	5.40 (m)	5.30 (brd, 5.0)
7	2.48 (m), 1.84 (m)	1.88 (m), 1.43 (m)
8	2.07 (m)	1.88 (m)
9	1.80 (m)	0.89 (m)
10		
11	1.58 (m), 1.60 (m)	1.45 (m), 1.40 (m)
12	2.29 (m), 1.49 (m)	2.79 (m), 2.73 (m)
13		
14		1.08 (m)
15	2.36 (dd, 13.0, 7.5), 1.90 (13.0, 5.5)	2.04 (m), 1.43 (m)
16	5.42 (m)	4.94 (m)
17	2.89 (brt, 7.0)	1.93 (dd, 8.5, 6.5)
18	1.12 (s)	0.89 (s)
19	1.14 (s)	1.06 (s)
20	2.36 (m)	2.36 (quint, 6.0)
21	1.35 (d, 7.0)	1.32 (d, 7.0)
22		
23	2.07 (m), 2.00 (m)	2.03 (m), 2.00 (m)
24	2.07 (m), 1.73 (m)	2.07 (m), 1.73 (m)
25	1.94 (m)	1.93 (m)

Table 4.23 (Continued)

position	δ_H (ppm)	
	DMW4	**DMW5**
26	3.93 (dd, 9.0, 7.0), 3.64 (dd, 9.0, 6.5)	3.93 (dd, 10.0, 7.0), 3.61 (dd, 10.0, 6.0)
27	1.01 (d, 6.0)	0.99 (d, 6.0)
Glucose		
1'	5.00 (d, 7.0)	5.02 (d, 7.5)
2'	4.24 (m)	4.26 (m)
3'	4.24 (m)	4.26 (m)
4'	4.12 (t, 7.0)	4.14 (t, 7.5)
5'	3.86 (m)	3.88 (m)
6'	4.47 (brd, 11.0), 4.34 (m)	4.48 (d, 12.0, 2.5), 4.35 (d, 12.0, 6.0)
Rhamnose		
1"	6.33 (brs)	6.35 (brd, 1.0)
2"	4.76 (m)	4.76 (m)
3"	4.60 (dd, 9.0, 3.0)	4.60 (dd, 9.0, 3.5)
4"	4.32 (m)	4.31 (t, 9.0)
5"	4.97 (dd, 9.0,7.0)	4.97 (m)
6"	1.76 (d, 6.0)	1.76 (d, 6.5)
Glucose		
1"'	4.86 (brd, 7.0)[a]	4.80 (d, 7.5)
2"'	4.00 (t, 7.0)	3.99 (t, 7.5)
3"'	4.20 (m)	4.20 (m)
4"'	4.20 (m)	4.20 (m)
5"'	3.91 (m)	3.91 (m)
6"'	4.52 (brd, 12.0) 4.34 (m)	4.53 (d 12.0, 2.5), 4.32 (d, 12.0, 6.0)

Table 4.24 Comparison of ^{13}C NMR and HMBC spectral data between DMW4, DMW5 and 26-*O*-β-D-glucopyranosyl-22-hydroxyfurost-5-ene-3β,26-diol 3-*O*-α-L-rhamnopyranosyl (1 → 2)-β-D-glucopyranoside (Agrawal *et al*., 1985) (recorded in pyridine-d_5)

position	**δ_C (ppm)**		
	DMW4	**DMW5**	**Agrawal *et al*., 1985**
1	37.8	37.6	
2	30.3	30.3	
3	78.0	78.1	
4	39.1	40.0	
5	140.5	140.96	
6	122.4	121.8	
7	26.8	32.4	
8	35.7	31.8	
9	43.7	50.4	
10	37.5	37.2	
11	20.4	21.2	
12	32.0	39.1	
13	45.5	40.9	
14	86.4	56.7	
15	40.1	32.5	
16	81.8	81.2	
17	60.7	63.9	
18	20.1	16.5	
19	19.4	19.4	
20	40.9	40.7	
21	16.7	16.4	
22	111.0	110.7	
23	37.3	37.2	
24	28.4	28.4	
25	34.3	34.3	

Table 4.24 (Continued)

position	δ_C (ppm)		
	DMW4	**DMW5**	**Agrawal *et al*., 1985**
26	75.3	75.3	
27	17.5	17.5	
Glucose			
1'	100.4	100.5	100.5
2'	77.9	77.9	79.5
3'	79.7	79.7	78.1
4'	71.9	71.96	72.1
5'	78.2	78.25	77.9
6'	62.9	62.9	62.9
Rhamnose			
1"	102.0	102.0	101.8
2"	72.6	72.6	72.4
3"	72.9	72.9	72.8
4"	74.2	74.2	74.2
5"	69.5	69.5	69.3
6"	18.7	18.7	18.5
Glucose			
1"'	104.96	104.97	104.7
2"'	75.2	75.2	75.1
3"'	78.6	78.7	78.5
4"'	71.8	71.8	71.9
5"'	78.5	78.5	78.1
6"'	62.8	62.8	63.0

4.6.6 Compound DMW6

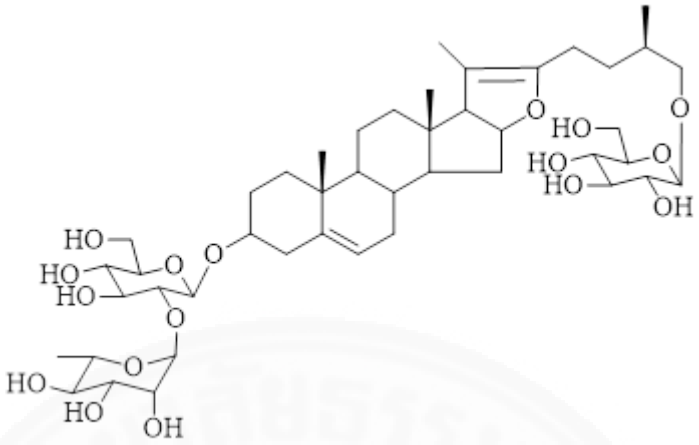


Figure 4.26 Chemical structure of DMW6

Compound DMW6 was obtained as a brown viscous solid. After measuring ^{1}H and ^{13}C NMR it decomposed. However, this compound was related to compound DMW5, except the hydroxyl group at C-22 disappeared (**Table 4.25**, **Figure 4.26**). By comparison of ^{13}C NMR with the previous reported data (Zheng *et al*., 2014), compound DMW6 was established as 3β, 26-dihydroxy-25 (*R*)-furosta-$\Delta^{5,20(22)}$-diene-3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-β-D-glucopyranoside.

Table 4.25 Comparison of ^{13}C NMR spectral data between DMW5, DMW6 and 3β, 26-dihydroxy-25 (*R*)-furosta-$\Delta^{5,20(22)}$-diene-3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-β-D-glucopyranoside (Zheng *et al*., 2014) (recorded in pyridine-d_5)

position	**δ_C (ppm)**		
	DMW5	**DMW6**	**Zheng *et al*., 2014**
1	37.6	37.6	37.9
2	30.3	30.3	30.6
3	78.1	78.0	78.7
4	40.0	39.1	39.4
5	140.96	141.0	141.2
6	121.8	121.8	122.2
7	32.4	32.5	32.8
8	31.8	31.5	31.8
9	50.4	50.4	50.7
10	37.2	37.2	37.5
11	21.2	21.3	21.6
12	39.1	39.7	40.0
13	40.9	43.5	43.8
14	56.7	55.0	55.3
15	32.5	34.6	34.9
16	81.2	84.5	84.8
17	63.9	64.6	64.9
18	16.5	14.2	14.5
19	19.4	19.5	19.8
20	40.7	103.6	103.9
21	16.4	11.8	12.1
22	110.7	152.5	152.8
23	37.2	33.6	33.8

Table 4.25 (Continued)

position	δ_C (ppm)		
	DMW5	**DMW6**	**Zheng *et al*., 2014**
24	28.4	23.8	24.1
25	34.3	31.5	31.8
26	75.3	75.0	75.3
27	17.5	17.4	17.7
Glucose			
1'	100.5	100.5	100.3
2'	77.9	77.9	78.9
3'	79.7	79.7	80.0
4'	71.96	71.9	72.2
5'	78.25	78.3	78.2
6'	62.9	62.8	63.0
Rhamnose			
1"	102.0	102.1	102.4
2"	72.6	72.6	73.0
3"	72.9	72.9	73.2
4"	74.2	74.2	74.5
5"	69.5	69.5	69.9
6"	18.7	18.7	19.0
Glucose			
1"'	104.97	104.9	105.3
2"'	75.2	75.2	75.6
3"'	78.7	78.7	78.7
4"'	71.8	72.0	72.1
5"'	78.5	78.5	78.7
6"'	62.8	63.0	63.0

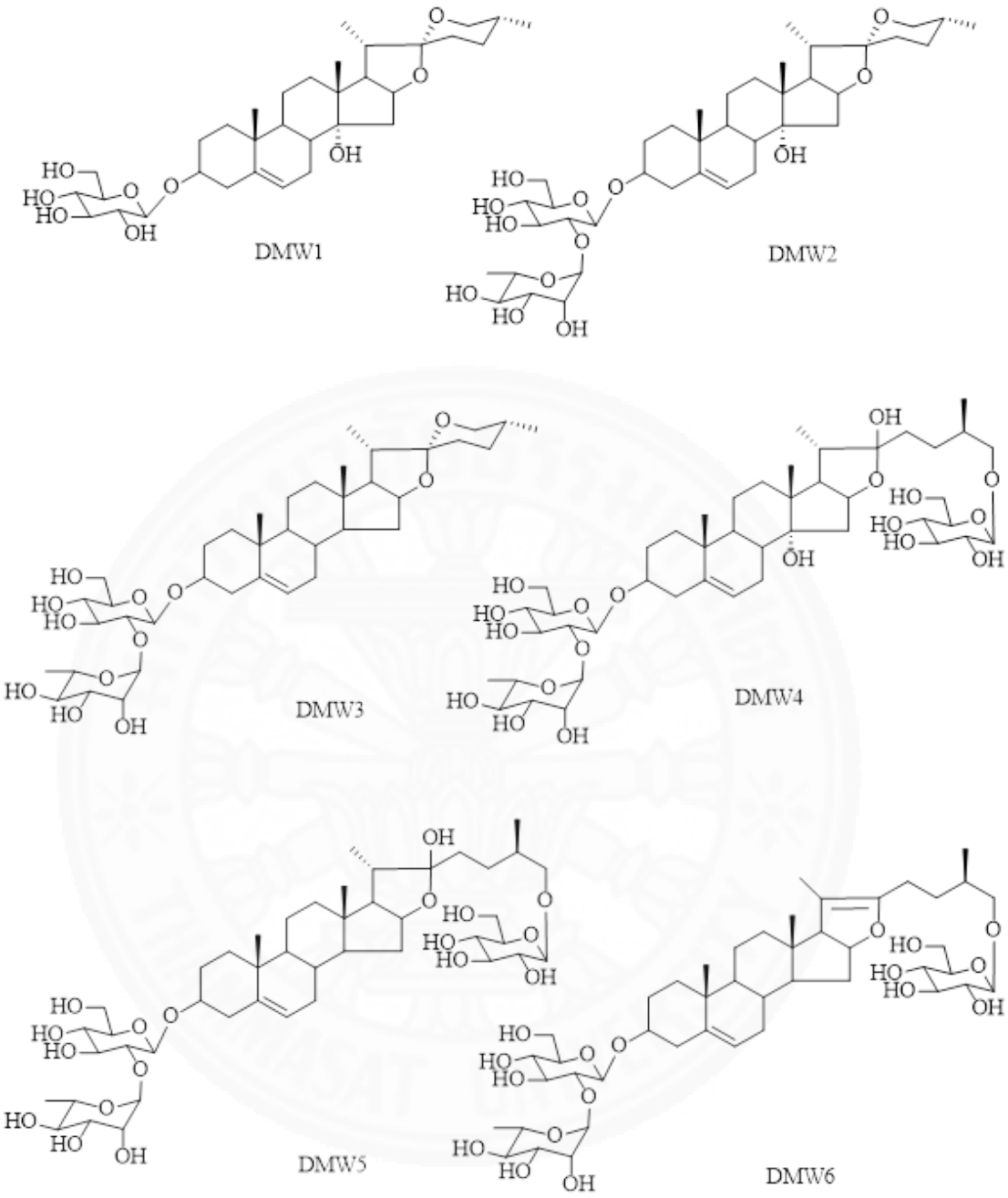


Figure 4.27 Chemical structures of DMW1-DMW6 of water extract of *Dioscorea membranacea*

4.7 *In vitro* cytotoxic activity of isolated compounds from *Dioscorea membranacea*

The ethanolic, water extracts and all isolated compounds were dissolved in DMSO with the stock solution at concentration of 10 mg/mL and 1 mg/mL, respectively. The final concentration of DMSO when diluted with medium is less than 1% w/v before cytotoxic testing assay.

The cytotoxicity against cholangiocarcinoma (KKU-M156), hepatocellular carcinoma (HepG2), breast cancer (MCF-7) and ductal carcinoma (T47D) of all isolated compounds of the ethanolic and water extracts were determined using SRB assay (Skehan *et al*., 1990). The result showed a napthofuranoxepin (DME2) exhibited the highest potent against KKU-M156 (IC_{50} = 1.67 ± 0.21 µM), HepG2 (IC_{50} = 2.87 ± 0.10 µM), MCF-7 (IC_{50} = 2.17 ± 0.01 µM) and T47D (IC_{50} = 1.70 ± 0.17 µM) and had no toxicity against normal cell (HaCaT) with IC_{50} >100 µM (**Table 4.26**). An order of potency of these twelve compounds were DME2 >DME3 >DMW3 >DME4 >DME5 >DMW1 >DME1 >DME6, DME7, DMW2, DMW4, DMW6. A phenanthraquinone (DME3) showed strong activity against four cell lines but had higher cytotoxic effect in normal cells than other compounds. The selective index value (SI) indicating the safety of isolated compound for anticancer therapy were calculated by obtaining the ratio of IC_{50} (µM) value of normal cells to IC_{50} (µM) value of cancer cell, DME2 showed high specificity for liver cancer (HepG2), cholangiocarcinoma (KKU-M156), breast cancer (MCF-7 and (T47D) cells with the ratio of >99.78, >58.06, 76.92 and 98.04, respectively (**Table 4.27**). By comparison between DME2 and positive control which are paclitaxel and vincristine sulfate, DME2 exhibited less cytotoxicity against KKU-M156 and HepG2 cells than positive control, but also showed less toxic against normal cell. For a positive control as tamoxifen exhibited high potency cytotoxic against breast cancer (MCF-7) and ductal carcinoma (T47D) with IC_{50} = 1.88 ± 0.37 vs 0.46 ± 0.01 µM, but less toxic effect on normal cell with the SI value of >14.9 and >58.82, respectively. This result showed that DME2 had high potency cytotoxic against breast cancer cells as well as drug tamoxifen. Additionally, compound DME2 also showed high cytotoxic against colon cancer (Caco-2, LS-174T and SW480) (Jaiaree *et al*., 2013), lung cancer (COR-L23)

and breast cancer (MCF-7) cells (Itharat *et al*., 2003) with IC_{50} < 4 µg/mL. The cytotoxic mechanism of DME2 was reported only against breast cancer cells might be involved in p53 and the intrinsic apoptotic pathway (Saekoo *et al*., 2010). Thus, DME2 should be continued to determine molecular mechanism of another type of cancer cell lines.

The result from this study showed a novel steroid (epipanthogenin B, DME7) and one known steroid (panthogenin B, DME6) which were isolated from chloroform fraction of ethanolic extract of *D. membranacea* had no cytotoxic against cholangiocarcinoma (KKU-M156), hepatocellular carcinoma (HepG2), breast cancer (MCF-7 and T47D) cells and one normal cell (HaCat) with IC_{50} > 100 µM. However, in the previous study by the other reported that the crude ethanolic extract of *D. membranacea* significantly interfered with cytochrome P450-mediated hepatic metabolism such as the potent inhibitory activities on CYP2D6, CYP3A4, and CYP2C19 (Sumsakul *et al*., 2015). Thus, these steroid compounds from this plant should be studied on human cytochrome P450 and compared with plant extract.

The result of water extract showed six steroids saponin (DMW1-DMW6) in which prosapogenin A of dioscin (DMW3) showed high cytotoxicity against four cell lines but had toxic on two normal cells; human keratinocyte cell line (SVK-14) and human fibroblast (HF) (IC_{50} = 3.0 ± 0.6 µM (2.17 µg/mL) vs 4.0 ± 0.1 µM (2.9 µg/mL) which was described in the reported previously (Itharat and Ooraikul, 2007). Compound DMW1 showed weak activity, while DMW2, DMW4 and DMW6 showed no activity (IC_{50} >50 µM). There is no test for DMW5 because of it has small amount <0.5 mg.

The structure activity relationship between DMW1 and DMW3 showed that DMW1 has one hydroxy group located at C-14 and a glucose attached at C-3 showed less toxicity than DMW3 which has a fructose instead at C-3 and hydroxyl group at C-14 disappeared has high activity. However, the structure of compound DMW2 was closely related to DMW3 except for a hydroxy group appeared at C-14, it has reduced toxicity (IC_{50} >50 µM) which were described in section 4.9.

Table 4.26 Cytotoxicity of isolated compounds (IC_{50} as µg/mL and **µM**) from crude ethanolic and water extract of *Dioscorea membranacea* against four human cancer cells and one normal cell (n=3)

Compound	**Cell lines (IC_{50} as µg/mL and µM)**				
	KKU-M156	**HepG2**	**MCF-7**	**T47D**	**HaCaT**
DME1	>50	48.53±1.18	47.97±2.04	48.15±1.69	>50
	(**>100**)	(**>100**)	(**>100**)	(**>100**)	(**>100**)
DME2	0.50±0.21*	0.86±0.10*	0.65±0.01*	0.51±0.17*	>50
	(**1.67**)	(**2.87**)	(**2.17**)	(**1.70**)	(**>100**)
DME3	0.98±0.11	9.33±0.64	2.76±0.05	0.70±0.09	2.44±0.07
	(**3.46**)	(**32.84**)	(**9.68**)	(**2.46**)	(**8.58**)
DME4	22.62±0.22	25.71± 2.32	39.98±1.61	8.00±0.18	6.79±5.33
	(**83.14**)	(**94.49**)	(**>100**)	(**29.43**)	(**24.96**)
DME5	30.70±0.42	29.81± 3.52	21.83±2.99	13.68±0.89	14.78±5.24
	(**>100**)	(**>100**)	(**75.77**)	(**47.48**)	(**51.31**)
DME6	>50	>50	>50	>50	>50
	(**>100**)	(**>100**)	(**>100**)	(**>100**)	(**>100**)
DME7	>50	>50	>50	>50	>50
	(**>100**)	(**>100**)	(**>100**)	(**>100**)	(**>100**)
DME	30.49±0.82	38.97±2.04	13.19±2.86	25.09±4.90	71.43±0.41

Table 4.26 (Continued)

Compound	**Cell lines (IC_{50} as µg/mL and µM)**				
	KKU-M156	**HepG2**	**MCF-7**	**T47D**	**HaCaT**
DMW1	24.76±3.44 (**41.77**)	39.38±4.48 (**66.43**)	38.95±1.27 (**65.71**)	33.47±3.56 (**56.46**)	>50 (>**84.41**)
DMW2	>50	>50	>50	>50	>50
DMW3	7.23±0.32 (**10.01**)	6.42±0.70 (**8.89**)	6.15±0.52 (**8.51**)	6.51±0.03 (**9.01**)	NT
DMW4	>50	>50	>50	>50	>50
DMW5	NT	NT	NT	NT	NT
DMW6	>50	>50	>50	>50	>50
PC[a]	0.0001± 0.0[b]	0.0001± 0.0[b]	NT	NT	0.0001± 0.0[b]
VS[a]	0.0026±0.001	0.012± 0.0005	0.00095±0.1	NT	0.000007±0.0
	(**0.002**)	(**0.01**)	(**0.0010**)		(**0.000007**)
Tam[a]	NT	NT	0.70±0.37 (**1.88**)	0.17±0.01 (**0.46**)	>10 (>**26.92**)
DMW	0.001±0.00	0.25±0.16	0.07±0.01	0.03±0.01	0.15±0.03

[a] paclitaxel (PC), vincristine sulfate (VS) and tamoxifen (Tam) as positive control, [b]IC_{50} as nM,

NT = no test because of its small amount (< 1.0 mg), **P* <0.05 compared with HaCat cell line

DME = ethanolic extract of *Dioscorea membranacea*, DMW = water extract of *Dioscorea membranacea*

Table 4.27 The ratio of IC_{50} (μM) values of normal cell/ IC_{50} (μM) values of cancer cells of all isolated compounds and the crude extract

compound	**Selective index or SI (ratio IC_{50} normal cell/ IC_{50} cancer cell)**			
	KKU-M156	**HepG2**	**MCF-7**	**T47D**
DME1	>1.00	>1.03	>1.04	>1.04
DME2	> 99.78	> 58.06	>76.92	>98.04
DME3	2.48	0.26	0.88	3.48
DME4	1.14	0.26	0.17	0.85
DME5	0.97	0.50	0.68	1.08
DME6	>1.00	>1.00	>1.00	>1.00
DME7	>1.00	>1.00	>1.00	>1.00
DMW1	2.02	1.27	1.28	>1.50
DMW2	>1.00	>1.00	>1.00	>1.00
DMW3	NT	NT	NT	NT
DMW4	>1.00	>1.00	>1.00	>1.00
DMW5	NT	NT	NT	NT
DMW6	>1.00	>1.00	>1.00	>1.00
PC[a]	1.00	1.00	NT	NT
VS[a]	0.004	0.0007	0.007	NT
Tam[a]	NT	NT	>14.29	>58.82
DME	2.34	1.83	5.42	2.85
DMW	150	0.6	2.14	5

[a] paclitaxel (PC), vincristine sulfate (VS) and tamoxifen (Tam) as positive control,

DME = ethanolic extract of *Dioscorea membranacea*

DMW = water extract of *Dioscorea membranacea*

NT = no test

4.8 *In vitro* estrogenic and anti-estrogenic activities of all isolated compounds from water extract of *Dioscorea membranacea*

All of eleven isolated compounds were tested estrogenic and anti-estrogenic effects using estrogen-responsive breast cancer cell lines MCF-7 and T47D with increasing concentrations ranging from 0.01 μM to 10 μM, and their stimulatory activity was determined by comparing their effects with a positive control, estradiol (E2) at concentrations ranging from 1 to 100 pM. For anti-estrogenic activity based on T47D cell proliferation was investigated for eleven compounds to support Thai traditional doctor used *D. membranacea* as an ingradient for treatment of cancer patients. Estradiol (E2) at 100 pM was used initially to enhance cell proliferation. Tamoxifen (anti-estrogen drug) was used as a positive control and it suppressed E2-enhance cell proliferation over 90% at the concentration lower than 10 μM (**Table 4.28**).

Regarding eleven pure compounds, there is no compounds showed any estrogenic activity because they did not enhanced T47D cell proliferation by them selft through 0.1-10 μM. The effect of steroid spirostane saponin (DMW1-DMW3 and DMW6), napthofuranoxepins (DME1 and DME2), phenanthraquinone (DME3), dihydrophenanthrenes (DME4 and DME5) and steroids (DME6 and DME7) on breast cancer cell proliferation were tested in T47D cells (**Table 4.28**).

The dihydrophenanthrene (DME4) and steroid spirostane saponin (DMW1) showed strong inhibition against T47D cell at low concentrations for their $iEqE_1$ values of <0.1 μM followed by DMW6 ($iEqE_1$= 0.27 μM) and hieghest activity than tamoxifen ($iEqE_1$ = 9 μM). Compounds DME1, DME6, DME7 and DMW3 showed 50% suppressive E2-enhanced T47D cell proliferation at concentrations lower than 0.1 μM against E2-enhanced T47D cell proliferation. Compounds DME2, DME3, DME5 and DMW2 showed 50% suppressive activity at concentration s of 3.15, 7.27, 4.73 and 5.81 μM, respectively. These compounds showed mild activity. Compounds DME1, DME2, DME3, DME5, DME6, DME7, DMW2 and DMW3 suppressed E2-enhanced T47D cell proliferation dose-dependently. However, cytotoxicity of compounds DME1, DME2 and DMW3 were observed at concentration of 10 μM against T47D cell. Almost all compound showed cytotocixity

at concentration of 10 μM. An order of potency of these eleven ccompounds were DME4, DMW1 >DMW6 > DME1, DME6, DME7, DMW3 >DME2, DME3, DME5, DMW2 inhibited T47D cell proliferation.Thus, compounds DME4, DMW1 and DMW6 might be developed as anti-cancer drug for breast cancer patients in the future.

Table 4.28 Inhibitory activities (μM) of isolated compounds of water extract of *Dioscorea membranacea* against E2-enhance cell proliferation

Compound	**T47D**		
	$iEqE_{50}$[a] (μM)	**$iEqE_{10}$[a] (μM)**	**$iEqE_1$[a] (μM)**
DME1	<0.1	>10	>10
DME2	3.15	6.46	7.21
DME3	7.27	10	>10
DME4	<0.1	<0.1	<0.1
DME5	4.73	5.46	5.63
DME6	<0.1	10	>10
DME7	<0.1	5.38	7.15
DMW1	4.79	<0.1	<0.1
DMW2	5.81	10	>10
DMW3	<0.1	5.35	9.54
DMW6	>10	2.46	0.27
tamoxifen[b]	0.1	0.8	9

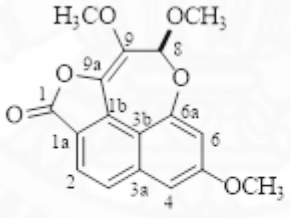
[a] $iEqE_{50}$, $iEqE_{10}$, and $iEqE_1$ represent the concentrations of the compounds (μM) that inhibited cell proliferation enhanced by 100 pM of E2 to equivalent levels of those induced by 50 pM, 10 pM, and 1 pM E2 treatment, respectively. These values were determined by linear regression analysis atleast four different concentrations. Mild inhibition (M), more than 50% inhibition through the concentrations tested. Strong inhibition (S), more than 90% inhibition through the concentrations tested.

[b] positive control

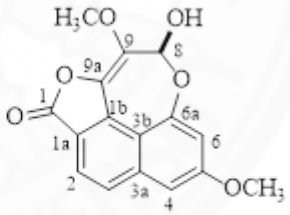
4.9 Structure-activity relationships (SAR) for cytotoxic activity of pure compounds

The thirteen compounds can be divided into four main groups, naphthopyrones, quinones, phenanthrene and steroids such as sterol and steroid saponin. In assessing structure-activity relationships (**Table 4.27**) showed that the pyrone which has the hydroxy group and the quinone derivatives were potent and selective with the cell lines tested.

In the previous described, both dioscorealide A (DME1) and dioscorealide B (DME2) are a naptho-α-pyrone derivatives. Dioscorealide B showed selective cytotoxic activity paralleled with antioxidant activity but dioscorealide A has 20 times less activity than dioscorealide B. Both compounds have same analog but dioscorealide A differs from dioscorealide B only at C-8 (-OMe and -OH) which affected the activity. The hydroxyl group of dioscorealide B appears to have high potential for cell death since methylation, as in dioscorealide A, reduces cytotoxic activity considerally (Itharat, 2002).



dioscorealide A
(DME1)

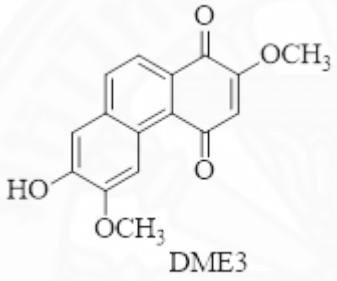


dioscorealide B
(DME2)

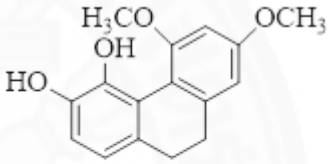
It is possible that *in vivo* dioscorealide A has a similar cytotoxicity activity to dioscorealide B, if it is metabolised by demethylation to dioscorealide B. Dioscorealide B is an important anticancer compound against breast, liver and bile duct cancer cells in this plant extract and it showed a greater cytotoxicity value than the crude extract (**Table 4.26**). In addition, quinone groups, such as benzoquinone, naphthoquinone and anthraquinone compounds are often found to have anticancer and cytotoxic activity (Itharat, 2002). Thus, dioscoreanone which is a naphthoquinone, is expected to have cytotoxic activity. The diference type analog between dioscorealide

B and dioscoreanone were showed no activity of LDH assay implies that the cell membrane is not affected (Itharat, 2002).

For the phenanthrene group, which are 9, 10-dihydrophenenthrene derivatives such as 5,6-dihydroxy-2,4 dimethoxy-9,10-dihydrophenanthrene (DME4) and 2, 5, 6-trihydroxy-3, 4-dimethoxy, 9, 10- dihydrophenanthrene (DME5) had no cytotoxicity. By comparison of cytotoxicity between dioscoreanone and 9, 10-dihydrophenanthrene (DME4 and DME5) which were similar analog structure, shows that the quinone group exhibited cytotoxicity while 9, 10-dihydrophenanthrene decreased activity.



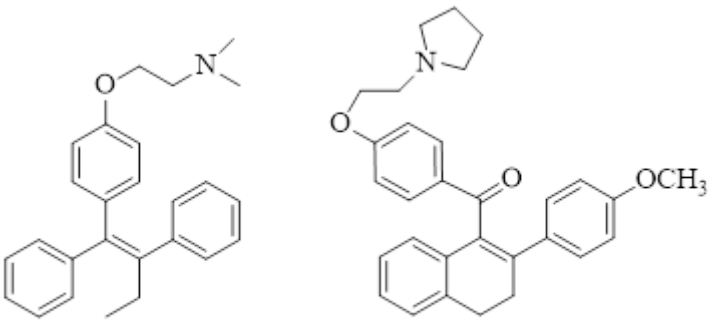
DME3



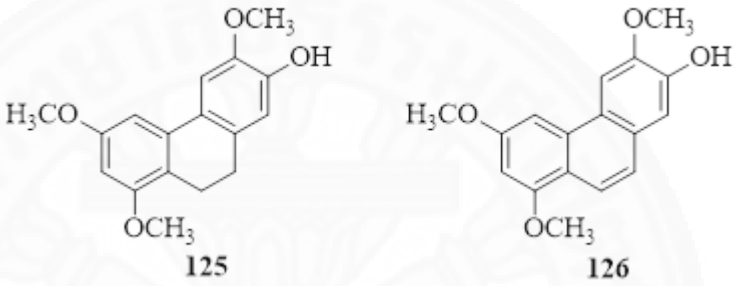
DME4

However, dihydrophenanthrene (DME4) showed strong inhibition against T47D cell proliferation at low concentrations for their $iEqE_1$ values of <0.1 μM better than anti-estrogen drug tamoxifen ($iEqE_1$ = 9 μM), which is a stilbenoid group. In the previous report, synthesized trioxifen found it to be a better anti-estrogen than tamoxifen, in possessing diminished agonist character (Durani *et al*., 1989).

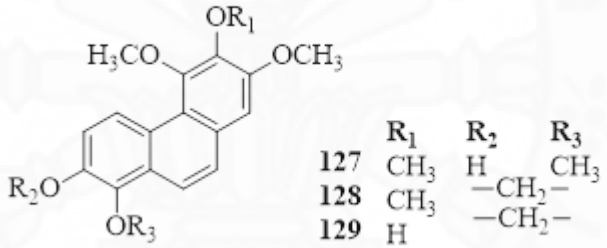
In addition, tamoxifen also showed strong cytotoxic activity against breast cancer cells (MCF-7 and T47D). In this case, some phenanthrene or stilbene derivertives were also showed cytotoxic activity such as dihydrophenthrene (**125**), phenanthrene (**126**, **127** and **129**) showed specific cytotoxic effect against cervix adenocarcinoma (HeLa) cells using MTT assay, except for **128**. Compounds **127** and **129** exhibited significant cell growth inhibitory effects with IC_{50} = 8.52 and 3.64 μM, respectively (Kovács *et al*., 2007). From this result, the structure of dioscoreanone (DME3), which was related with **127** and **129** also showed higher cytotoxic activity than dihydrophenanthrene derivatives (DME4, DME5 and **126**).



tamoxifen trioxifen

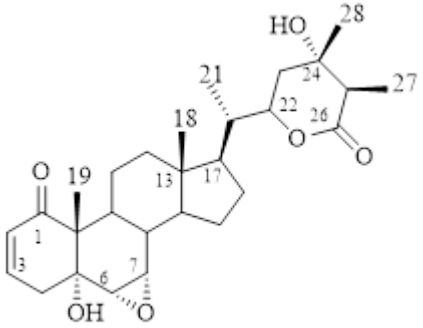


125 **126**

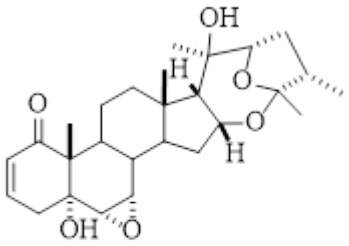


	R_1	R_2	R_3
127	CH_3	H	CH_3
128	CH_3	$-CH_2-$	
129	H	$-CH_2-$	

For the sterol groups, which are unusual steroids including panthogenin B (DME6) and epipanthogenin B (DME7) showed no cytotoxicity against four cell lines of this study (breast, liver and bile duct cancer cells) (IC_{50} >50 μM). These core-structures similar to those of withanolide analog, dioscorolide A, which were reported from the rhizomes of *Dioscorea japonica* and showed cytotoxicity against SK-OV-3 (ovary malignant ascites) and SK-MEL-2 (skin melanoma) cells (IC_{50} = 7.1 vs 6.3 μM) (Kim *et al*., 2011).

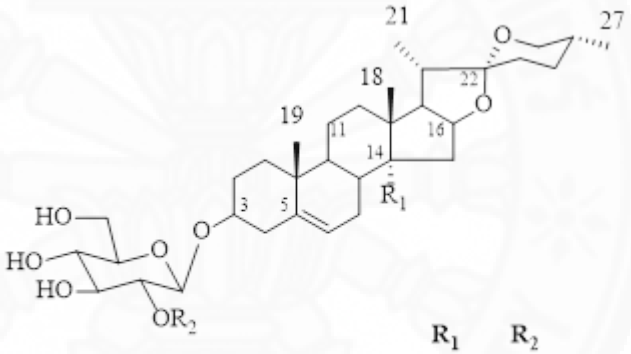


dioscorolide A



panthogenin B
(DME6)

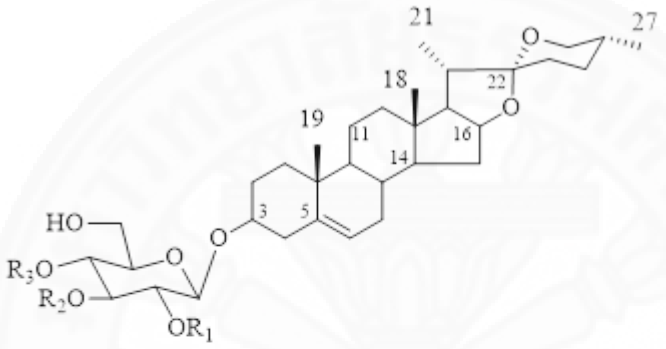
It is worth nothing that the water extract of *D. membranacea* contains mainly steroid saponins; these may be classified two types as spirostane (diosgenin) and furostane. If the aglycon part is a spirostane type it showed stronger cytotoxicity than furostane type and the sugar moieties contribute to the cytotoxicity.



	R_1	R_2
DMW1	OH	H
DMW2	OH	Rha
DMW3	H	Rha

In the previous studied of structure activity relationships (SAR) for cytotoxicity of steroidal saponin against human promyelocytic leukemia cells (HL-60) (Mimaki *et al.,* 2001). The result shows that diosgenin β-D-glucoside (**130**) exhibited no cytotoxic activity against HL-60 cell (IC_{50} >20 μg/mL) but the appearance of α-L-rhamnosyl group at C-2 (**131** or DMW3) of the glucosyl moiety increased activity (IC_{50} =1.8 μg/mL). Further addition of an α-L-rhamnosyl, an α-L-arabinofuranosyl or a β-D-glucosyl, to C-3 or C-4 of the inner glucosyl moiety either gave no potent on the activity or slightly increased the activity (**132-137,** IC_{50} = 0.5-3.3 μg/mL); a β-D-galactosyl attached at C-3 of the glucosyl moiety led to decrease activity (**137**, IC_{50} = 9.2 μg/mL). Comparison between diglycoside in which α-L-rhamnosyl group attached

at C-3 (**138**) and C-4 (**139**) of the inner glucosyl had no cytotoxicity activity (IC_{50} >20 μg/mL), but increased activity when attached only at C-2 (**131**). It was concluded that the three-dimensional (3-D) structure of the diglycoside moiety contributed to the activity. Diglycosides existed in the conformation having a vertical orientation against the steroid plane of the aglycon showed cytotoxic activity but glycosides which have attachment at C-3 and C-4 have sugars in the same plane with the steroid skeleton which had no cytotoxic activity (Mimaki *et al.,* 2001) (**figure 4.25**).

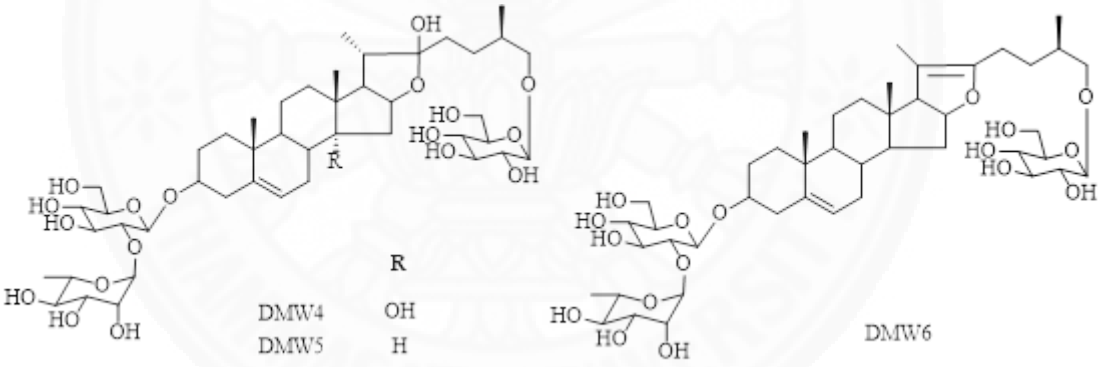


	R_1	R_2	R_3	IC_{50} (mg/mL)
130	H	H	H	>20
131	Rha	H	H	1.8
132	Rha	Glc	H	2.1
133	Rha	H	Glc	1.8
134	Rha	Rha	H	2.0
135	Rha	H	Rha	3.3
136	Rha	H	Ara	0.5
137	Rha	Gal	H	9.2
138	H	Rha	H	>20
139	H	H	Rha	>20

Figure 4.28 Cytotoxic activity of the steroidal saponins (diosgenyl saponin) against HL-60 cells and structure-activity relationships

In this study, the spirostane type saponin yield 14α-hydroxyl group to yield 25 (*R*)-spirost-5-en-3β,14α-diol-3-*O*-β-D-glucopyranoside or polygodoside E (DMW1) composed a non-deoxysugar or glucose at C-3 showed no cytotoxicity (IC_{50} > 50 μM), whereas deoxysugar, rhamnose attached to C-2 yield 25 (*R*)-spirost-5-en-3β, 14α-diol -3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-*β*-D-glucopyranoside or dracaenoside F (DMW2) also showed no cytotoxic activity. Surprisingly, DMW3 which lost of hydroxyl group at C-14 to give diosgenin-3-*O*-α-L-rhamnosyl (1→2)-β-

D-glucopyranoside or prosapogenin A of dioscin (DMW3) showed the highest cytotoxicity against breast, liver and bile duct cancer cells (IC_{50} < 10 μM) (**Table 4.26**). By comparison cytotoxicity with those previous report, prosapogenin A of dioscin or DMW3 was isolated from *Triteleia lacteal* showed cytotoxicity against HL-60 cell lines (IC_{50} = 1.8 μg/mL at exposure 72 h) (Mimaki *et al.,* 2001). This report related the result of DMW3 on cytotoxicity against three cancer cell lines. It showed cytotoxic activity against breast, liver and bile duct cancer cell lines (**Table 4.26**) because IC_{50} at exposure time 72 were identical. In addition, If α-L-rhamnose attached at C-3 of DMW3 to give dioscin (**130**) and β-D-glucosyl appeared at C-2 to give gracillin (**131**) they also showed higher cytotoxicity than prosapogenin A of dioscin with IC_{50} = 1.0, 1.2 and 7.0 μM, respectively (Hu *et al.*, 1996). The result was concluded that the hydroxyl group at C-14 in aglycon part reduced cytotoxicity but sugar moieties contributed to the cytotoxicity.



The cytotoxicity of furostane type saponin showed no activity against four cell lines (IC_{50} >50 μM) but they showed no activity for 26-*O*-β-D-glucopyranosyl-22-hydroxyfurost-5-ene-3β, 26-diol-3-*O*-α-L-rhamnopyranosyl (1→2)-β-D-gluco-pyranoside or DMW5 and it can not conclude the SAR in this study.

In addition, the structures of DMW1 and DMW6 showed strong anti-estrogenic activity against T47D cell proliferation (iEqE1 = <0.1 vs 0.27 μM), while estradiol (E2) enhance cell proliferation. These steroidal structures were similar but difference in ring A, in which estradiol (**122**) has aromatic ring except for DMW1 and DMW6.

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

Five Thai medicinal plants called 'Hua-Khao-Yen'; *D. birmanica, D. membranacea, S. corbularia, S. glaba* and *P. herbacea,* were used as an ingredient in many Thai traditional medicine preparation for cancer treatment. The crude ethanolic and water extracts were tested for *in vitro* cytotoxic activity against breast cancer (MCF-7 and T47D), cholangiocarcinoma (KKU-M156) and hepatocellular carcinoma (HepG2) cells, and they were also tested etrogenic/anti-estrogenic activities. The ethanolic extract of *D. membranacea* exhibited cytotoxic moderately against all cell lines but the best activity when compared with another species. In addition, water extract of *D. membranacea* showed the highest potency cytotoxic against all cell lines but had no cytotoxicity on normal cells. Thus, these results can predict that the ethanolic and water extracts of *D. membranacea* had anti-estrogenic activity. Furthermore, the isolation of active compounds of the crude ethanolic and water extracts of *D. membranacea* were investigated. This is the first report of these five plants for their cytotoxicity against bile duct cancer or cholangiocarcinoma cells and estrogenic/anti-estrogenic activity against E2 enhance cell proliferation.

The bioassay-guided fractionation of the rhizome of ethanolic extract of *D. membranacea* led to the isolation of two napthofuranoxepins [dioscorealide A (DME1) and dioscorealide B (DME2)], phenanthraquinone [dioscoreanone (DME3)], two phenanthrene [5,6-dihydroxy-2,4-dimethoxy-9,10-dihydrophenanthrene (DME4) and 2,5,6-trihydroxy-3,4-dimethoxy, 9, 10-dihydrophenanthrene (DME5)] and two steroids [panthogenin B (DME6) and a novel epipanthogenin B (DME7)]. The crude water extract of *D. membranacea* was purified by HPLC technique to give six steroid saponin compounds (DMW1-DMW6) and DME1-DME7 were also investigated. Six known steroids saponin; three spirostane type steroids [25 (*R*)-spirost-5-en-3β,14α-diol-3-*O*-β-D-glucopyranoside or polygodoside E (DMW1), 25 (*R*)-spirost-5-en-3β, 14α-diol -3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-β-D-glucopyranoside or 25 (*R,S*)-dracaenoside F (DMW2) and diosgenin-3-*O*-α-L-rhamnosyl (1→2)-β-D-

glucopyranoside or prosapogenin A of dioscin (DMW3)] and three furostane type steroids [26-*O*-β-D-glucopyranosyl- 25(*R*)-furost-5-en-3β,14α,22, 26-tetrol -3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside or 25 (*R*, *S*)-dracaenoside N (DMW4), 26-*O*-β-D-glucopyranosyl-22-hydroxyfurost-5-ene-3β,26-diol 3-*O*-α-L- rhamno-pyranosyl (1→2)-β-D-glucopyranoside (DMW5) and 3β, 26-dihydroxy-25(*R*)-furosta-$\Delta^{5,20(22)}$-diene-3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-β-D-glucopyranoside (DMW6)]. The cytotoxicity against cholangiocarcinoma (KKU-M156), hepatocellular carcinoma (HepG2), breast cancer (MCF-7 and T47D) of all isolated compounds of the ethanolic and water extracts were determined using SRB assay. The result showed that dioscorealide B (DME2) exhibited the greatest potency against breast cancer, liver and bile duct cancer or cholangiocarcinoma with the cytotoxicity value more than crude extract, which has IC_{50} value more than 100 times and it had also no cytotoxicity against normal cell (HaCaT). Whereas dioscoreanone (DME3) also showed high activity as well as DME2 but it had also cytotoxicity on normal cell (HaCaT). Prosapogenin A of dioscin (DMW3) showed strong cytotoxic activity against these four cell lines (IC_{50} = 6-7 μg/mL), unfortunately this compound was isolated and got small amout yield so we can not test cytotoxic activity against HaCat normal cell. However, it has ever been reported and showed cytotoxicity on two normal cells (Itharat and Ooraikul, 2007). For their estrogenic and anti-estrogenic activity against E2-enhance T47D cell proliferation, all crude extracts and compounds showed no estrogenic activity against T47D cell. The crude ethanolic extract (DME) showed anti-estrogenic activity at 0.1 μg/mL, and crude water extract (DMW) was recognized the anti-estrogenic activity at 100 times less concentration (< 0.01 μg/mL). Dihydrophenanthrene (DME4) which has weak cytotoxic activity and specific for breast cancer T47D cell (IC_{50} = 8 μg/mL) showed anti-estrogenic activity by strong suppressing E2 cell proliferation more than 99% inhibition at concentration less than 0.1 μM. In the same way, polygodoside E (DMW1) which showed no or slightly cytotoxicity against breast cancer cell (MCF-7 and T47D) but it is specific to be anti-estrogen agent by strong suppressing E2 cell proliferation more than 99% inhibition at concentration less than 0.1 μM. and higher activity than tamoxifen ($iEqE_1$ = 9 μM).

In the previous work, these four active cytotoxic compounds (DME2, DME3, DMW1 and DMW3) have been studied for others activity such as anti-inflammatory activity for nitrix oxide inhibitory activity and DME3 showed high potent inhibitory effect on TNF-α release (Tewtrakul *et al*., 2007). For the anti-allergic activity, dioscorealide B showed the highest activity against β-hexosaminidase release with the IC_{50} = 5.7 μM, followed by dioscoreanone (IC_{50} = 7.7 μM) and dioscorealide A (IC_{50} = 27.9 μM), while DMW1 showed no (IC_{50} >100 μM) (Tewtrakul *et al*., 2006). However, both ethanolic and water extract of *D. membranacea* showed significant increase immune function, but dioscorealide B had no effect (Panthong *et al*., 2014). The cytotoxicity of dioscorealide B was evaluated against two human breast cancer cell lines, MCF-7 and MDA-MB 468 by SRB assay. RT-PCR and Caspase-Glo® assay were used to further elucidate its cytotoxic mechanism. Dioscorealide B showed cytotoxic effect on MCF-7 (IC_{50} = 2.76 μM) and MDA-MB 468 (IC_{50} = 9.93 μM). The mRNA level for p53, p21 and Bax were increased while Bcl-2 was decreased after the treatment. MCF-7 treated with dioscorealide B showed the induction of apoptosis via the activation of caspase-9 and -7. The mechanisms of dioscorealide B might be involved in p53 and the intrinsic apoptotic pathway (Saekoo *et al*., 2010).

Thus further investigation on dioscorealide B should carried out for its mechanism against liver and bile duct cancer and may provide for discovery new anticancer molecules. Because of dioscorealide B has a high yield with high cytotoxicity and non toxic on normal cells, therefore it could be a marker in analysis of cytotoxic activity of extracts from Hua-Khao-Yen, are used by Thai traditional doctors. However, dioscorealide B also showed anti-estrogenic activity moderately by suppressing E2 cell proliferation at 50% inhibition (3.15 μM) but it can be anti-cancer agent for inhibit breast cancer cell.

According to the ethanolic and water extract of *D. membranacea* exhibited the anti-estrogenic activity as well as tamoxifen. Therefore, further study of both extracts and active isolated compounds [5,6-dihydroxy-2,4-dimethoxy-9,10-dihydrophenanthrene (DME4), polygodoside E (DMW1) and 3β, 26-dihydroxy-25 (*R*)-furosta-$\Delta^{5,20(22)}$-diene 3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-β-D-glucopyranoside

(DMW6)] should be investigated in anti-estrogenic activity such as the competitive binding of test compound to estrogen receptors (ERs) and the molecular mechanism shoulde be studied on the activation of growth factor signaling pathway, such as EGF receptor tyrosine kinase, which related to breast cancer cell proliferation. In addition, this data can support Thai traditional use of this plant to treat breast cancer patient.

In addition for the recommendation of this study, plant harvesting of root and rhizome part should be collected in summer season because it produced a high yield and high active ingredients but collecting rhizome or root part in the rainy season makes less quality or weak activity.

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APPENDICES

APPENDIX A
CHEMICAL REAGENTS FOR CELL CULTURE

1. Minimum essential medium (MEM)

MEM powder medium	9.5	g
Sodium bicarbonate ($NaHCO_3$)	2.2	g

Add into 1 L of sterile deionized water and adjust to pH 7.2-7.4 with 10% HCl or 10% NaOH and sterilized by filtration.

2. Dulbeco's modified eagle medium (DMEM)

DMEM powder medium	13.4	g
Sodium bicarbonate ($NaHCO_3$)	3.7	g

Add into 1 L of sterile deionized water and adjust to pH 7.2-7.4 with 10% HCl or 10% NaOH and sterilized by filtration.

3. HAM's F12 medium

Nutrient mixture F-12 Ham powder medium	11.1	g
Sodium bicarbonate ($NaHCO_3$)	1.2	g

Add into 1 L of sterile deionized water and sterilized by filtration.

4. RPMI 1640 medium

RPMI 1640 powder medium	10.4	g
Sodium bicarbonate ($NaHCO_3$)	2.0	g

Add into 1 L of sterile deionized water and adjust to pH 7.2-7.4 with 10% HCl or 10% NaOH and sterilized by filtration.

5. Phosphate buffer saline

PBS	1	tablet

Add into 100 mL of deionized water

APPENDIX B
CHEMICAL REAGENTS FOR ESTROGENIC AND ANTI-ESTROGENIC ASSAY

1. PR (-) RPMI-1640 medium

PR (-) RPMI-1640 powder medium	10.36	g
Sodium bicarbonate ($NaHCO_3$)	2.0	g

Add into 1 L of sterile deionized water and sterilized by filtration.

2. DCC-FBS

(1) Dextran-coated charcoal (DCC)	1	mL
(2) Fetal bovine serum (FBS)	10	mL

Add (1) into (2), stirred slowly for 2 hr at 20° C and centrifuged (1600 × g, 20 min). After that the DCC-FBS was sterilized by filtration through a 0.22 μm filter.

APPENDIX C

NMR SPECTRUM OF ISOLATED COMPOUNDS

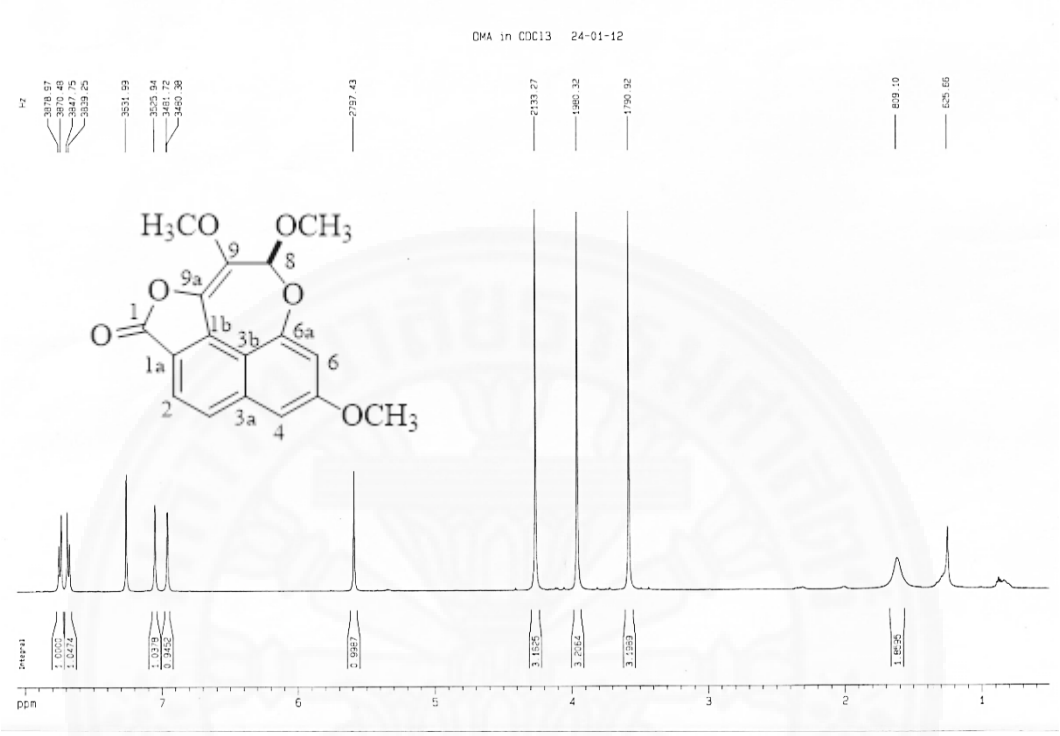


Figure A1 ^{1}H NMR spectrum of DME1 (500 MHz, in $CDCl_3$)

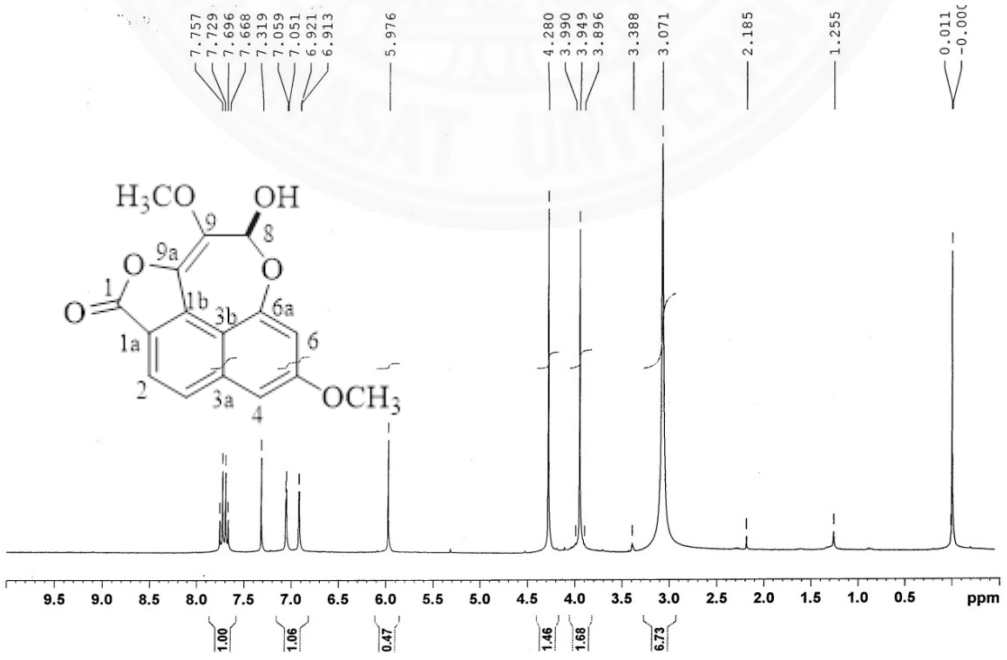


Figure A2 ^{1}H NMR spectrum of DME2 (400 MHz, in $CDCl_3$+ CD_3OD)

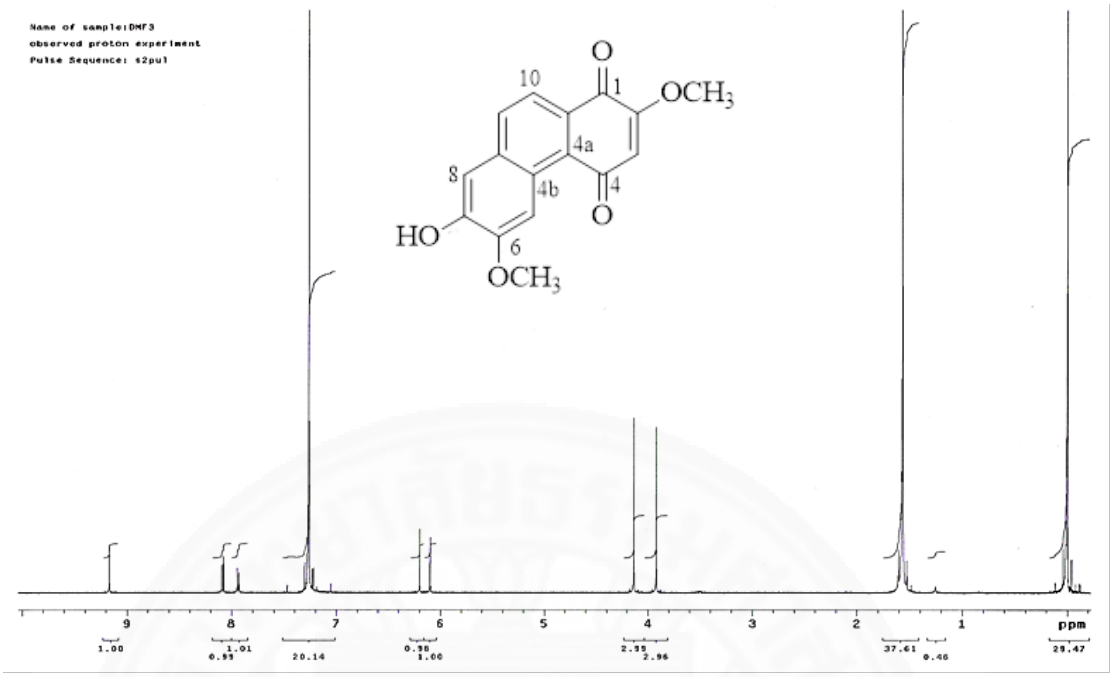


Figure A3 ^{1}H NMR spectrum of DME3 (500 MHz, in $CDCl_3$)

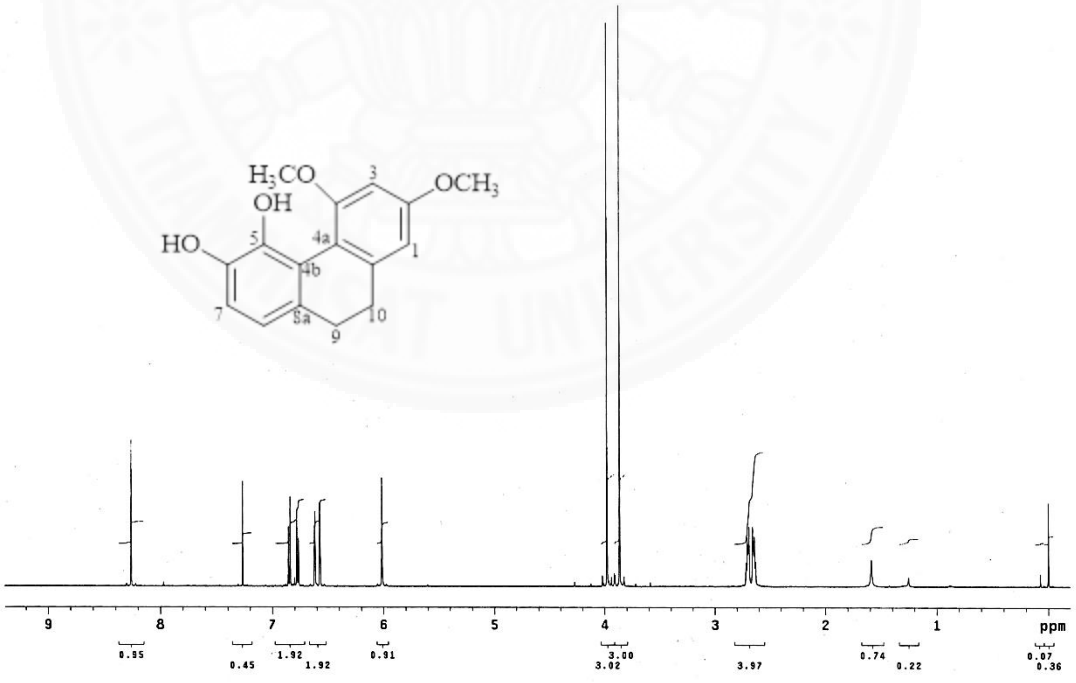


Figure A4 ^{1}H NMR spectrum of DME4 (500 MHz, in $CDCl_3$)

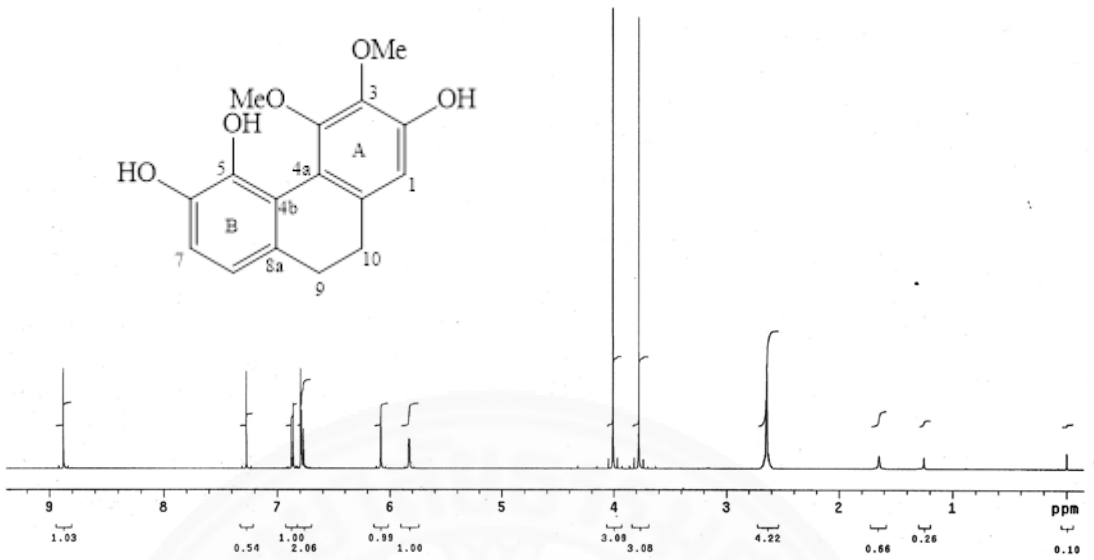


Figure A5 ^{1}H NMR spectrum of DME5 (500 MHz, in $CDCl_3$)

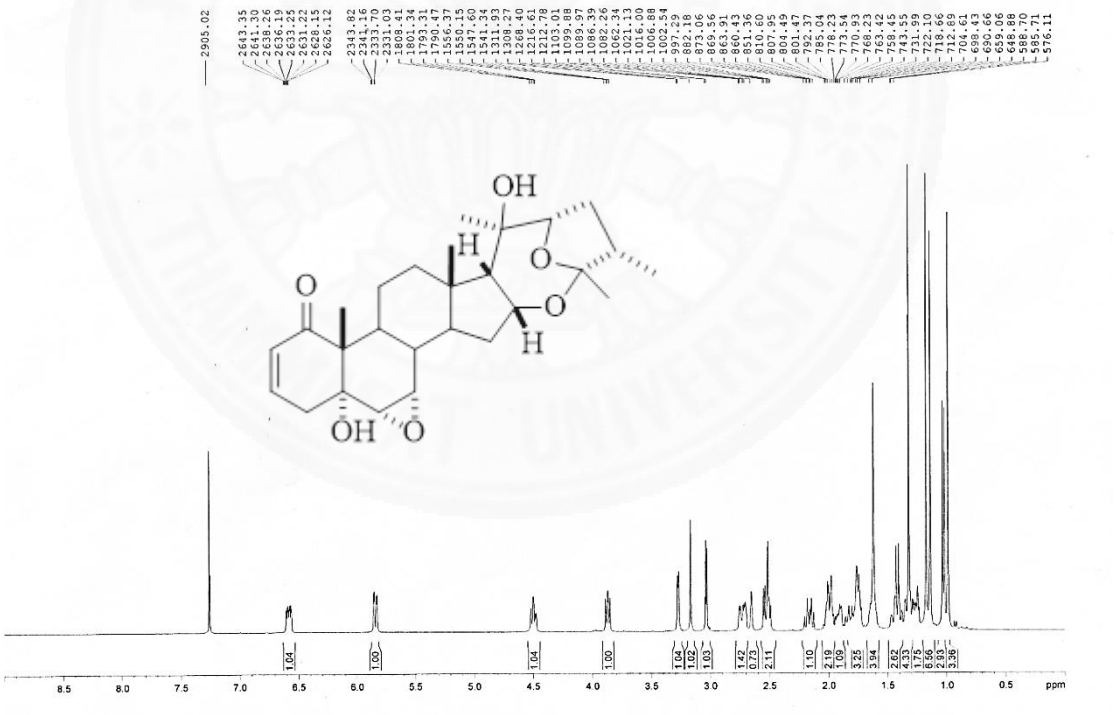


Figure A6 ^{1}H NMR spectrum of DME6 (400 MHz, in $CDCl_3$)

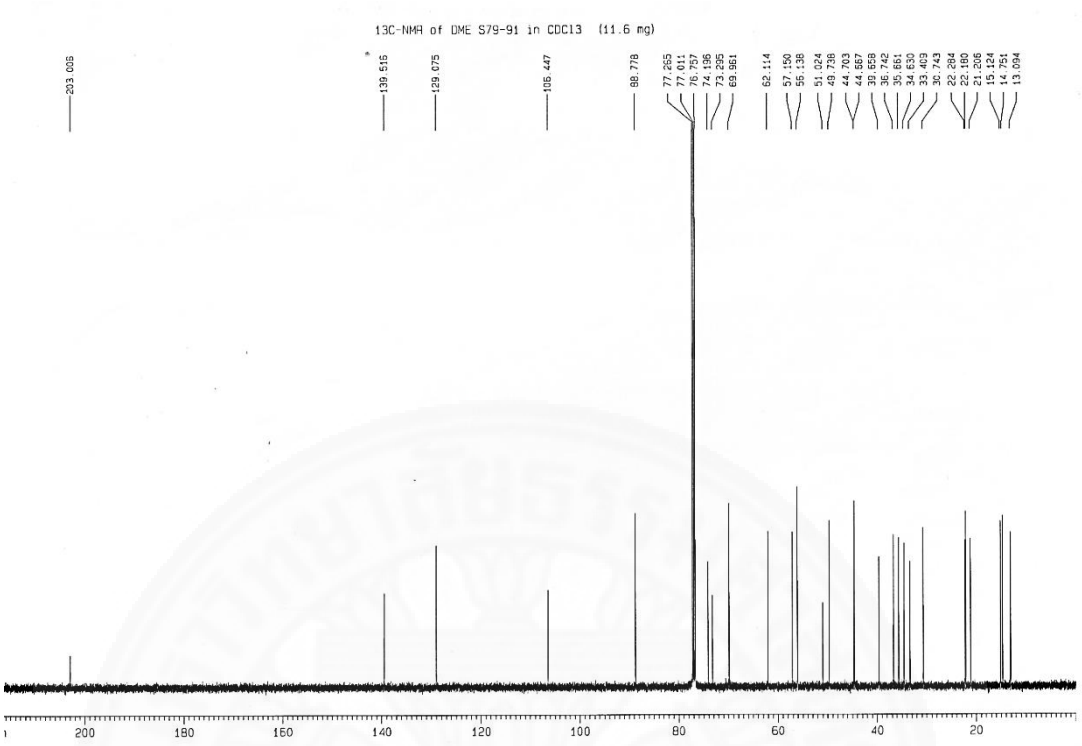


Fig.A7 ^{13}C NMR spectrum of DME6 (100 MHz, in $CDCl_3$)

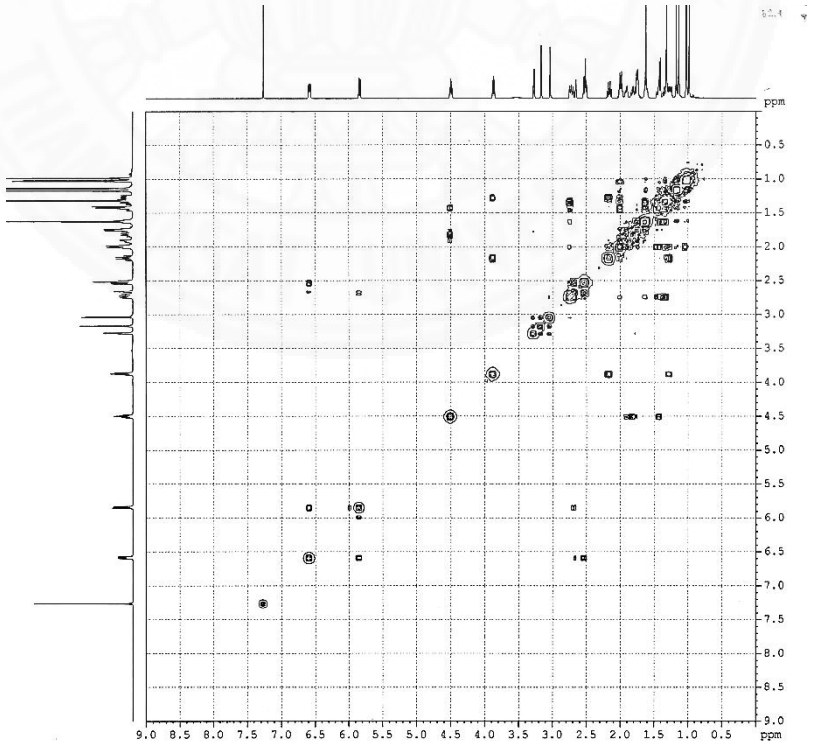


Figure A8 COSY spectrum of DME6

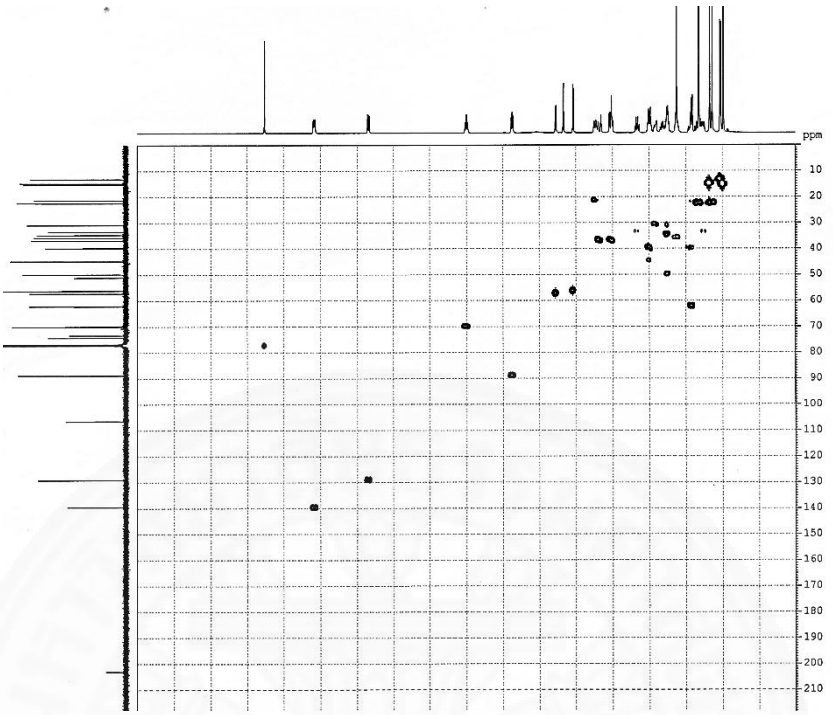


Figure A9 HMQC spectrum of DME6

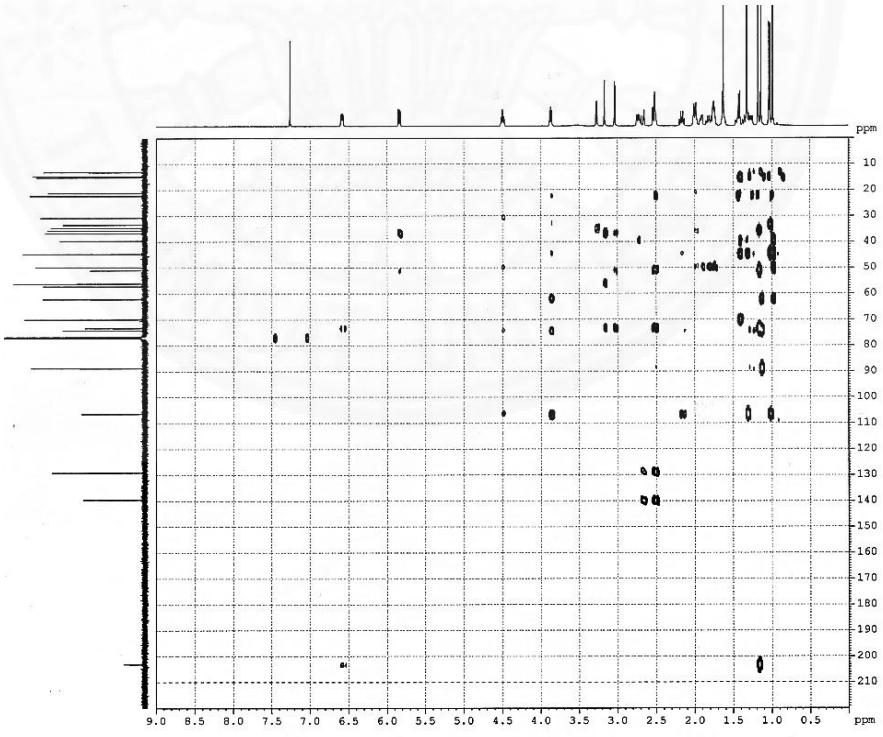


Figure A10 HMBC spectrum of DME6

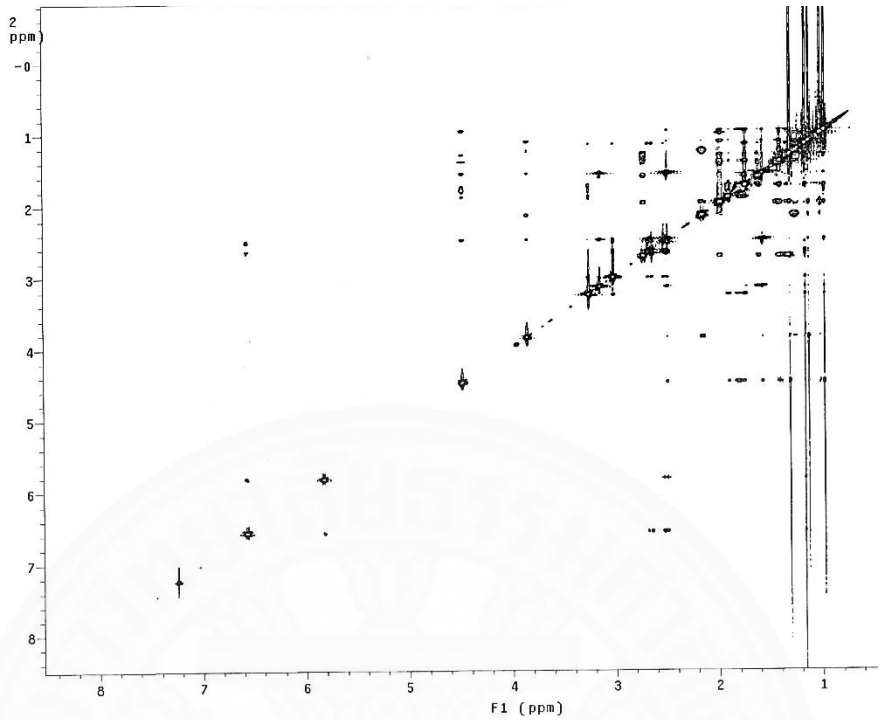


Figure A11 NOESY spectrum of DME6

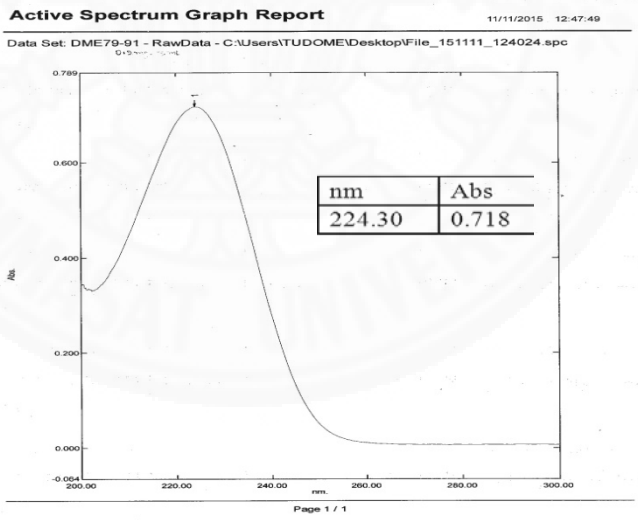


Figure A12 UV spectrum of DME6

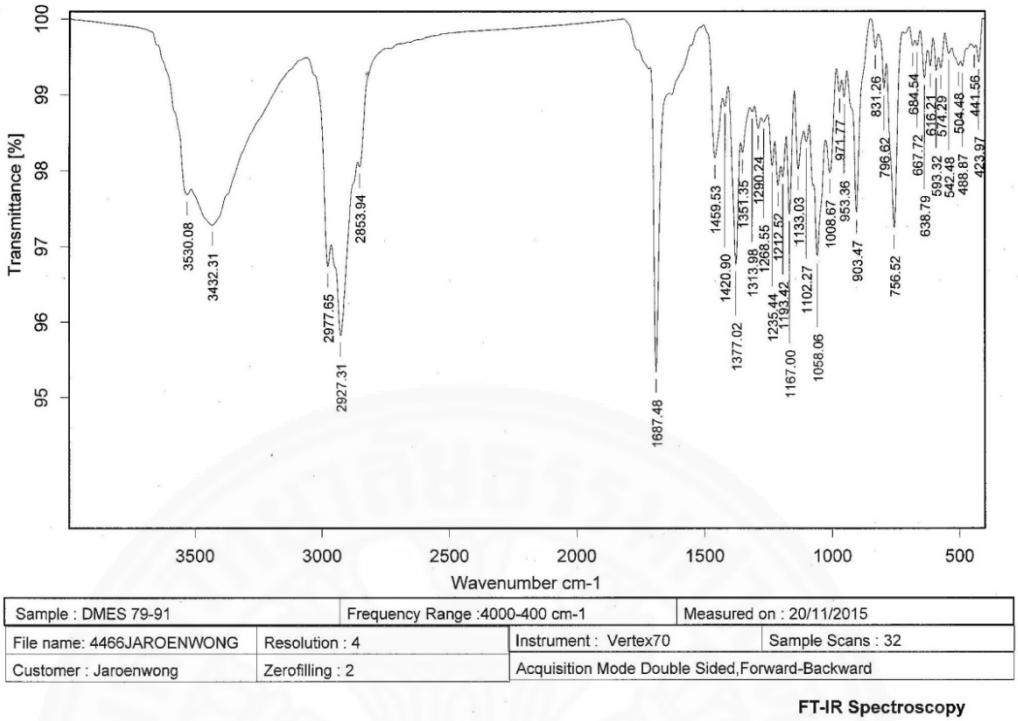
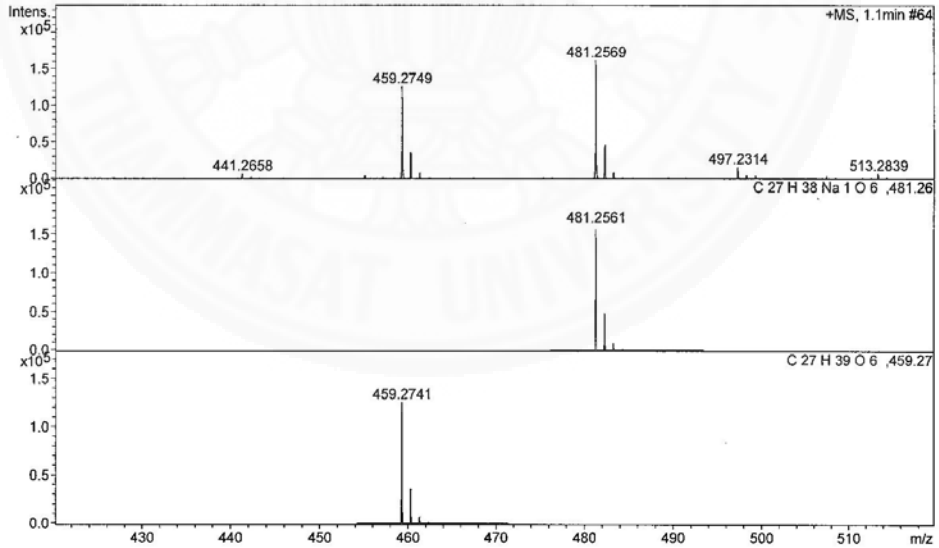


Figure A13 IR spectrum of DME6



Footnotes: *m/z* : 513.2839 (6), 497.2314 (12), 481.2569 (100), 459.2749 (76) and 441.2658 (6)

Figure A14 HR-ESI-TOF- MS spectrum of DME6

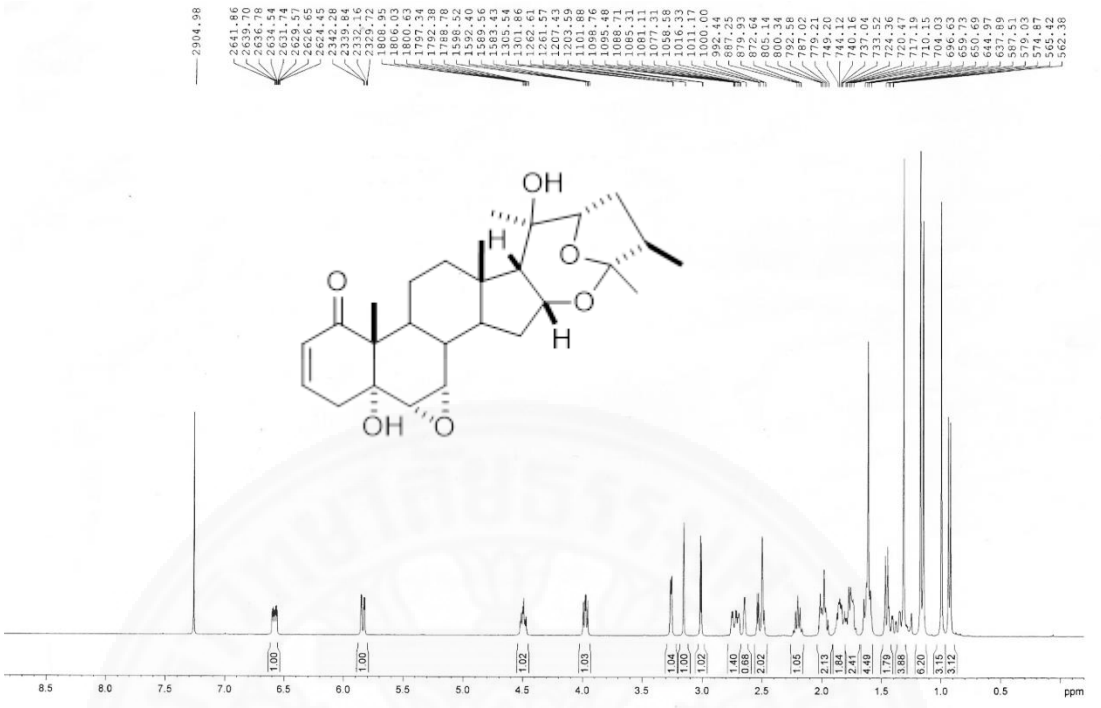


Figure A15 ^{1}H NMR spectrum of DME7 (400 MHz, in $CDCl_3$)

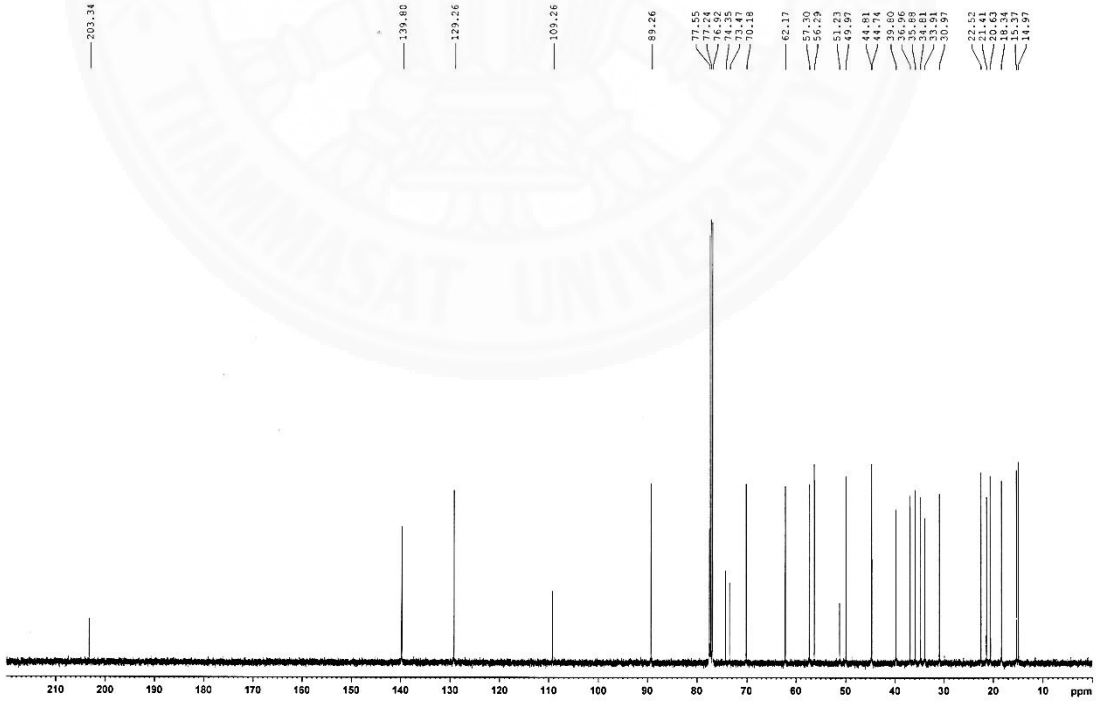


Figure A16 ^{13}C NMR spectrum of DME7 (100 MHz, in $CDCl_3$)

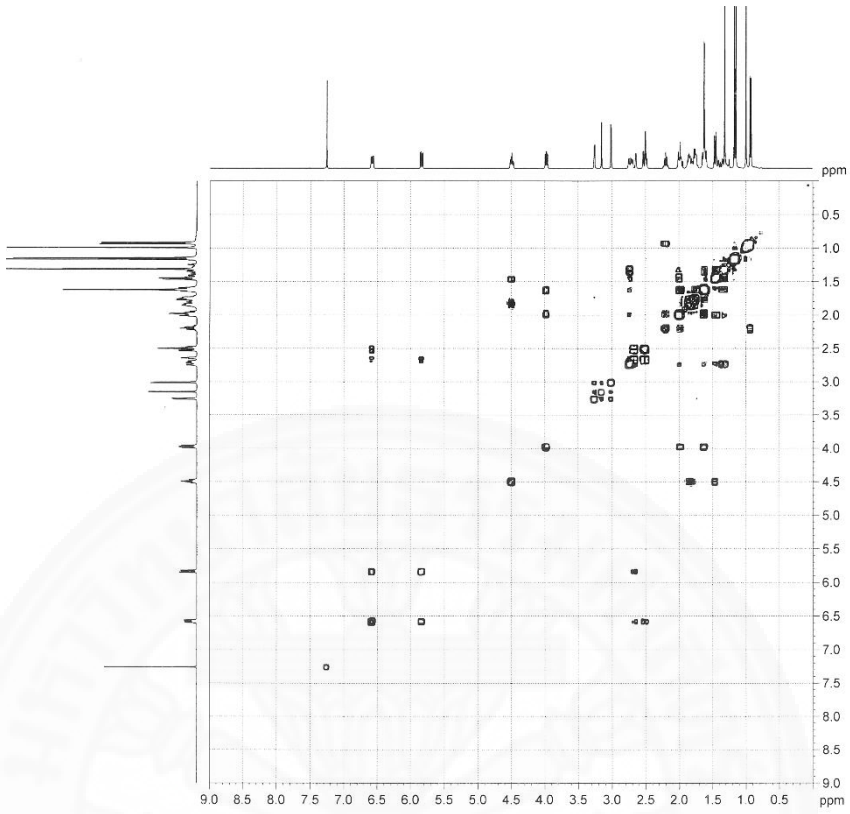


Figure A17 COSY spectrum of DME7

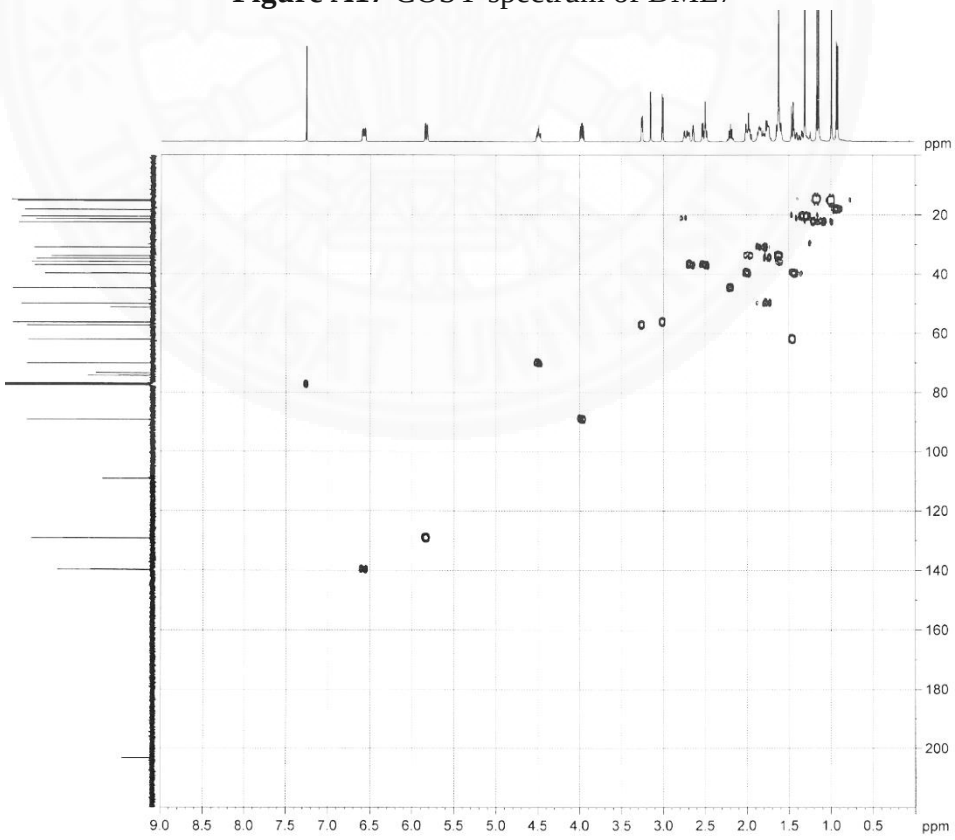


Figure A18 HMQC spectrum of DME7

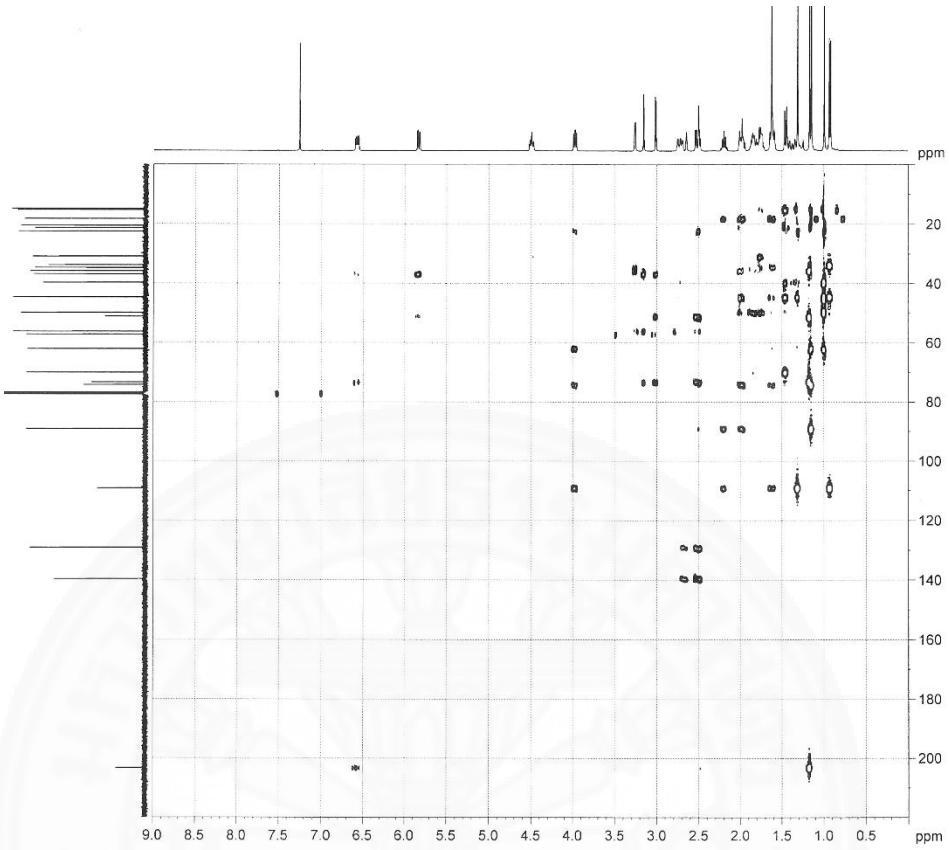


Figure A19 HMBC spectrum of DME7

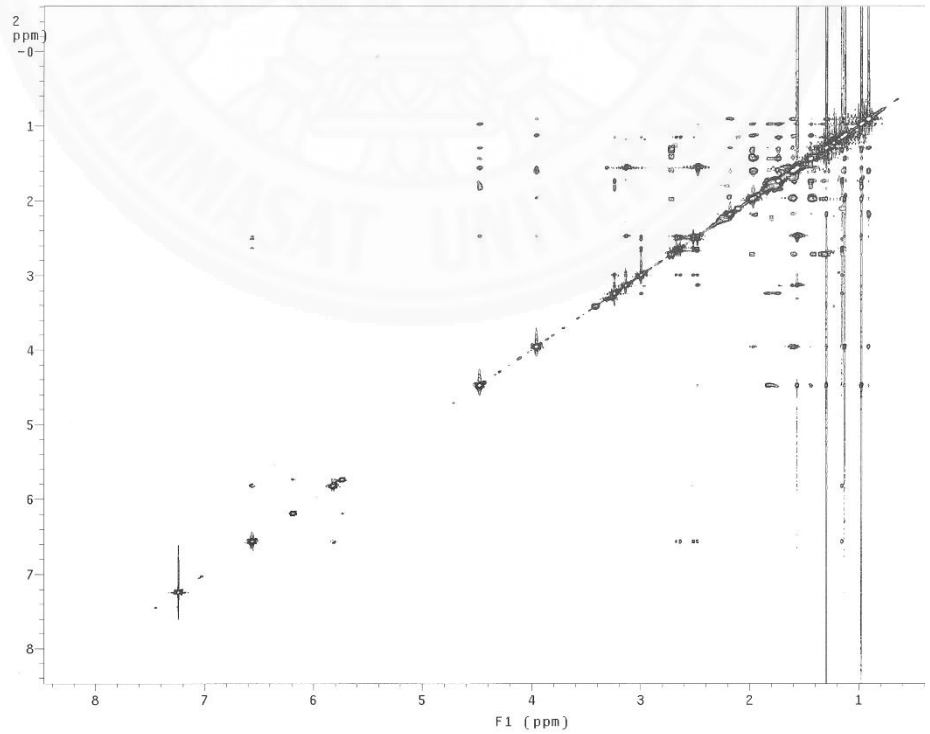


Figure A20 NOESY spectrum of DME7

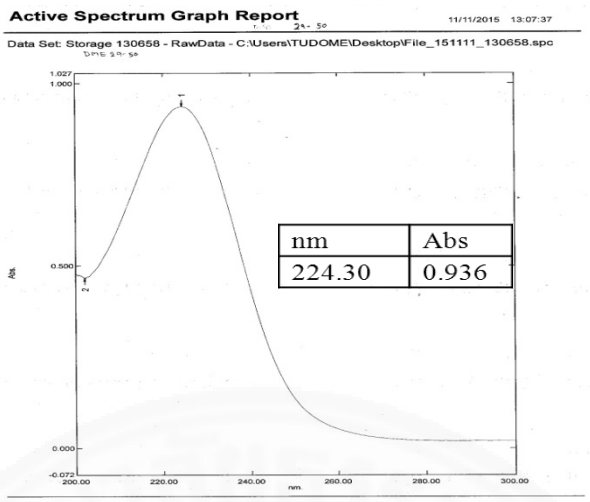


Figure A21 UV spectrum of DME7

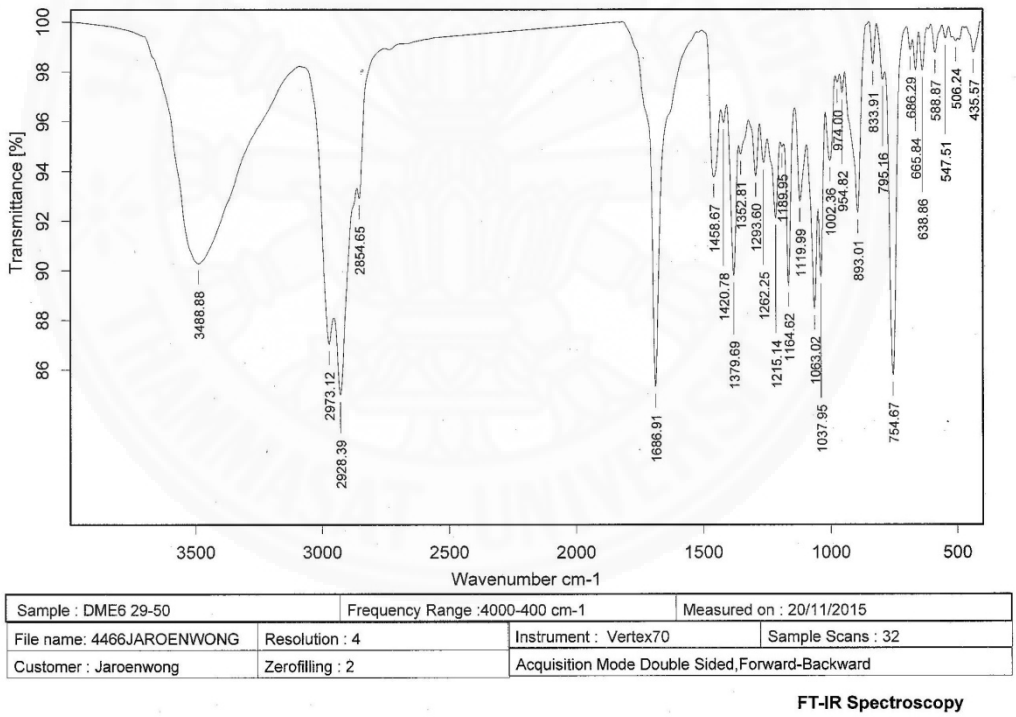
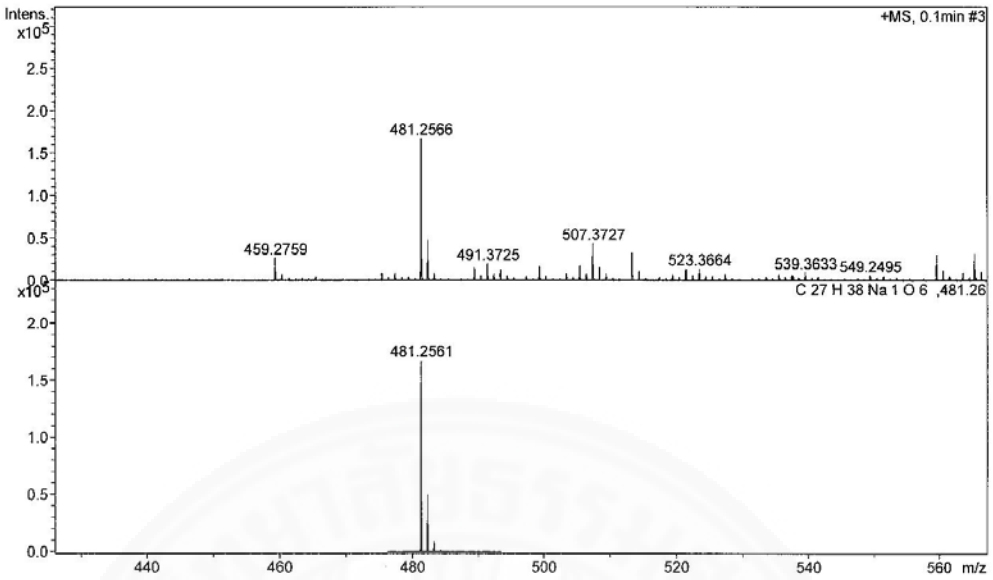


Figure A22 IR spectrum of DME7



Footnote : *m/z* : 523.3664 (6), 507.3727 (29), 491.3725 (12), 481.2566 (100) and 459.2759 (18)

Figure A23 HR-ESI-TOF- MS spectrum of DME7

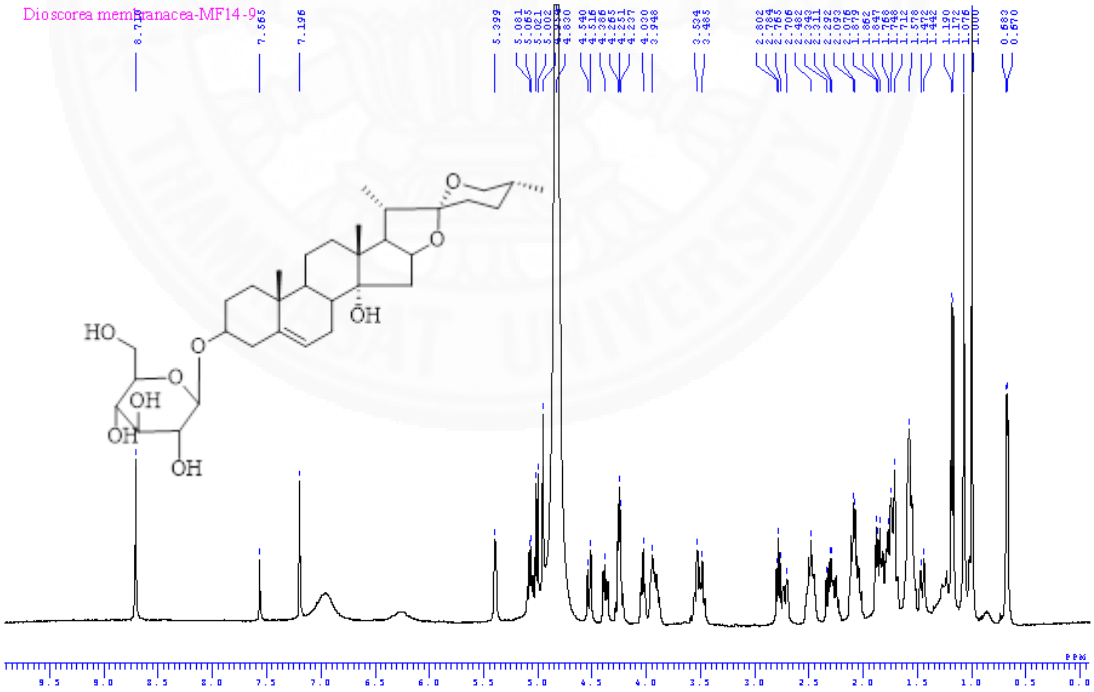


Figure A24 ^{1}H NMR spectrum of DMW1 (400 MHz, in pyridine-d_5)

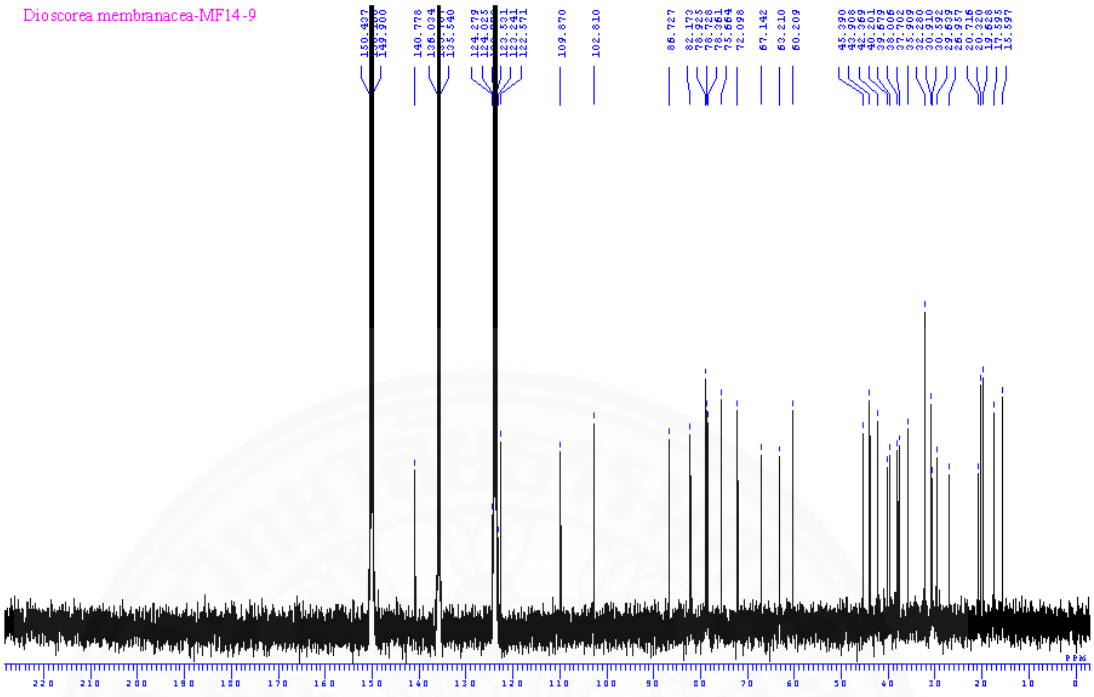


Figure A25 ^{13}C NMR spectrum of DMW1 (100 MHz, in pyridine-d_5)

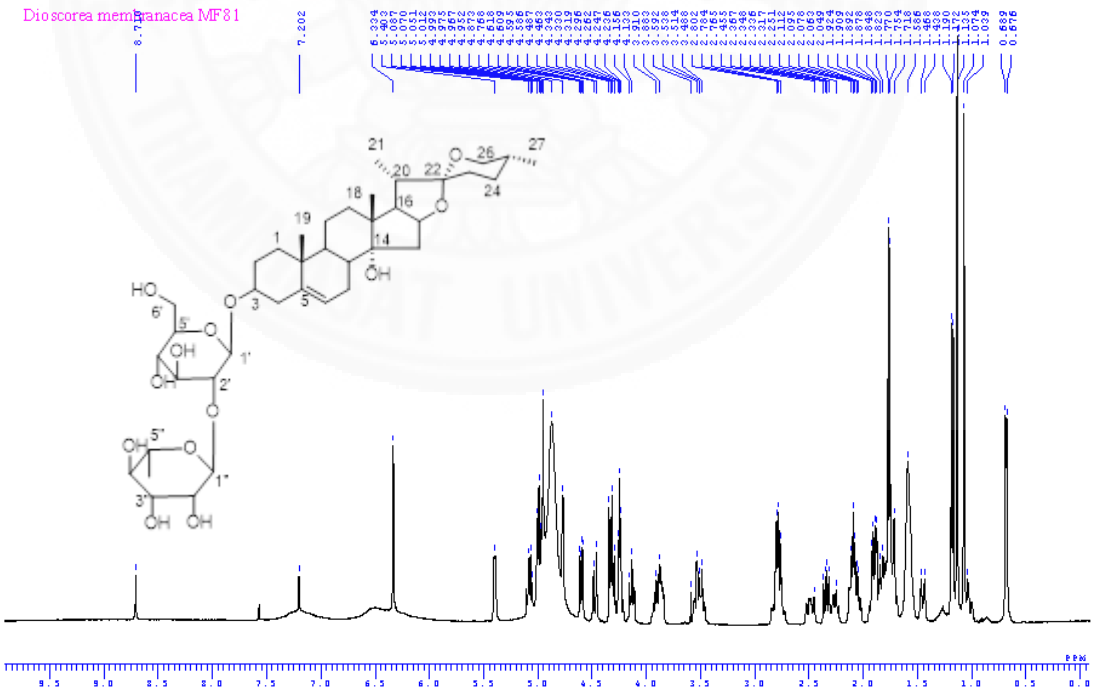


Figure A26 ^{1}H NMR spectrum of DMW2 (400 MHz, in pyridine-d_5)

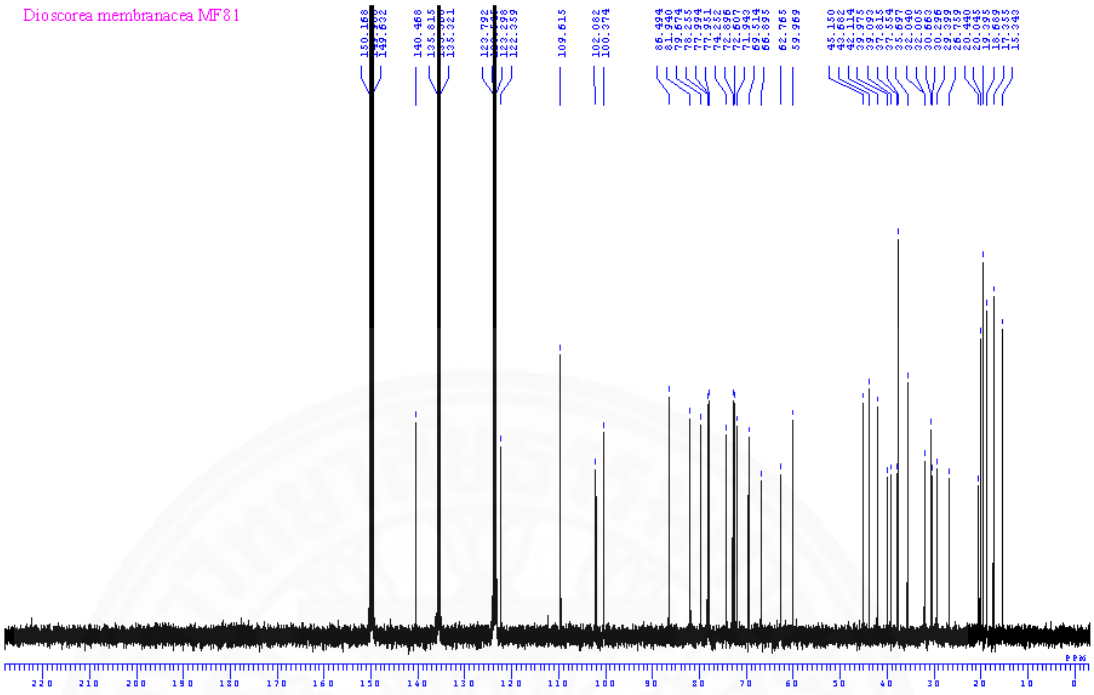


Figure A27 ^{13}C NMR spectrum of DMW2 (100 MHz, in pyridine-d_5)

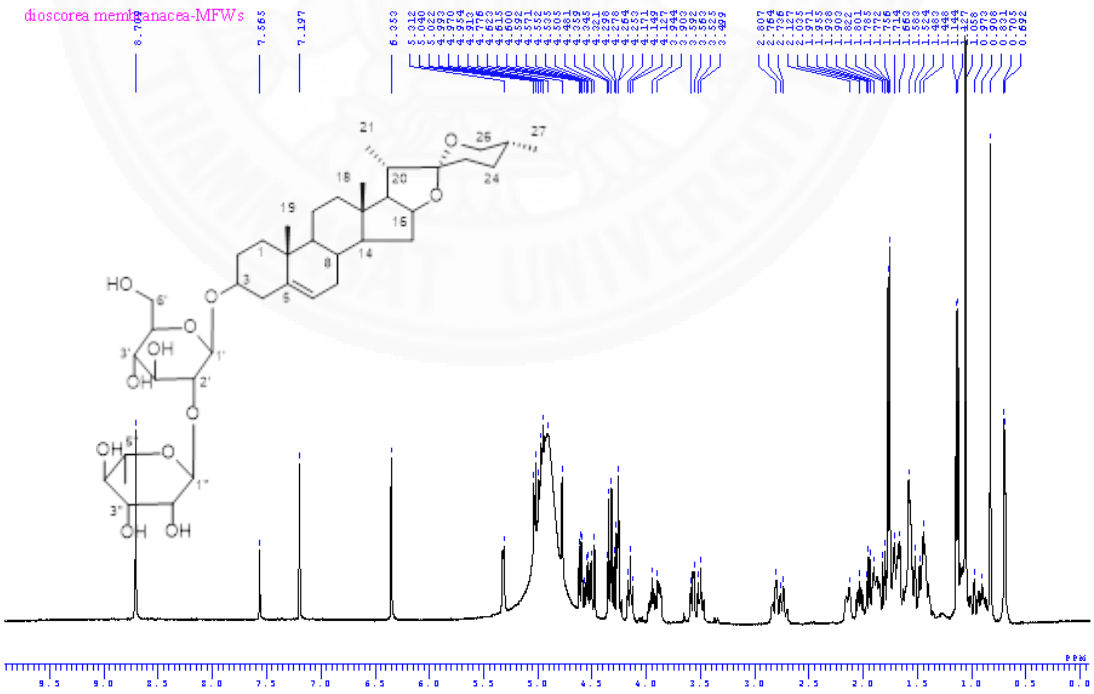


Figure A28 ^{1}H NMR spectrum of DMW3 (400 MHz, in pyridine-d_5)

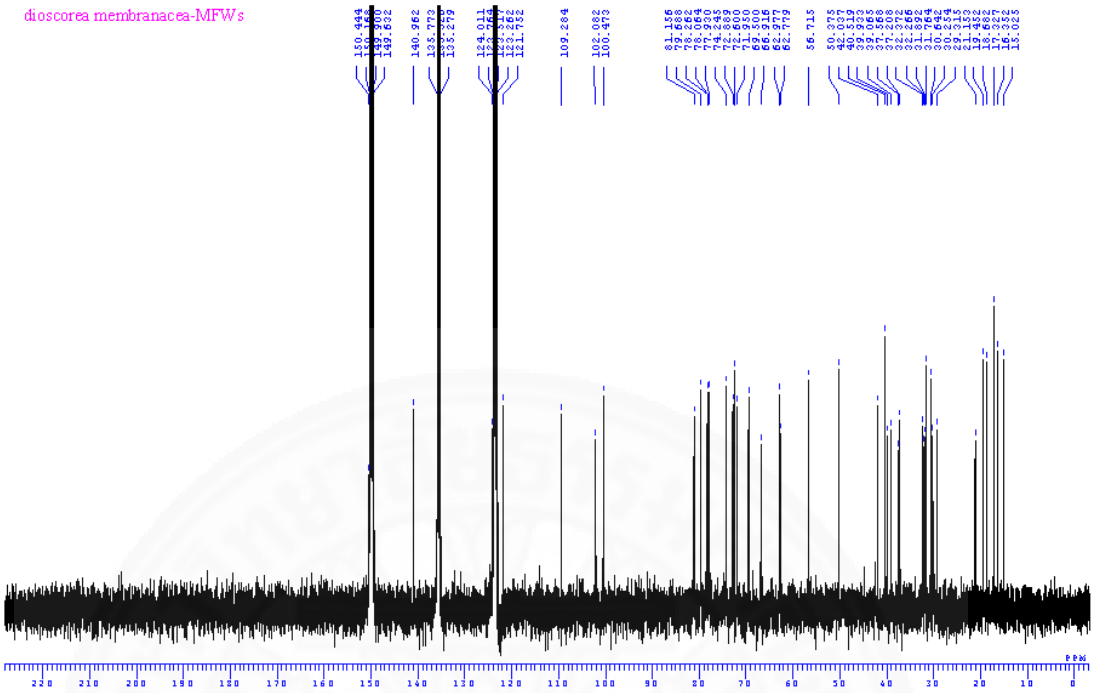


Figure A29 ^{1}H NMR spectrum of DMW3 (100 MHz, in pyridine-d_5)

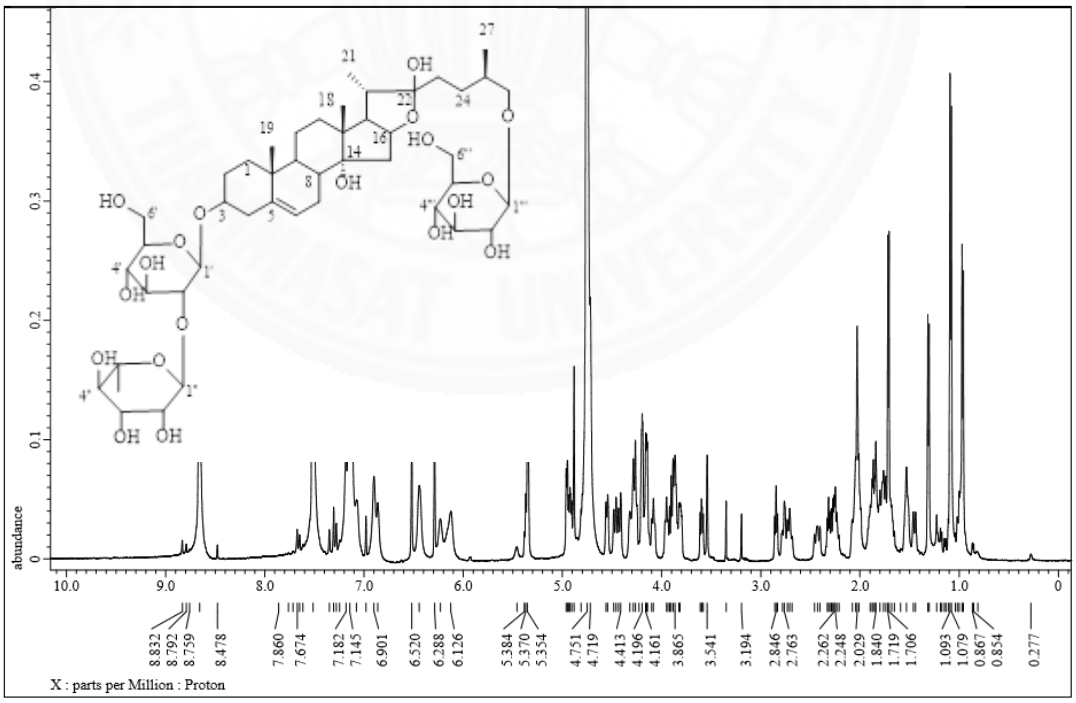


Figure A30 ^{1}H NMR spectrum of DMW4 (500 MHz, in pyridine-d_5)

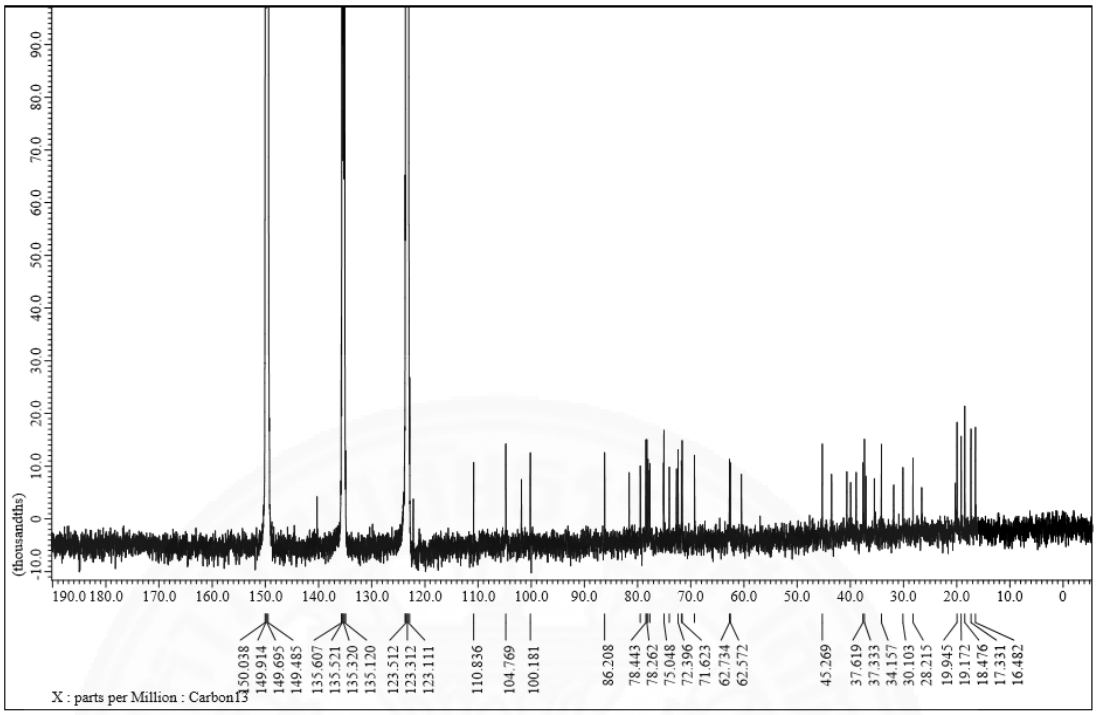


Figure A31 ^{13}C NMR spectrum of DMW4 (125 MHz, in pyridine-d_5)

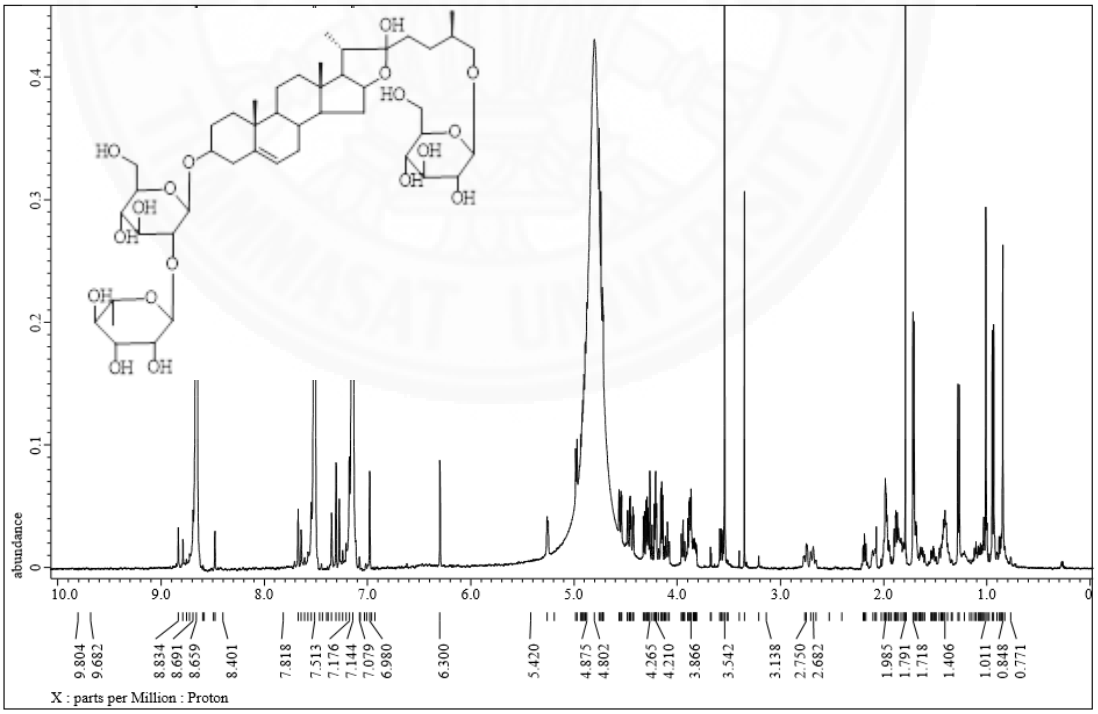


Figure A32 ^{1}H NMR spectrum of DMW5 (500 MHz, in pyridine-d_5)

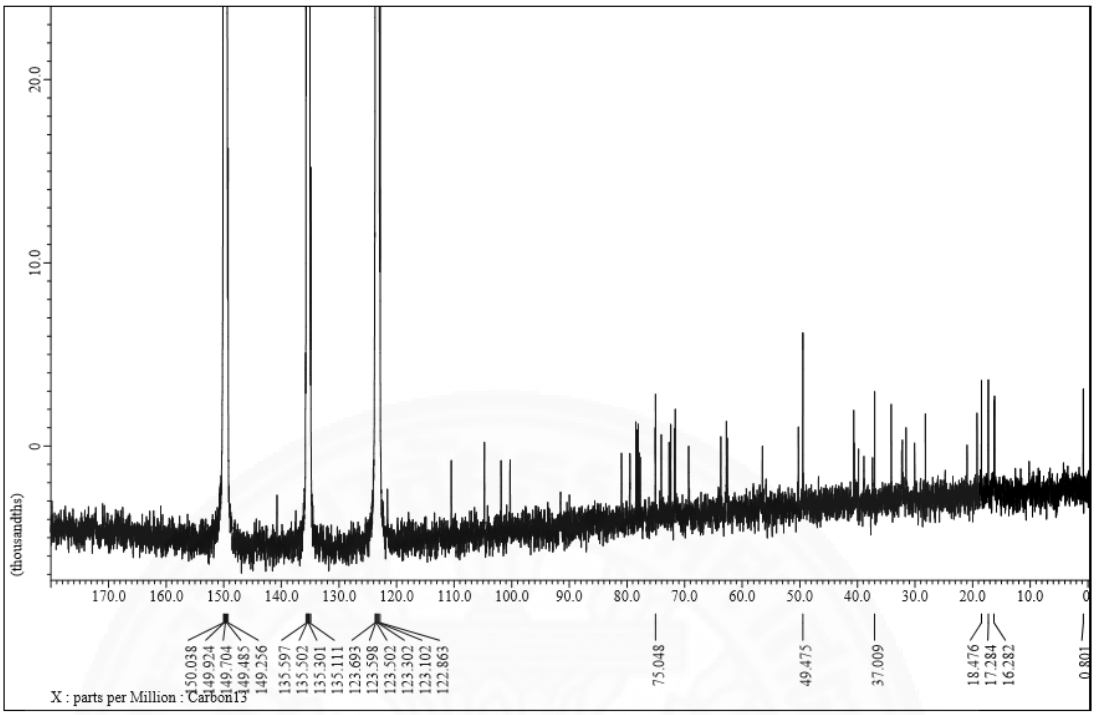


Figure A33 ^{13}C NMR spectrum of DMW5 (125 MHz, in pyridine-d_5)

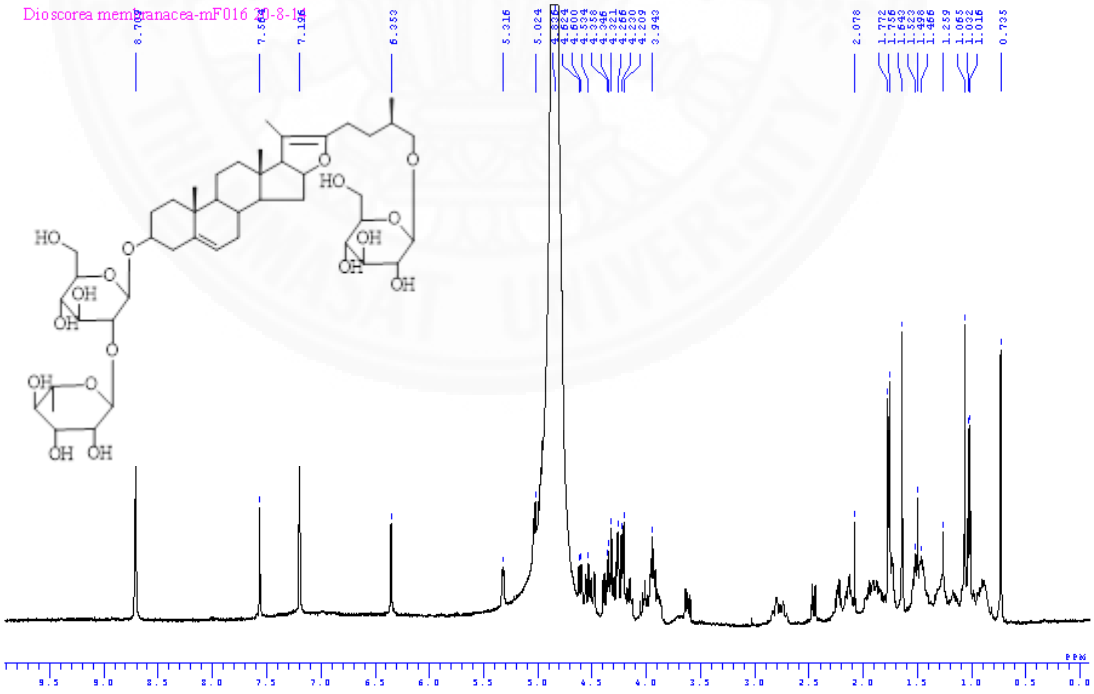


Figure A34 ^{1}H NMR spectrum of DMW6 (400 MHz, in pyridine-d_5)

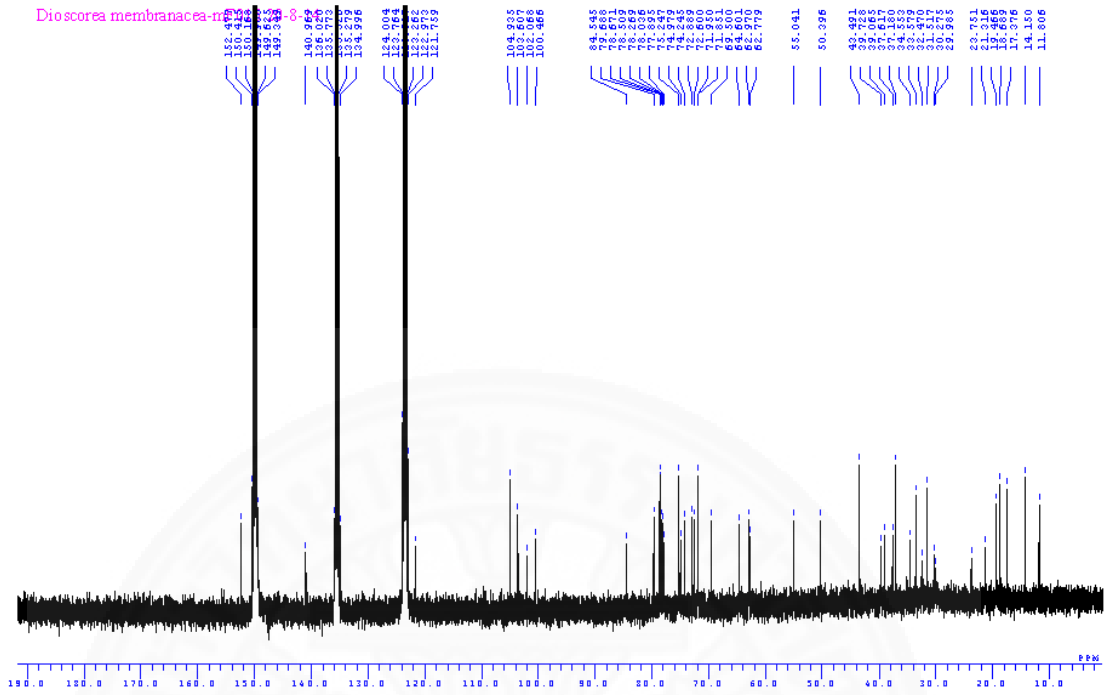


Figure A35 ^{13}C NMR spectrum of DMW6 (100 MHz, in pyridine-d_5)

BIOGRAPHY

Name	Miss Pakakrong Thongdeeying
Date of Birth	October16, 1980
Educational Attainment	1999-2002: Bachelor of Sciences in Chemistry 2003-2005: Master of Sciences in Organic Chemistry
Work Position	Assistance Researcher on Department of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University
Scholarship	2013: Ph.D. Scholarship of Thammasat Universty

Publications

Thongdeeying, P., Itharat, A. Umehara, K., Ruangnoo, S. (2016). A novel steroid and cytotoxic constituents from *Dioscorea membranacea* Pierre against hepatocellular carcinoma and cholangiocarcinoma cells. J. Ethnopharmacol. 194, 91-97. (Ph.D. publication)

Itharat, A., **Thongdeeying, P.**, Ruangnoo, S. (2014). Isolation and characterization of a new cytotoxic dihydrophenanthrene from *Dioscorea membranacea* rhizomes and its activity against five human cancer cell lines. J. Ethnopharmacol. 156, 130-134. (Ph.D. publication)

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Panthong, S., Ruangnoo, S., **Thongdeeying, P.**, Sriwanthana, B., Itharat, A. (2014). Immunomodulatory activity of *Dioscorea membranacea* Pierre rhizomes and of its main active constituent Dioscorealide B. BMC Complement. Altern. Med. 14, 403.

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Work Experiences

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