## รายงานวิจัยฉบับสมบูรณ์

# โครงการการศึกษาหาสารออกฤทธิ์ต้านเซลล์มะเร็ง ต้านเชื้อไวรัสเอดส์ และต้านการอักเสบจากต้นมหาพรหมราชินี

# Investigation of cytotoxic, anti-HIV-1 and anti-inflammatory constituents from *Mitrephora sirikitiae* (Annonaceae)

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สัญญาเลขที่ MRG5580047

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| ผู้วิจัย | สังกัด |
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| ณัฏฐินี อนันตโชค | ภาควิชาเภสัชวินิจฉัย คณะเภสัชศาสตร์ มหาวิทยาลัยมหิดล |



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วัตถุประสงค์ของงานวิจัยนี้คือการศึกษาหาสารและฤทธิ์ทางชีวภาพของส่วนผสมของใบและกิ่งและส่วนลำต้นของต้นมหาพรหมราชินี (*Mitrephora sirikitiae*) การศึกษาทางเคมีสารสกัดด้วยเมทานอลของต้านมหาพรหมราชินีด้วยวิธีทางโครมาโตกราฟฟี และการตกผลึก พบว่าสามารถแยกได้สารที่เป็นที่รู้จักทั้งหมด 20 ชนิด โดยพิสูจน์โครงสร้างทางเคมีด้วยวิธีทางสเปกโทรสโคปีได้แก่ อินฟราเรดสเปกโทรสโคปี อัลตราไวโอเลตและวิสิเบิลสเปกโทรสโคปี นิวเคลียร์แมกเนติกเรโซแนนซ์สเปกโทรสโคปี และแมสสเปกโทรเมตรี สารสกัดส่วนใบและกิ่งพบสารกลุ่มลิกแนน 5 ชนิด คือ epieudesmin (**1**), phyllegenin, magnone A, forsythialan B และ 2-(3,4-methylenedioxyphenyl)-6-(3,5-dimethoxyphenyl)-3,7-dioxabicyclo[3.3.0]octane สารกลุ่มไดไฮโดรเบนโซฟิวแรนลิกแนน 1 ชนิด คือ 3′,4-*O*-dirnethylcedrusin สารกลุ่มเฟลโวนอยด์กลัยโคไซด์ 1 ชนิดคือ quercetin 3,7-dimethylether 3'-O-$\alpha$-L-rhamnopyranosyl-(1$\rightarrow$2)-$\beta$-glucopyranoside สารกลุ่มสเตอรอลกลัยโคไซด์ 1 ชนิด คือ stigma-5-en-3-O-$\beta$-glucopyranoside สารกลุ่มแอลคาลอยด์ 5 ชนิด คือ liriodenine, dicentrinone, N-*trans*-feruloyltyramine, oxo-*O*-methylpukateine และ epiberberine ส่วนสารสกัดด้วยเมทานอลของส่วนลำต้นพบสารกลุ่มลิกแนน 1 ชนิด คือ (-)-epieudesmin สารกลุ่มไดเทอร์พีนอยด์ 3 ชนิด คือ kaurenoic acid, trachyloban-19-oic-acid และ ciliaric acid สารกลุ่มสเตอรอล 3 ชนิด คือ $\beta$-sitosterol, stigmasterol, stigma-5-en-3-O-$\beta$-glucopyranoside และสารกลุ่มแอลคาลอยด์ 3 ชนิด คือ liriodenine , oxo-*O*-methylpukateine, 5-methoxy-4-methyl-1H-1-aza-2,9,10-anthracenetrione และ stepharanine การศึกษาความเป็นพิษต่อเซลล์ของสารที่แยกได้พบว่า สาร magnone A และ 3′,4-*O*-dirnethylcedrusin เป็นพิษต่อเซลล์มะเร็งเต้านมชนิด MCF-7 และ สาร 3′,4-*O*-dirnethylcedrusin ยังเป็นพิษต่อเซลล์มะเร็งปอดชนิด A549 นอกจากนี้พบว่าสาร liriodenine และ oxo-*O*-methylpukateine เป็นพิษต่อเซลล์ทุกชนิดที่ใช้ทดสอบ และสาร 5-methoxy-4-methyl-1*H*-1-aza-2,9,10-anthracenetrione เป็นพิษต่อเซลล์สี่ชนิดคือ เซลล์มะเร็งเม็ดเลือดขาว (P-338) เซลล์มะเร็งลำไส้ใหญ่ (HT-29) เซลล์มะเร็งเต้านม (MCF-7) และเซลล์มะเร็งปอด (A 549) ดังนั้นสาร magnone A, 3′,4-*O*-dirnethylcedrusin และ 5-methoxy-4-methyl-1*H*-1-aza-2,9,10-anthracenetrione มีผลจำเพาะเจาะจงต่อเซลล์มะเร็งบางชนิด ซึ่งน่าจะสามารถนำไปศึกษากลไกการออกฤทธิ์และการปรับโครงสร้างเพื่อพัฒนาเป็นยาต้านมะเร็งต่อไปในอนาคต

**คำหลัก :** มหาพรหมราชินี / ลิกแนน / แอลคาลอยด์ / ความเป็นพิษต่อเซลล์

## Abstract

**Project Code :** MRG5580047

**Project Title :** Investigation of cytotoxic, anti-HIV-1 and anti-inflammatory constituents from Mitrephora sirikitiae (Annonaceae)

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**Project Period :** 3 years

The objective of this study were the chemical and biological investigation of the mixture of leaves and twigs as well as the stem of *Mitrephora sirikitiae.* The phytochemical investigation of methanol extracts of *M. sirikitiae* by chromatographic techniques and recrystrallization led to the isolation of twenty known compounds determined by means of spectroscopic methods, including IR (Infrared Spectroscopy), UV (Visible-Ultraviolet Spectroscopy), NMR (Nuclear Magnetic Resonance Spectroscopy) and MS (Mass Spectrometry). Five lignans, (-)-epieudesmin, phyllegenin, magnone A, forsythialan B, and 2-(3,4-methylenedioxyphenyl)-6-(3,5-dimethoxyphenyl)-3,7-dioxabicyclo[3.3.0]octane, one dihydrobenzofuran lignan, 3′,4-*O*-dirnethylcedrusin, one flavonoid glycoside, quercetin 3,7-dimethylether 3'-O-$\alpha$-L-rhamnopyranosyl-(1→2)-$\beta$-glucopyranoside, one steroidal glycoside, stigma-5-en-3-O-$\beta$-glucopyranoside, and five alkaloids, liriodenine, dicentrinone, N-*trans*-feruloyltyramine, oxo-*O*-methylpukateine, and epiberberine were isolated from the extract of leaves and twigs. Furthermore, one lignan, (-)-epieudesmin, three diterpenoids, kaurenoic acid, trachyloban-19-oic-acid, and ciliaric acid, three sterols, $\beta$-sitosterol, stigmasterol, stigma-5-en-3-O-$\beta$-glucopyranoside, and three alkaloids, liriodenine, oxo-O-methylpukateine, 5-methoxy-4-methyl-1H-1-aza-2,9,10-anthracenetrione and stepharanine were found from the extract of stem.

The evaluation of cytotoxicity of the isolated compounds found that magnone A and 3′,4-*O*-dirnethylcedrusin showed strong cytotoxicity against MCF-7 cells and 3′,4-*O*-dirnethylcedrusin was also toxic to A549 cells. Liriodenine and oxo-*O*-methylpukateine exhibited cytotoxic activity against all tested cell lines. Moreover, 5-methoxy-4-methyl-1*H*-1-aza-2,9,10-anthracenetrione could inhibit the growth of four tested cell lines, P-338, HT-29, MCF-7, and A 549 cell lines. Therefore, magnone A, 3′,4-*O*-dirnethylcedrusin and 5-methoxy-4-methyl-1*H*-1-aza-2,9,10-anthracenetrione had specific effect to some cell lines, therefore, they have been shown to possess an effective anti-cancer agent. It should be studied for their mechanisms of action and structure modification in order to develop a new anti-cancer drug in the future.



**Keywords :** *MITREPHORA SIRIKITIAE* / LIGNAN / ALKALOID / CYTOTOXICITY

# CONTENTS

# CHAPTER I
# INTRODUCTION

## 1.1 Introduction to the research problem and its significance

Cancer and AIDS are threatened diseases. A lot of Thai people die from the diseases every years because many of them can not get to expensive drugs. In many cases the drugs available at present can not cure the diseases and appear to cause serious side effects. Moreover, inflammation is another suffering symptom, almost everyone has encountered. Most of the drugs used to treat it also cause inverse effects. Therefore, drug development to improve their activities as well as to reduce the side effects is needed. According to the problems above and Thailand have been rich and diverse biodiversity in plants. The aim of my project is to search for new lead compounds from plants in order to develop to new effective drugs.

The plants in the genus *Mitrephora* belonging to the family Annonaceae are very interesting for studying their chemicals and biological activities. The previous reports from many literatures showed that these plants have a variety of types of chemical constituents, such as alkaloids, terpenoids, steroids, flavonoids, and lignanamides. Some species of this genus were reported to promising cytotoxic, antimicrobial, anti-plasmodial and anti-PAF activities.

This project will focus on the investigation of chemical constituents and biological activities such as cytotoxic, anti-HIV-1 and anti-inflammatory activities of *M. sirikitiae* collected in Thailand. It is an interesting species because it is a new record recently reported in Thailand and has not been investigated before. Crude methanol extracts of the stems and the combination of leaves and twigs of *M. sirikitiae* have been submitted for the biological activities, including cytotoxic, anti-inflammatory, anti-HIV-1, anti-microbial and antioxidant activities. The preliminary results showed that the methanol extract from the leaves and twigs showed moderate to potent cytotoxicity against P-388 (murine lymphocytic leukemia), KB (human nasopharyngeal carcinoma), Col-2 (human colon cancer), MCF-7 (human breast cancer), Lu-1 (human lung cancer) cell lines with the $ED_{50}$ value of 0.67, 5.1, 12, 2.58, 3.92 $\mu$g/mL, respectively. Both methanol extracts of stems and the combination of leaves and twigs exhibited anti-inflammatory activity via ethyl phenylpropiolate (EPP)-induced ear edema in rats, as well as they also showed anti-HIV-1 activities in the syncytium assay with $EC_{50}$ values <7.8 $\mu$g/mL and SI values from >4.28 (stem) and >2.11 (leaves and twigs) and could inhibit HIV-1 reverse transcriptase enzyme with the inhibition values of 80.27% (stem) and 64.27% (leaves and twigs) at the concentration 200 $\mu$g/mL.

Consequently, the investigation of the biological constituents from *M. sirikitiae* is promising to the discovery of new lead compounds which may be further developed to anticancer, anti-HIV-1 and anti-inflammatory drugs. Therefore, this project will contribute significantly to a scientific basis of natural product chemistry. This scientific data will be use as information for further study the development of the derivatives which have more activities and less toxicity. The researches on the investigation of the biological chemicals from natural product are important for increasing the alternative route of the medical treatment and new therapeutic agents which will be beneficial for improving human health and the quality of life.

### 1.2 Literature review

*Mitrephora* is one of genera in Annonaceae family [1]. It consists of 48 species of shrubs and small to large trees. This genus is widely distributed in southeast Asia, southern India, and north-western Australia. Eight species of *Mitrephora* have previously been recorded from Thailand, *M. maingayi* Hook. f. & Thomson (Nang-Dang), *M. wangii* Hu (Lumduandoi), *M. tomentosa* Hook .f. & Thomson (Mapuan), *M. vulpine* C.E.C. Fischer (Mapuantai), *M. winitii* Craib (Mahaphrom), *M. alba* Ridl (Phromkoa), *M. keithii* Bidl (Kray) and *M. sirikitiae* Weersooriya, Chalermglin & R.M.K. Saunders (Maha Phrom Rachini) [2].

Many plants in the genus *Mitrephora* have been revealed that they composed of many types of compounds, such as terpenoids, alkaloids, lignans, lignanamides, phenolic compounds, flavonoids and polyacetylenic compounds. Moreover, this genus exhibited many biological properties, including antimicrobial, anticancer, antimalarial and antiplatelet activating factor (PAF) receptor binding.

Terpenoids (Figure 1) have been found from many plants in the genus *Mitrephora*, including *M. maingayi*, *M. alba*, *M. glabra*, *M.celebica*, *M. thorelii*, *M. tomentosa* and *M. zippeliana.* Chemical investigation of *M. maingayi* led to the isolation of many terpenoids, e.g. isoelemicin (**1**) from the extract of bark [3] as well as spathulenol (**2**), (+)-pimaric acid (**3**), (−)-16-kauren-19-oic acid (**4**), and didymooblongin (**5**) from the extract of leaves and stem [4]. In addition, several diterpenoids, *ent*-8$\beta$-hydroxyprimar-15-en-18-oic acid (**6**), *ent*-15,16-dihydroxypimar-8(14)-en-18-oic acid (**7**), *ent*-pimara-8(14),15-dien-8-oic acid (**8**), *ent*-3$\beta$-hydroxytrachyloban-18-oic acid (**9**), *ent*-3$\beta$-hydroxytrachyloban-18-ol (**10**), methyl *ent*-3$\beta$-hydroxytrachyloban-18-ote (**11**), *ent*-trachyloban-18-oic acid (**12**), *ent*-trachyloban-3$\beta$,19-diol (**13**), *ent*-trachyloban-3$\beta$,18-diol (**14**), and *ent*-trachyloban-3$\beta$-ol (**15**), were found in the hexane extract of *M. alba*. All compounds were evaluated for cytotoxicity against three cancer cells, NCI-H187 (human small cell lung cancer), KB (human carcinoma of the nasopharynx), and MCF7 (human breast cancer). Methyl *ent*-3$\beta$-hydroxytrachyloban-18-ote (**11**), *ent*-trachyloban-3$\beta$,18-diol (**14**), and *ent*-3$\beta$-hydroxytrachyloban-18-ol (**10**) showed cytotoxic activity against NCI-H187 cell line with $IC_{50}$ of 47.2, 49.8, and 55.9 μM, respectively. Moreover, *ent*-trachyloban-3$\beta$,18-diol (**14**) had moderate activity against KB cell lines with $IC_{50}$ value of 62.1 μM. Ellipticine and doxorubicin were used as positive controls with $IC_{50}$ values of 4.3 and 0.2 μM against NCI-

H187 cells and $IC_{50}$ values of 1.8 and 1.1 μM against KB cells. [5] Three terpenoids, 4-epi-kaurenic acid (**16**), mitrekaurenone (**17**), and methylmitrekeurenate (**18**), were found in the stem and bark extract of *M. glabra* [6]. Moreover, four *ent*-trachylabanes, *ent*-trachyloban-18-oic acid (**12**), mitrephorone A-C (**19-21**), were isolated from *M. glabra*. All compounds were evaluated for cytotoxicity against a panel of cancer cells, KB, MCF-7, NCI-H187, H460 (human large cell lung carcinoma), SF-268 (human astrocytoma), and antimicrobial activity. Mitrephorone A (**19**) showed moderate cytotoxicity against all tested cancer cell lines. Mitrephorone B (**20**) had a modest cytotoxic activity against KB cells, but was not toxic to the other cell lines. Mitrephorone C (**21**), and *ent*-trachyloban-18-oic acid (**12**) lacked discernible cytotoxicity against any of four cancer cell lines. *Ent*-trachyloban-18-oic acid (**12**) was inactive in antimicrobial activity against *Micrococcus luteus*, while the other compounds (**19-21**) were inactive in antimicrobial activities against bacteria (*Micrococcus luteus* and *Mycobacterium smegmatis*), a yeast (*Saccharomyces cerevisiae*), and a fungus (*Aspergillus niger*) [7]. In addition, Four terpenoids, *ent*-trachyloban-19-oic acid (**22**), *ent*-kaur-16-en-19-oic acid (**23**), 8(14),15-pimaradien-18-oic acid (**24**), and 7,15-pimaradien-18-oic acid (**25**) were isolated from the stem bark extract of *M. celebica*. Two of them were found to be responsible for the antimicrobial activity. Compounds **22** and **23** showed antibacterial activity against methicillin-resistant *Staphylococcus aureus* and *Mycobacterium smegmatis* [8]. The chemical constituents of *M. thorelii* were reported. One sesquiterpene, thorelinin (**26**) was found in the stem extract [9] while two clerodane-type diterpenes, 6$\alpha$,16,18-trihydroxycleroda-3(4),13(14)-dien-15,16-olide (**27**) and 16-hydroxycleroda-3(4), 13(14)-dien-15,16-olide (**28**) were isolated from the aerial parts of this plant. Compounds **27** and **28** exhibited inhibitory activity against the proliferation of human hepatoma BEL-7402 cells *in vitro*. Compound **28** also showed an anti-tumor effect against the growth of hepatoma H22 in mice [10]. Moreover, two diterpenes, 8$\beta$-hydroxypimar-15-en-18-oic acid (**29**), and *ent*-kaur-16-en-19-oic acid (**23**) have been isolated from the bark of *M. tomentosa* [11]. Monoterpenoids and sesquiterpenoids, spatulenol (**2**), limonene (**30**), $\alpha$-pinene (**31**), $\beta$-pinene (**32**), aromadendrene (**33**), $\alpha$-humulene (**34**), $\beta$-caryophyllene (**35**), caryophyllene oxide (**36**), bicyclogermacrene (**37**), $\alpha$-copaene (**38**), ar-curcumene (**39**), $\beta$-curcumene (**40**), and $\delta$-3carene (**41**), were obtained from the leaves of *M. zippeliana* [12].

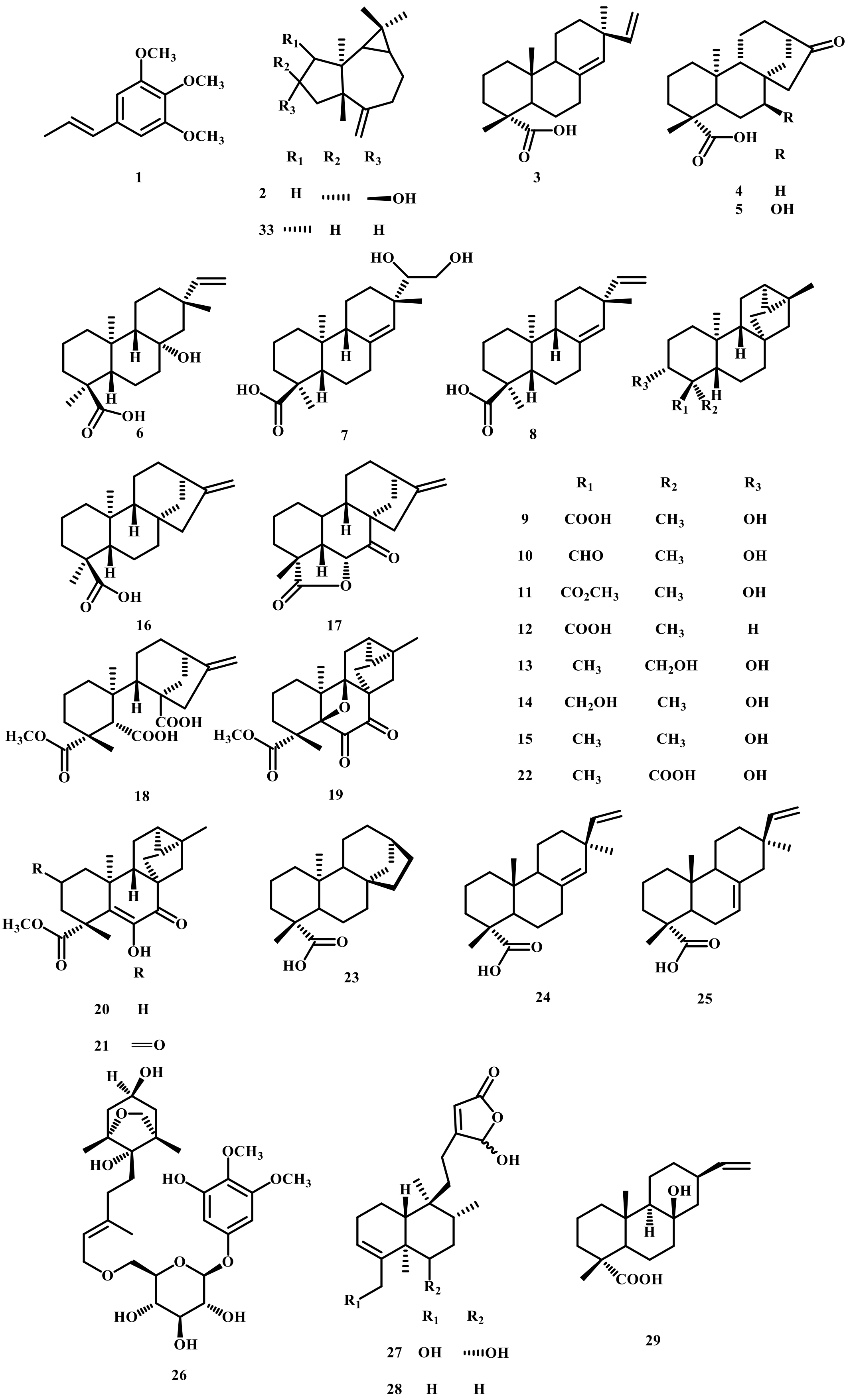
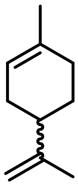



**Figure 1** Terpenoids isolated from *Mitrephora* genus



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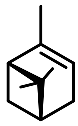

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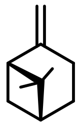

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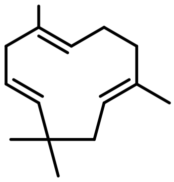

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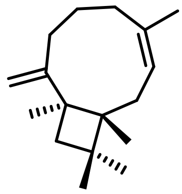

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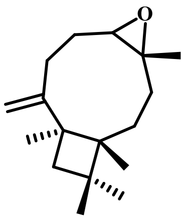

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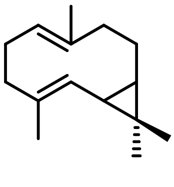

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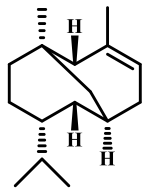

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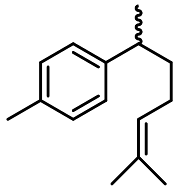

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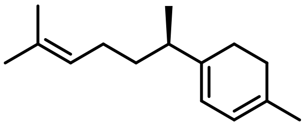

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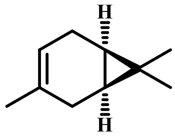
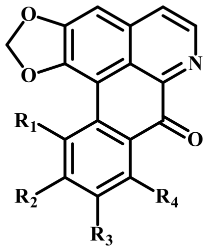


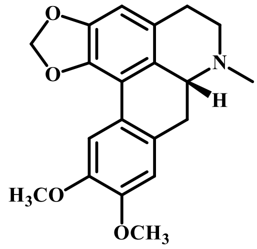
**41**

**Figure 1** Terpenoids isolated from *Mitrephora* genus (cont.)

Alkaloids (Figure 2) have been found from many species in the genus *Mitrephora*. *M. maingayi* was revealed to contain various alkaloids. Liriodenine (**42**) and oxostephanin (**43**) were isolated from the leaves of this plant [4]. Dicentrinone (**44**), dicentrinine (**45**), glaucine (**46**), and maingayinine (**47**) were isolated from the twigs [13]. Moreover, 1,2,3-trimethoxy-5-oxonoraporphine (**48**), 1,2-dimethoxy-3-hydroxy-5-oxonoraporphine (**49**), ouregidione (**50**), and 3-methoxy-cepharadione B (**51**) were separated from the bark [3]. Liriodenine (**42**) was also separated from the stem bark of *M. glabra* and showed a significant antimicrobial activity and cytotoxicity against four cell lines, including KB, MCF-7, NCI-H460, and SF-268 [6]. The chemical study of stem of *M. thorelii* [9] and twigs of *M. vulpine* [14] led to the isolation of liriodenine (**42**), and oxoputerine (**52**). In addition, *M. diversifolia* roots extract was reported to consist of azafluorenone alkaloids, 5,8-dihydroxy-6-methoxyonychine (**53**) and 5-hydroxy-6-methoxyonychine (**54**). Both of them showed antimalarial activity against the *Plasmodium falciparum* strains 3D7 and Dd2 [15]. The separation of the methanol extract of *M. wangii* gave two alkaloids, magnoflorine (**55**), and corytuberine (**56**) [16].



| | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| 42 | H | H | H | H |
| 43 | H | H | H | $OCH_3$ |
| 44 | H | $OCH_3$ | $OCH_3$ | H |
| 52 | $OCH_3$ | H | H | H |



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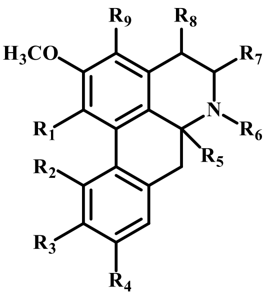
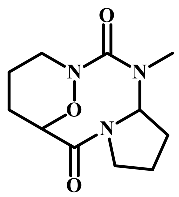


| | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | $R_6$ | $R_7$ | $R_8$ | $R_9$ |
|---|---|---|---|---|---|---|---|---|---|
| 46 | $OCH_3$ | H | $OCH_3$ | $OCH_3$ | ''''H | $CH_3$ | H | H | H |
| 48 | $OCH_3$ | H | H | H | ◄H | H | O | H | $OCH_3$ |
| 49 | $OCH_3$ | H | H | H | ◄H | H | H | H | OH |
| 56 | OH | OH | $OCH_3$ | H | ''''H | $CH_3$ | H | H | H |



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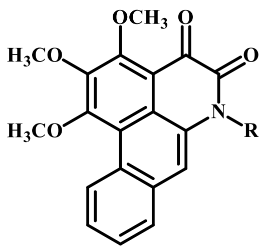
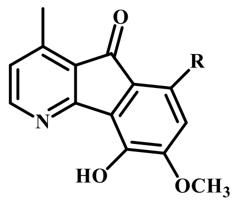
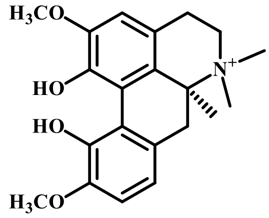


| | R |
|---|---|
| 50 | H |
| 51 | $CH_3$ |



| | R |
|---|---|
| 53 | OH |
| 54 | H |



55

**Figure 2** Alkaloids isolated from *Mitrephora* genus

Lignans (Figure 3), (+)-epieudesmin (**57**), eudesmin (**58**), and magnone A (**59**) have been found from the extract of *M. maingayi* [4]. The methanol extract of the twigs of *M. vulpina* was reported to consist of phylligenin (**60**). Phylligenin showed strong inhibitory effect on PAF receptor binding [14]. The methanol extract of *M. wangii* was found to consist of three dihydrobenzofuran neolignans, conocarpan (**61**), methyl conocarpan (**62**), and 3′-methoxy conocarpan (**63**) [16]. Lignanamides (figure 2.3), thoreliamide A-C (**64-66**), cannabisin F (**67**), and cannabisin G (**68**) were isolated from the stem of *M. thorelii* [9].

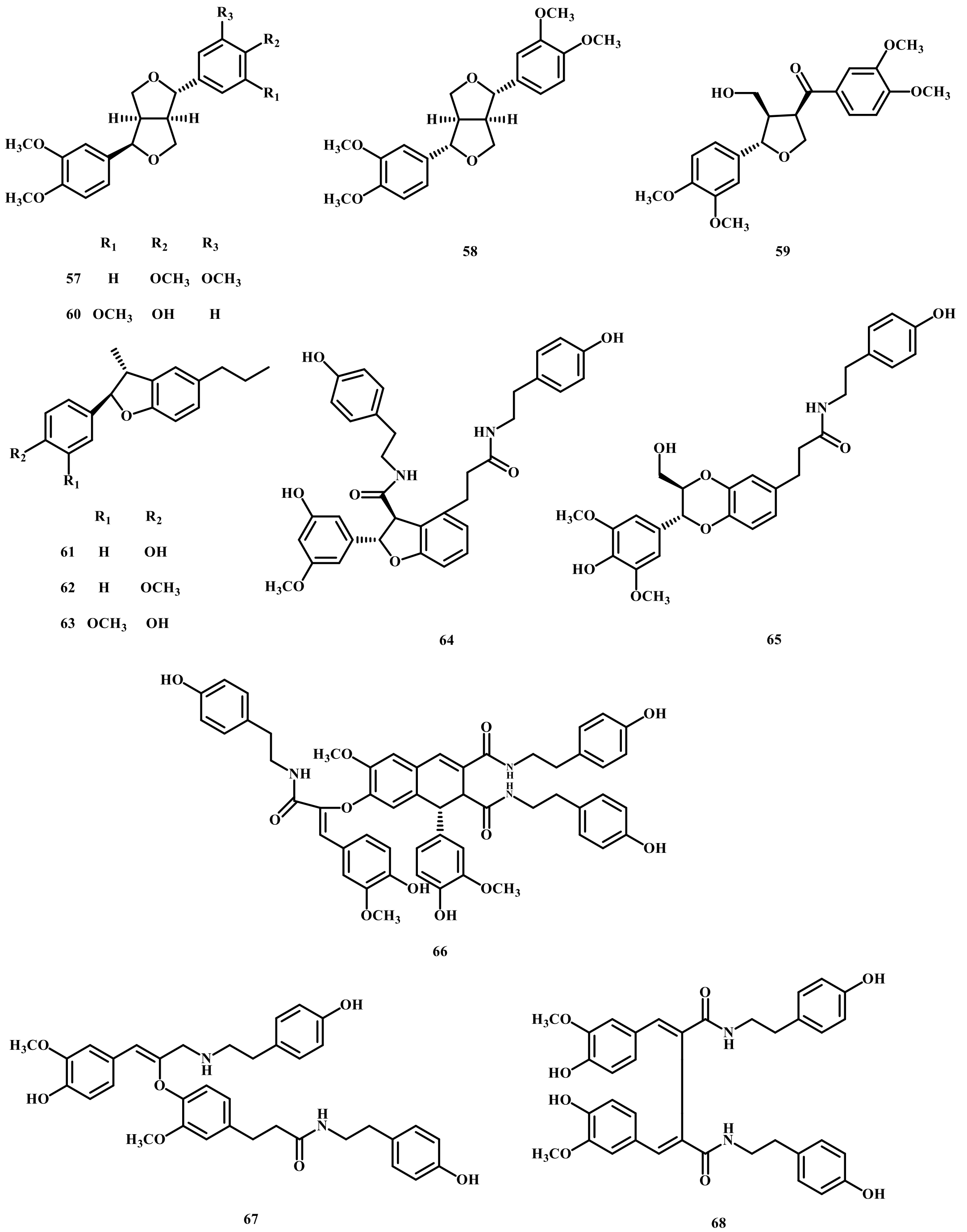



**Figure 3** Lignans and lignanamides isolated from *Mitrephora* genus

Polyacethylenic compounds (Figure 4) were isolated from *M. glabra*, *M. celebica*, *M. tomentosa*, and *M. wangii*. Oropheolide (**69**), 9,10-dihydrooropheolide (**70**), octadeca-9,11,13-triynoic acid (**71**), methyloropheate (**72**), and oropheic acid (**73**) were found in the stem bark of *M. glabra*. Among those, compound **70** and **72** exhibited antimicrobial activity [6]. The chemical study of the bark of *M. celebica* led to the isolation of oropheic acid (**73**), and 13,14,dihydrooropheic acid (**74**) [17]. 13,14-Dihydrooropheic acid (**74**) was also separated from the bark of *M. tomentosa*. Linoleic acid (**75**) was separated from the methanol extract of *M. wangii* [16].

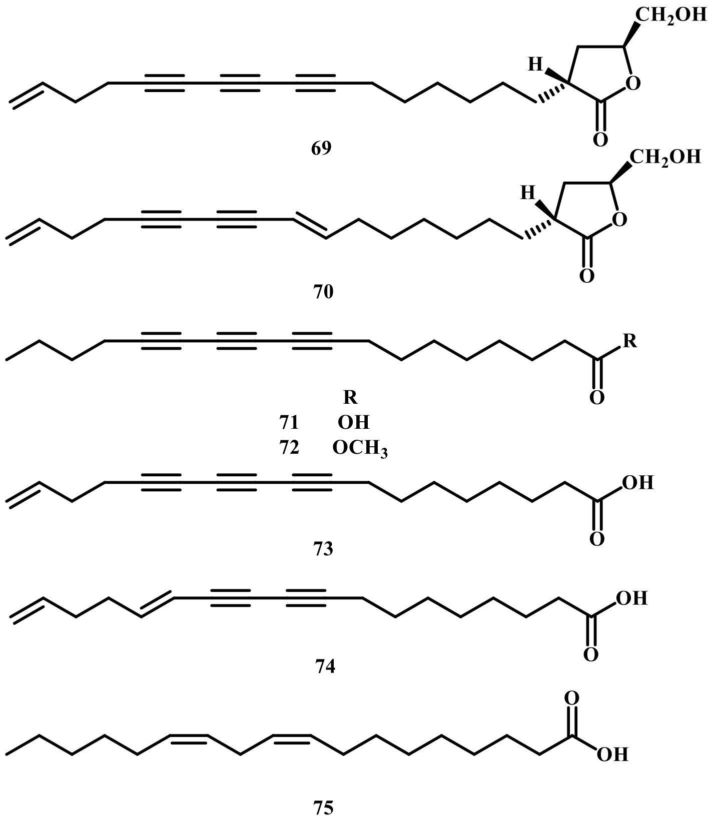
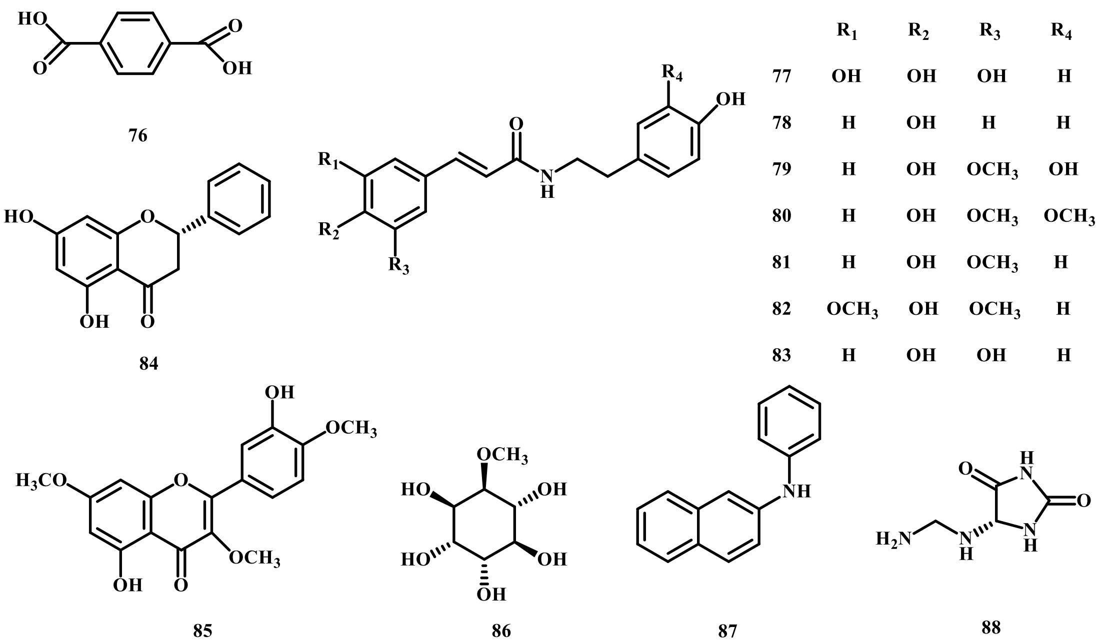



**Figure 4** Polyacethylenic compounds isolated from *Mitrephora* genus

Phenolic compounds (Figure 5) have been found in *M. maingayi*, *M. thorelii*, and *M. wangii*. One phenolic acid, terephthalic acid (**76**), was isolated from the twigs of *M. maingayi* [13]. The stem of *M. thorelii* has been reported to consist of six phenolic amides, *N*-*trans*-coffeoyltyramine (**77**), *N*-*trans*-coumaroyltyramine (**78**), *N*-*trans*-feruloyldopamine (**79**), *N*-*trans*-feruloyl-3-methyldopamine (**80**), *N*-*trans*-feruloyltyramine (**81**), and *N*-*trans*-sinapoyltyramine (**82**) [9]. In addition, two phenolic amide, *N*-caffeoyltyramine (**83**), and *N*-*trans*-coumaroyltyramine (**78**), were found in the methanol extract of *M. wangii* [16].

Furthermore, two flavonoids (Figure 5) found in the leaves [4] and twigs [13] of *M. maingayi*, were pinocembrin (**84**), and ayanin (**85**), respectively. In addition, quebrachitol (**86**), nepthylamine (**87**), and allantoin (**88**) were isolated from the twigs of *M. maingayi* [13]. Allantoin (**88**) and quebrachitol (**86**) were also found in methanol extract of *M. wangii* [16]. Moreover, quebrachitol (**86**) was found in twigs of *M. vulpina* and showed strong inhibitory effect on PAF receptor binding [14].



| | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| 77 | OH | OH | OH | H |
| 78 | H | OH | H | H |
| 79 | H | OH | $OCH_3$ | OH |
| 80 | H | OH | $OCH_3$ | $OCH_3$ |
| 81 | H | OH | $OCH_3$ | H |
| 82 | $OCH_3$ | OH | $OCH_3$ | H |
| 83 | H | OH | OH | H |

**Figure 5** Phenolic compounds and others isolated from *Mitrephora* genus

*Mitrepora sirikitiae* Weerasooriya, Chalermglin & R.M.K. Saunders is a new species in the genus *Mitrephora* and endemic to Thailand. This plant was given a royal name because it was found in 2004, the year of 6$^{th}$ cycle (72$^{nd}$ birthday) of the Queen of Thailand, Queen Sirikit. The common name of this plant is Maha Phrom Rachini.

**Figure 6** Flowers and fruits of *Mitrephora sirikitiae* [2]

*M. sirikitiae* was found on the peak of mountain (1,100 metres) in Mae-surin waterfall national park, Mae Hong Sorn Province. It is most similar to *M. winitii* (Mahaphrom), but differs in its larger flowers. It is small-sized tree, 4-6 metres in height. The leaf is 11-19 cm in long and 4-9 cm in wide. The flower, when in bloom, is 8-10 cm in diameter, making it the largest of the 8 species found in Thailand. There is an inner and outer layer of petals. At maturity, the flower has a distinctive smell and the inner petals become undulate, while the end still close in each other [2]. There is no report on chemical constituent and biological activity of this plant.

Preliminary biological screening studies of the methanol extracts of this plant showed that the extracts has anti-inflammatory, cytotoxic, anti-HIV-1, and amyloid-$\beta$ aggregation inhibitory activities. Consequently, this research focused on the investigation of bioactive compounds from the methanol extract of *M. sirikitiae* stem.

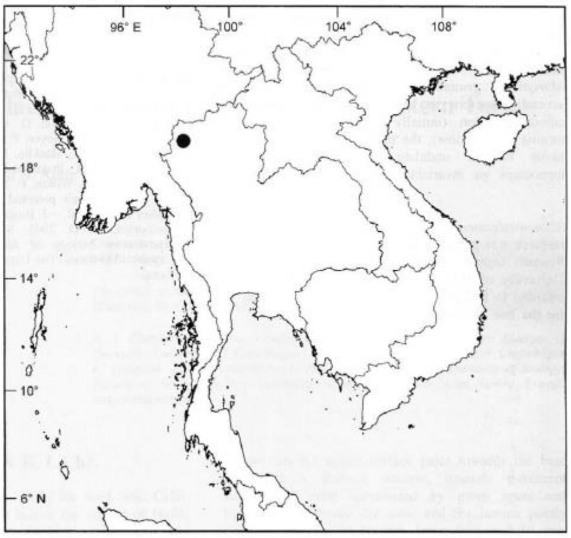



**Figure 7** Geographical distribution of *Mitrephora sirikitiae* in Mae Hong Sorn province [2]

### 1.3 Objectives

1.3.1 To isolate cytotoxic, anti-inflammatory and anti-HIV-1 constituents from the the methanol extract of stem and combination of leaves and twigs of Mitrephora sirikitiae by using chromatographic methods.

1.3.2 To elucidate the chemical structures of the isolated compounds by means of spectroscopic techniques.

# CHAPTER II

# MATERIALS AND METHODS

## 2.1 Material

### 2.1.1 Plant materials

The leaves, twigs and stems of *Mitrephora sirikitiae* Weerasooriya, Chalermglin & R.M.K. Saunders were collected from the peak of mountain in Mae-surin waterfall national park, Mae Hong Sorn Province, Thailand. The plant materials were prepared by drying at 45-50$^{o}$C and grinding to fine powder.

### 2.1.2 Chemicals and reagents

Alumina 60 basic 0.063-0.2 mm (70-230 mesh ASTM) for column chromatography, silica gel 60 $PF_{254}$ containing gypsum for chromatotron, silica gel 60 $GF_{254}$ and TLC aluminium sheets 20×20 cm were purchased from Merck (Darmstadt, Germany). Silica gel P60 (40-63 μm) and reversed phase silica gel C-18 (40-63 μm) were purchased from Silicycle (Quebec, Canada). Sephadex™ LH-20 was purchased from GE Healthcare Life Sciences (New Jersey, United States). The other chemicals were commercially available and analytical grade.

## 2.2 Methods

### 2.2.1 General methods

The melting points were determined on digital Electrothermal 9100. Optical rotations were measured with JASCO DIP-370 digital polarimeter, 50 mm microcell (1 mL). Ultraviolet spectra were obtained on SHIMADZU UV-2600 spectrophotometer. Infrared spectra were recorded using Alpha Bruker. Low resolution EI mass spectra (EI-MS) was obtained on Thermo Finnigan Polaris Q, and HR-ESI-MS was obtained on a Micromass model VQ-TOF2. The nuclear magnetic resonance experiments were carried out on Bruker AV 500 and Bruker Ascend 400 NMR spectrometers. $^{1}$H-NMR (400, 500 MHz), $^{13}$C-NMR (100, 125 MHz) and 2D correlation spectra were recorded as $\delta$ values in ppm using deuterated chloroform, deuterated methanol, and deuterated pyridine as solvents. Column chromatographic separation were carried out using aluminium oxide 60 basic 0.063-0.2 mm (70-230 mesh ASTM ) (Merck), silica gel P60 40-63 μm (SiliaFlash®; Silicycle), reversed phase silica gel C-18 40-63 μm (Silicycle) and Sephadex™ LH-20 (GE Healthcare Life Sciences) as packing materials. Moreover, the separation was also performed by preparative thin layer chromatography (PTLC) 20×20 cm silica gel 60 $F_{254}$, 1 mm (Merck), chromatotron (Herrison research, USA) and acid-base shaking techniques. TLC aluminum sheet of silica gel 60 PF 254 (Merck, layer thickness 0.2 mm) was used for analytical TLC. The TLC plates were detected under UV at wavelengths 254 and 366 nm and/or spraying with 30% sulfuric acid in methanol/or Dragendorff reagent and recorded by using CAMAG TLC Visualizer. Solvents for extraction, chromatography and recrystallization were distilled at their boiling point ranges prior to use.

### 2.2.2 Extraction

The powder of *M. sirikitiae* leaves and twigs (1.0 kg) as well as *M. sirikitiae* stems (2.0 kg) were extracted by maceration with distilled methanol, 6 L × 1 times and 4 L × 8 times (139 days) for leaves and twigs and 5 L × 1 times 4 L × 8 times (140 days) for stems. The solvent was removed by using a rotary evaporator (Buchi) and freeze dryer (FreeZone® Freeze Dry Systems; Labconco) to give the crude methanol extracts of the mixture of leaves and twigs 102.03 g and the crude methanol extracts of stems 72.37 g.

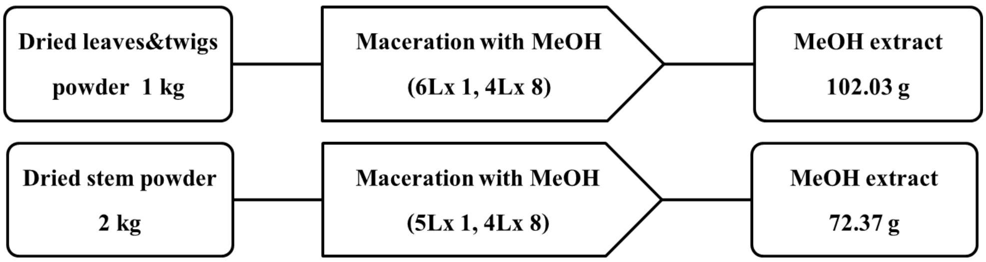



**Figure 8** Extraction of the mixture of leaves and twigs as well as the stem of *M. sirikitiae*

### 2.2.3 Isolation and purification

#### 2.2.3.1 Isolation and purification of the leaves and twigs extract

The methanol crude extract of leaves and twigs (90.0 g) was dissolved with 100 mL of MeOH and 1.2 L of water. The aqueous solution was further partitioned with hexane to give hexane fraction (20.71 g). The aqueous solution was continued to patition with ethyl acetate to yield EtOAc fraction (21.07 g). Finally, the aqueous solution was partitioned with butanol to give BuOH fraction (47.60 g) and aqueous fraction (24.20 g) (Figure 9).

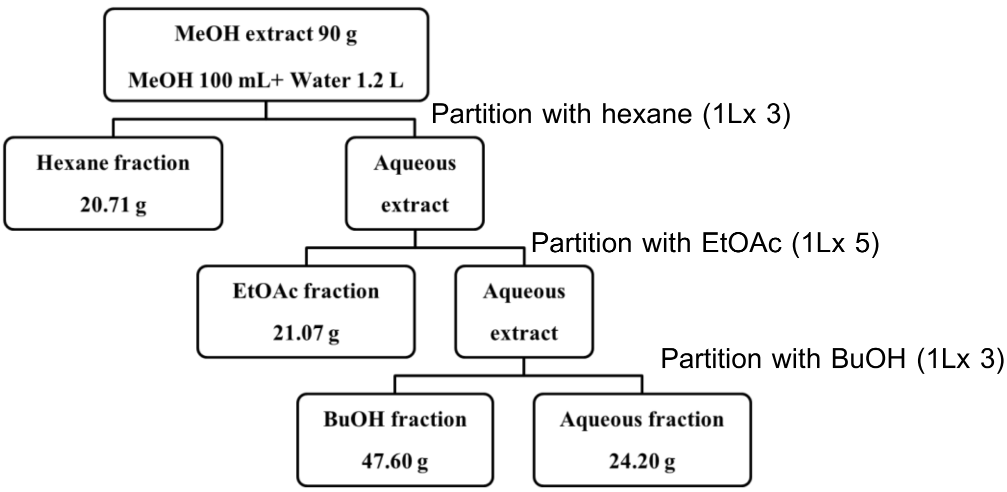



**Figure 9** The partition of methanol crude extract of leaves and twigs

The hexane and EtOAc fractions were combined and further fractionated by short column chromatography over silica gel eluting with a gradient system of hexane-acetone and MeOH to give nine fractions (A1-A9) (Figure 10).

The isolation of fraction A5 (8.66 g) by column chromatography over silica gel and eluting with gradient solvent of acetone-hexane and MeOH led to eight fractions (B1-B8). Fraction B5 was further isolated by silica gel column chromatography with MeOH-$CH_2Cl_2$ mixture to give eight fractions (C1-C8). Compound **1** (197.5 mg) was obtained as white crystals by recrystallization of fraction C4 (Figure 10). Fraction A6 (7.71 g) was separated by silica gel column chromatography with gradient solvent of MeOH-$CH_2Cl_2$ to give six fractions (D1-D6). Fraction D3 was isolated by silica gel column chromatography using gradient solvent of acetone-hexane and MeOH as mobile phase to give eight fractions (E1-E8). Compound **1** (20.1 mg) was obtained as white crystals by recrystallization of fraction E2. The recrystallization of fractions E3 and E4 led to the isolation of compound **2** as white crystals (Figure 11). Fractions D4, E6 and E7 were combined and separated by silica gel column chromatography eluting with EtOAc-hexane mixture and MeOH to give ten fractions (F1-F10). Compound **2** was also obtained from the recrystallization of fraction F7. Fraction F9 was rechromatographed over silica gel column chromatography using MeOH-$CH_2Cl_2$ to afford eight fractions (G1-G8). The purification of fraction G4 by recrystallization yielded compound **3** as white crystals. Fraction G5 was was continued to separate by column chromatography over Sephadex LH20 eluting with MeOH to give five fractions (H1-H5). The isolation of fractions H3 and H4 by PTLC gave four fractions (I1-I4) and compounds **3** and **4** were obtained from fraction I1 an I4, respectively (Figure 11). Compound **5** (25.8 mg) was obtained as a yellow solid from fraction A7. Fraction A7ML was further separated by silica gel column chromatography eluting with gradient systems of $CH_2Cl_2$-hexane and MeOH-$CH_2Cl_2$ to yield eight fractions (J1-J8). Compound **6** (51.9 mg) was obtained as a white solid from the recrystallization of fraction J6. Fraction J2 was separated by silica gel column chromatography eluting with gradient systems of $CH_2Cl_2$-hexane and MeOH-$CH_2Cl_2$ to give seven fractions (K1-K7). Fraction K5 was rechromatographed over silica gel column chromatography using MeOH-$CH_2Cl_2$ to afford six fractions (L1-L6). Compound **7** (13.9 mg) was obtained as yellow needles from fraction L3. Fraction K6 was isolated by column chromatography over Sephadex LH20 using MeOH as mobile phase to yield four fractions (M1-M4). Fraction M3 was further sepation by Sephadex LH20 column chromatography and crystallization to give 2.3 mg of compound **8** as a yellow solid (Figure 12). Fractions J3 and J4 were combined and separated by column chromatography over silica gel and eluting with gradient solvent of acetone-hexane and MeOH led to ten fractions (O1-O10). Fraction O6 was further separated by silica gel column chromatography eluting with gradient systems of $CH_2Cl_2$-hexane and MeOH-$CH_2Cl_2$ to yield seven fractions (P1-P7). Fraction P3 was isolated by Sephadex LH20 column chromatography using MeOH as mobile phase to yield five fractions (Q1-Q5). Fractions Q3 and I3 were combined and rechromatographed over silica gel column chromatography using MeOH-$CH_2Cl_2$ followed by recrystallization to give compound **4** (12.4 mg) as white crystals. Fraction O7 was separated by silica gel column chromatography with acetone-hexane mixture to give seven fractions (S1-S7). Fraction S5 was further separated by silica gel column chromatography eluting with gradient systems of MeOH-$CH_2Cl_2$ to yield six fractions (T1-T6). Fractions T3 and T5 were isolated by column chromatography over Sephadex LH20 using MeOH as mobile phase to yield three fractions (U1-U3) and six fractions (V1-V6), respectively. Fractions U2 and V2 were combined and rechromatographed by Sephadex LH20 column chromatography to give three fractions (W1-W3). Fraction W2 was further separated by silica gel column chromatography eluting with gradient systems of EtOAc-$CH_2Cl_2$ and MeOH-EtOAc to yield nine fractions (X1-X9). Compounds **9** (141.2 mg) and **10** (9.6 mg) were obtained as pale yellow semisolids from fractions X4 and X7, respectively. Fraction V3 was purified by Sephadex LH20 column chromatography eluting with MeOH to give pure compound **11** as a pale yellow semisolid (Figure 13). Fraction A8 (7.52 g) was isolated by silica gel column chromatography with MeOH-$CH_2Cl_2$ mixture to give eight fractions (Z1-Z8). Fraction Z3 was recrystallized to give compound **8** (2.4 mg) as yellow needles and fraction Z3ML was further subjected to silica gel column chromatography eluting with gradient systems of EtOAc-hexand and MeOH to yield seven fractions (AA1-AA7). Fraction AA7 was isolated by Sephadex LH20 column chromatography eluting with MeOH to yield seven fractions (AB1-AB6). The purification of a solid isolated from fraction AB4 by PTLC gave compound 8 (8.7 mg) as yellow needdles. Fraction AB4ML was separated by PTLC to yield four fractions (AC1-AC4). Fraction AC3 was further isolated by PTLC to give three fractions (AD1-AD3). The separation of fractions AD2 and AD3 by silica gel column chromatography with MeOH-$CH_2Cl_2$ mixture and followed by recrystallization to give compound **12** (5.7 mg) as yellow needles (Figure 14). Fraction Z7 was rechromatographed by column chromatography over silica gel eluting with MeOH-$CH_2Cl_2$ mixture and finally by recrystallization to give compound **13** as orange needles (Figure 15).

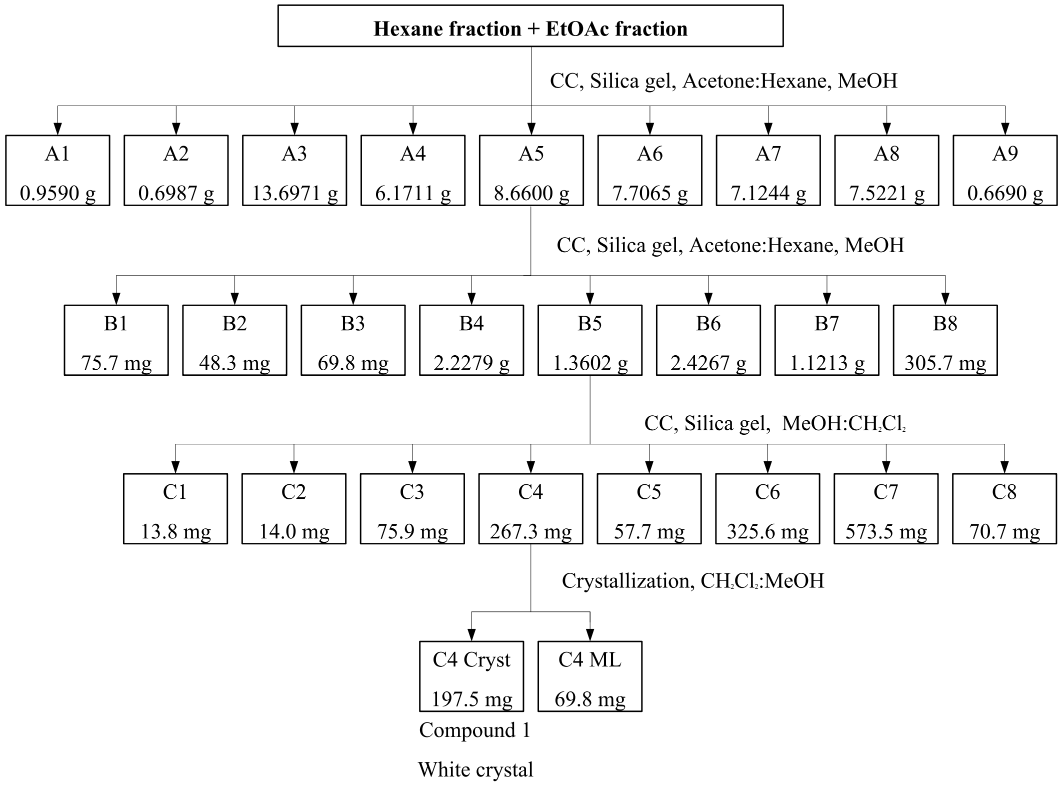



**Figure 10** The isolation of fraction A5

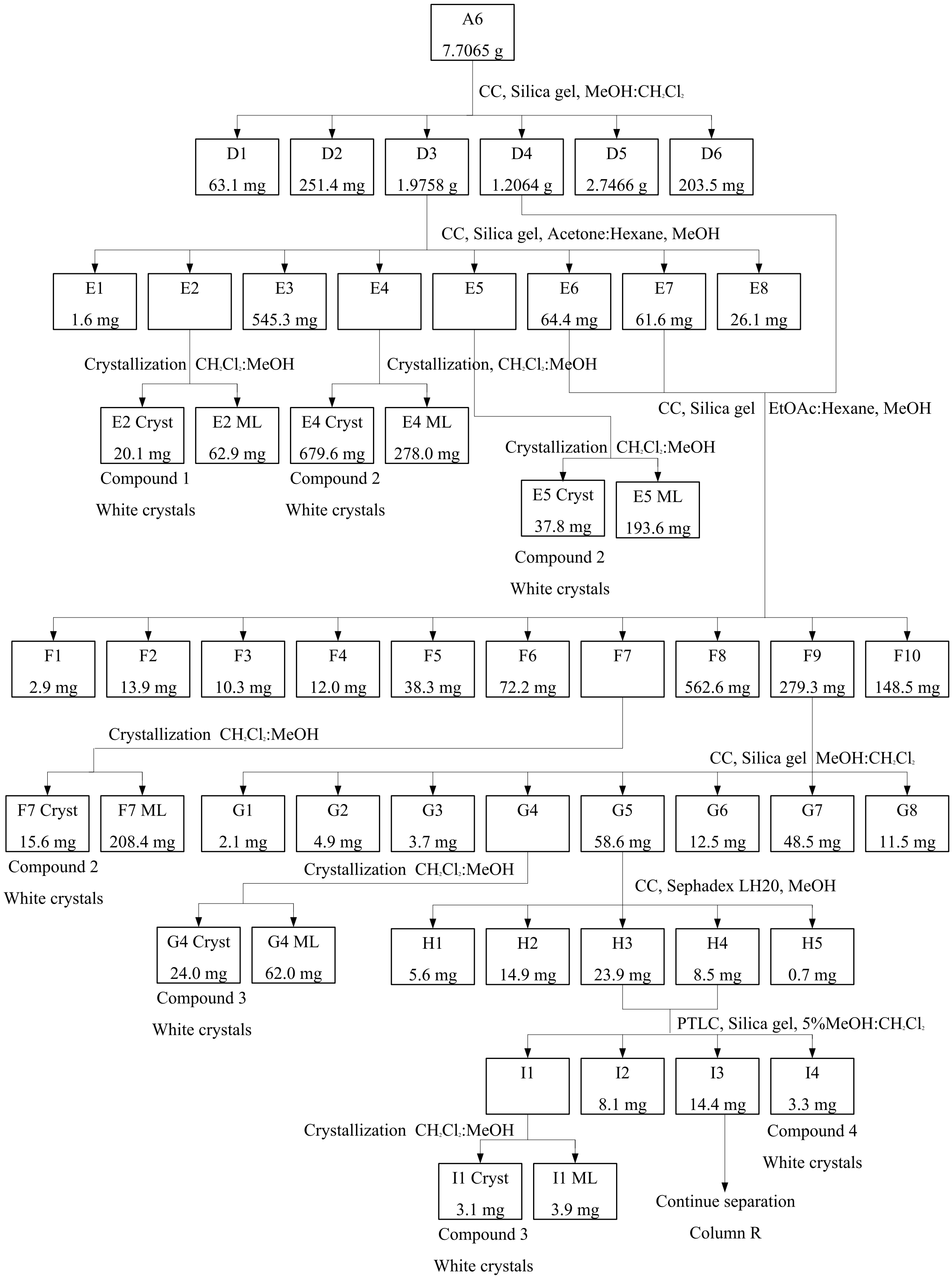



**Figure 11** The isolation of fraction A6

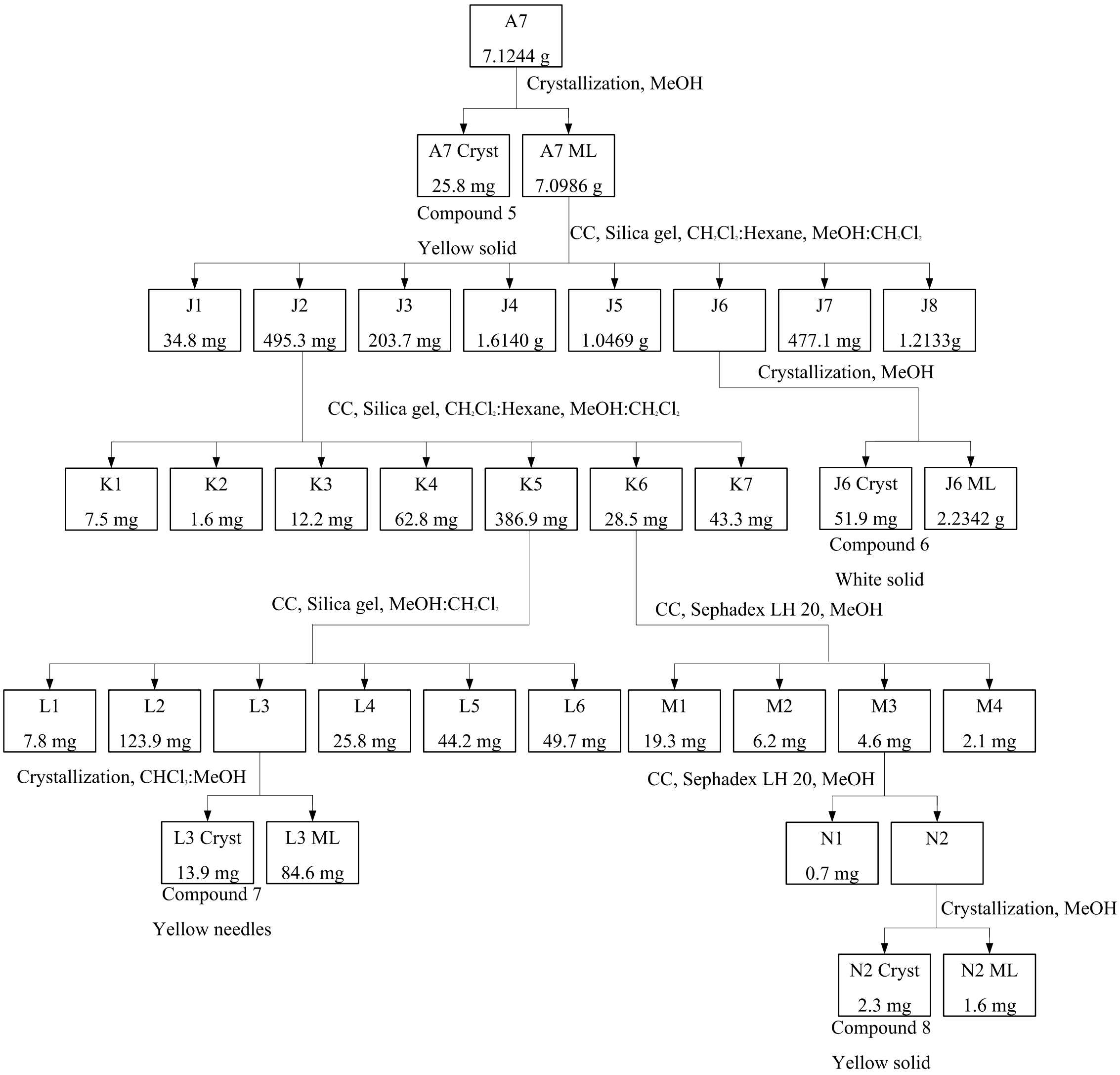



**Figure 12** The isolation of fraction A7

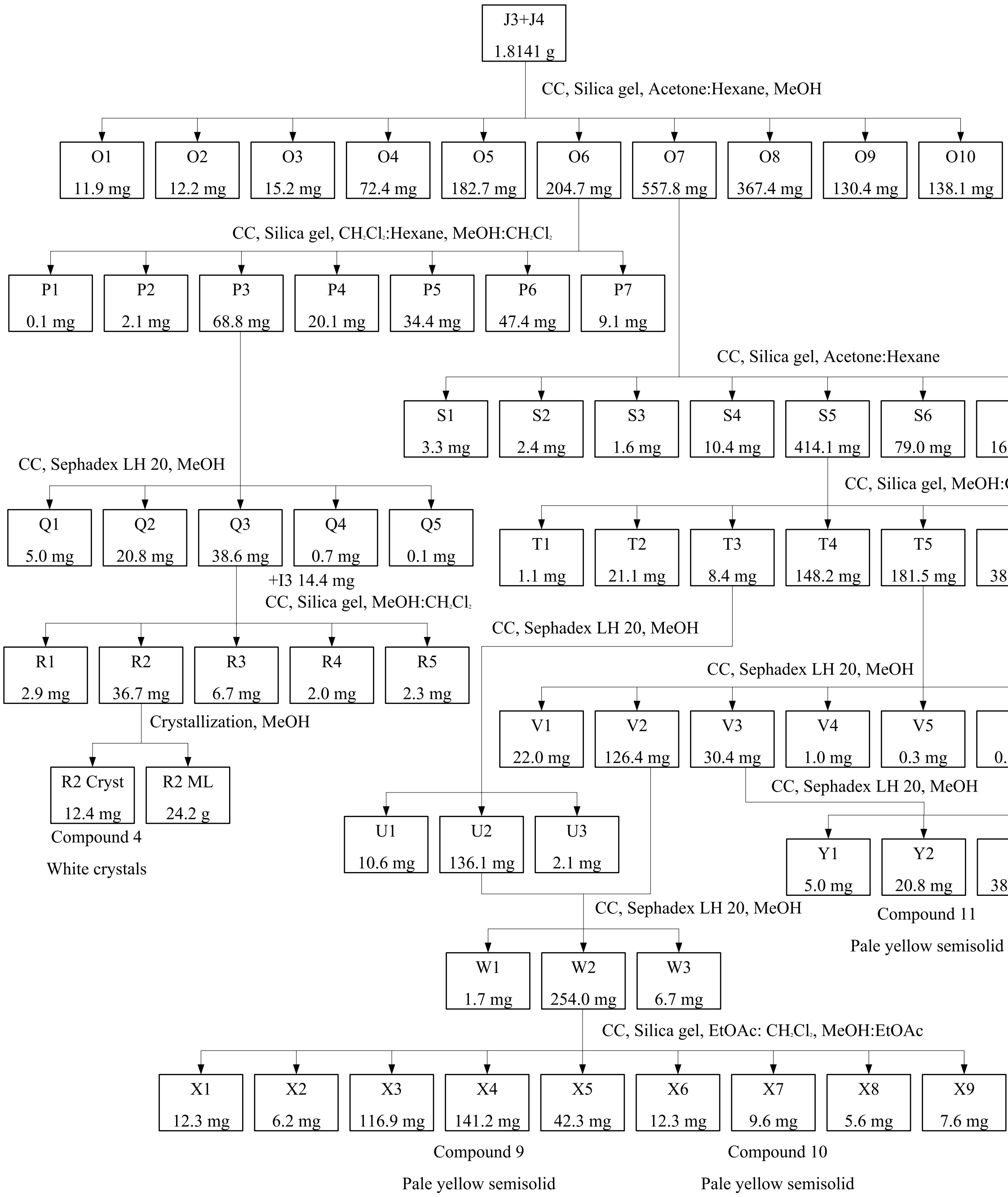



**Figure 13** The isolation of fractions J3 and J4

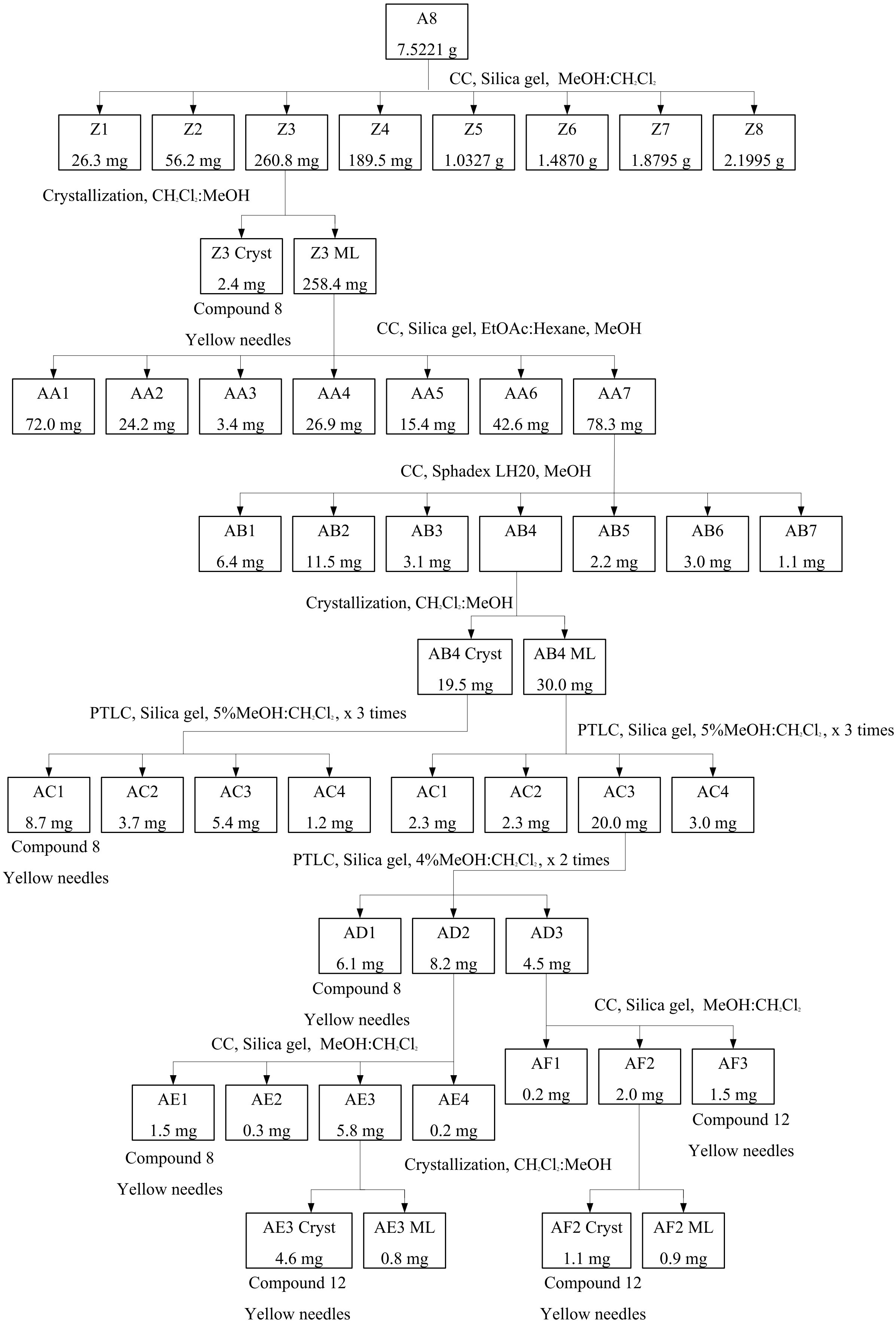



**Figure 14** The isolation of fraction A8

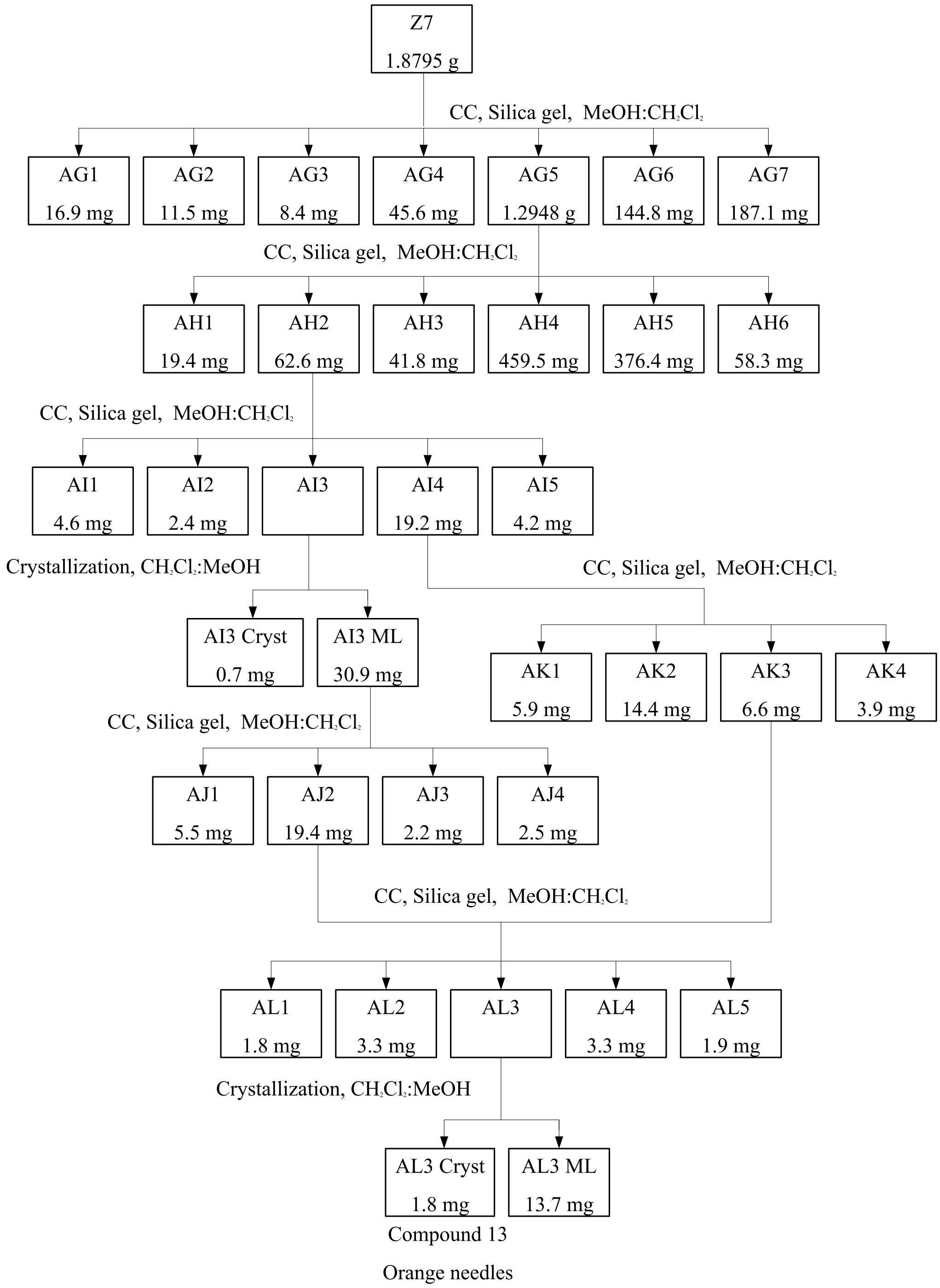



**Figure 15** The isolation of fraction Z7

### 2.2.3.2 Isolation and purification of the stem extract

The crude methanol extract was extracted by flash column chromatography over silica gel eluting with a gradient system of dichloromethane-methanol to give six fractions (A1-A6). Fraction A1 was further isolated by silica gel column chromatography, eluting with gradient systems of acetone-hexane and MeOH-acetone to give five fractions (B1-B5). Fraction B1 was recrystallized from hexane-MeOH to yield the mixture of compounds **14** and **15** as colorless crystals. Fraction A2 was separated by silica gel column chromatography using $CH_2Cl_2$-hexane and MeOH-$CH_2Cl_2$ gradient to afford nine fractions (C1-C9). The mixture of compounds **16** and **17** was obtained as white needles by recrystallization of fraction C4. Fraction C6 was isolated by silica gel column chromatography, eluting with $CH_2Cl_2$-hexane and MeOH-$CH_2Cl_2$ gradient to yield four fractions (D1-D4). Fraction D2 was separated by silica gel column chromatography, with $CH_2Cl_2$-hexane and MeOH-$CH_2Cl_2$ gradient as eluent, to give four fractions (E1-E4). Fraction E1 was rechromatographed over silica gel column chromatography, eluting with $Me_2CO$-hexane and MeOH-$Me_2CO$ gradient to afford four fractions (F1-F4). Fraction F2c1 and F2c2 were recrystallized from $CH_2Cl_2$-MeOH to yield compound **1** as white needles and compound **18** as a white powder, respectively. Fraction F2ML was purified by silica gel column chromatography, using acetone-hexane and MeOH-acetone gradient to give five fractions (G1-G5). Fraction G3c was separated by silica gel column chromatography, with acetone-hexane and MeOH-acetone gradient as eluent, to afford three fractions (H1-H3). Compound **1** was obtained as white needles by recrystallization of fraction H2c. Fraction E2ML was purified by silica gel column chromatography with MeOH-$CH_2Cl_2$ gradient to yield five fractions (I1-I5). Fractions I2 and I3ML were combined and further separated by silica gel column chromatography, with acetone-hexane and MeOH-acetone gradient as eluent, to give five fractions (J1-J5). Fractions E2c, I3c and J3c were combined and then purified by PTLC to give three fractions (K1-K3). Fractions K1 and K2 were identified to be two alkaloids, compounds **7** and **19**, respectively. Fraction D3 was separated by silica gel column chromatography, eluting with MeOH-$CH_2Cl_2$ gradient to give four fractions (L1-L4). Fraction L3 was recrystallized from $CH_2Cl_2$-MeOH to yield compound **7** as yellow needles. Fraction L3ML was purified by sephadex LH-20 column chromatography, with MeOH as eluent, to yield three fractions (M1-M3). Fraction A3 was separated by silica gel column chromatography, with acetone-hexane and MeOH-acetone gradient as eluent, to give eight fractions (N1-N8). Fraction N5 was recrystallized from $CH_2Cl_2$-MeOH to yield compound **6** as a white powder. Fractions N4, N5ml and N6 were combined and purified by silica gel column chromatography, eluting with MeOH-$CH_2Cl_2$ gradient to give six fractions (T1-T6). Compound **6** was recrystallized from fraction T5. Fractions B5 and N3 were combined and separated by sephadex LH-20 column chromatography, eluting with MeOH to give four fractions (O1-O4) then the fraction O2 was rechromatographed over sephadex LH-20 column chromatography using MeOH to afford two fractions (P1-P2). Fraction P2 was continued to separate by column chromatography over reverse phase silica gel C-18 using MeOH-$H_2O$ gradient as eluent to give three fractions (Q1-Q3). Fraction Q2 was purified by chromatotron over silica gel to afford two fractions (R1-R2). Fractions R2 and M3 were combined and purified by column chromatography over basic aluminiumoxide 60, using $CH_2Cl_2$-hexane and MeOH-$CH_2Cl_2$ gradient to yield 3 fractions (S1-S3). Fraction A4 was separated by silica gel column chromatography, eluting with MeOH-$CH_2Cl_2$ gradient to give three fractions

(U1-U3). Fraction U2 was continued to separate by column chromatography over silica gel, using $CH_2Cl_2$-hexane and MeOH-$CH_2Cl_2$ gradient, to give three fractions (V1-V3). Fraction V2 was separated by sephadex LH-20 column chromatography eluting with MeOH to give three fractions (W1-W3). Fractions S2 and W2 were combined and then separated by column chromatography over sephadex LH-20 eluting with MeOH to give two fractions (X1-X2). Fraction X2 was purified by PTLC to give three fractions (Y1-Y3). Compound **12** was obtained as orange needles by recrystallization of fraction Y1. Fraction U2 was further purified by acid-base extraction to afford four fractions (Z1-Z4). Fraction Z1ml was continued to separate by column chromatography over reverse phase silica gel C-18, eluting with MeOH-$H_2O$ to give three fractions (AA1-AA3). Fraction AA2 was further purified by PTLC to give three fractions (AB1-AB3). Fraction AB1 was recrystallized to yield compound **20**. Fraction Z2 was purified by column chromatography over sephadex LH-20 using $CH_2Cl_2$-MeOH to give four fractions (AC1-AC4). Fractions AC3 and AC4 were combined and further separate by PTLC to give compound **20** as yellow needles.

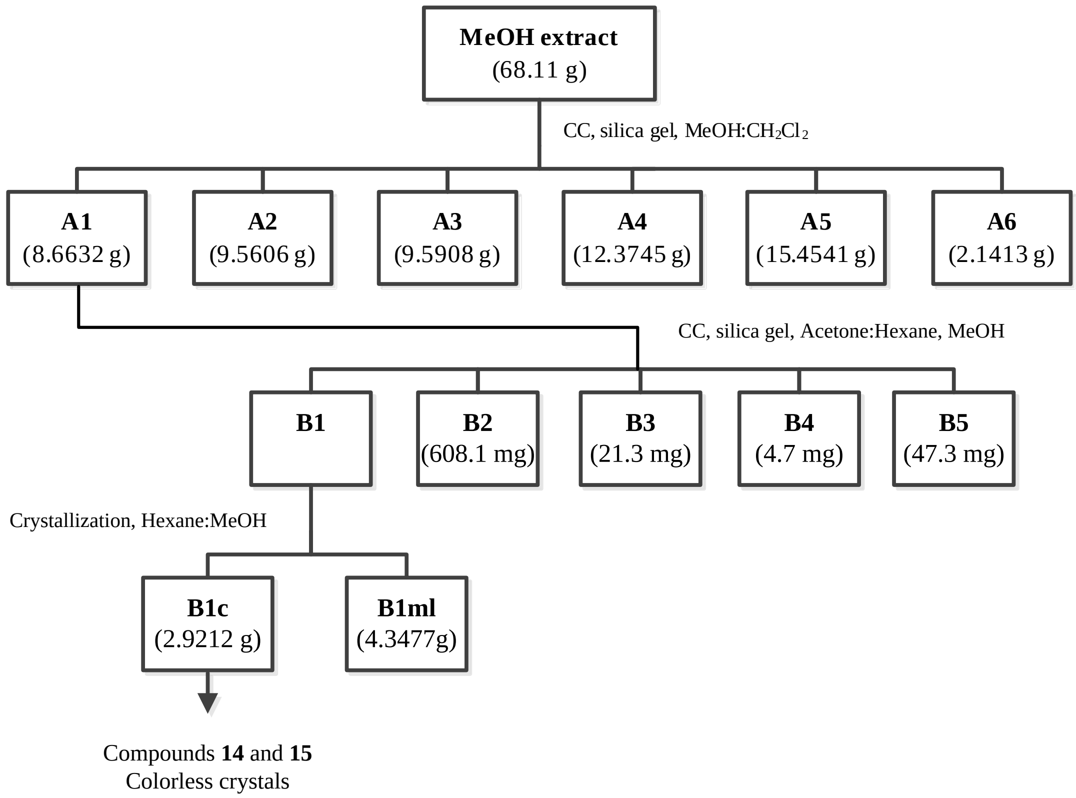



**Figure 16** The isolation of MeOH extract and fraction A1

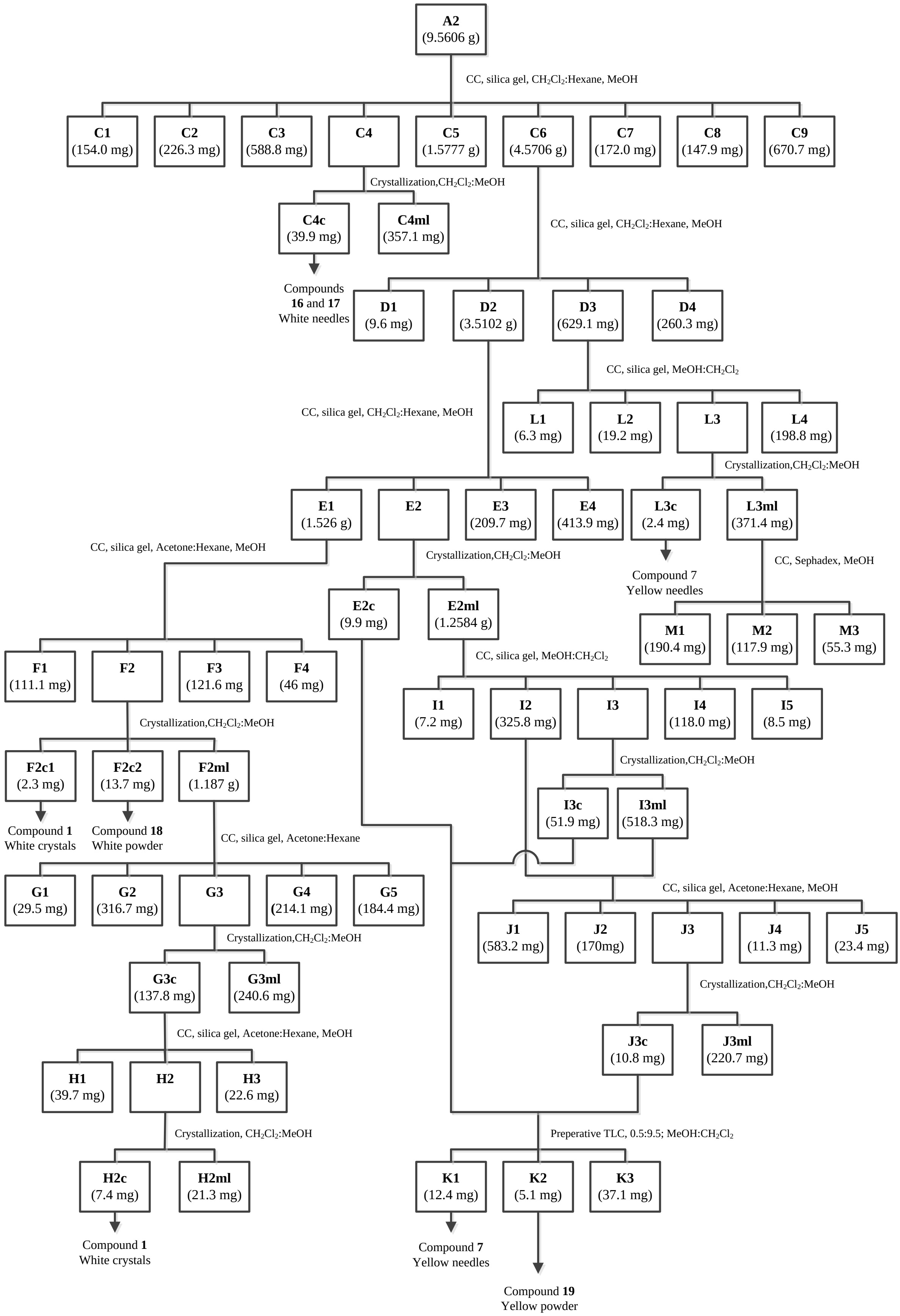



**Figure 17** The isolation of fraction A2

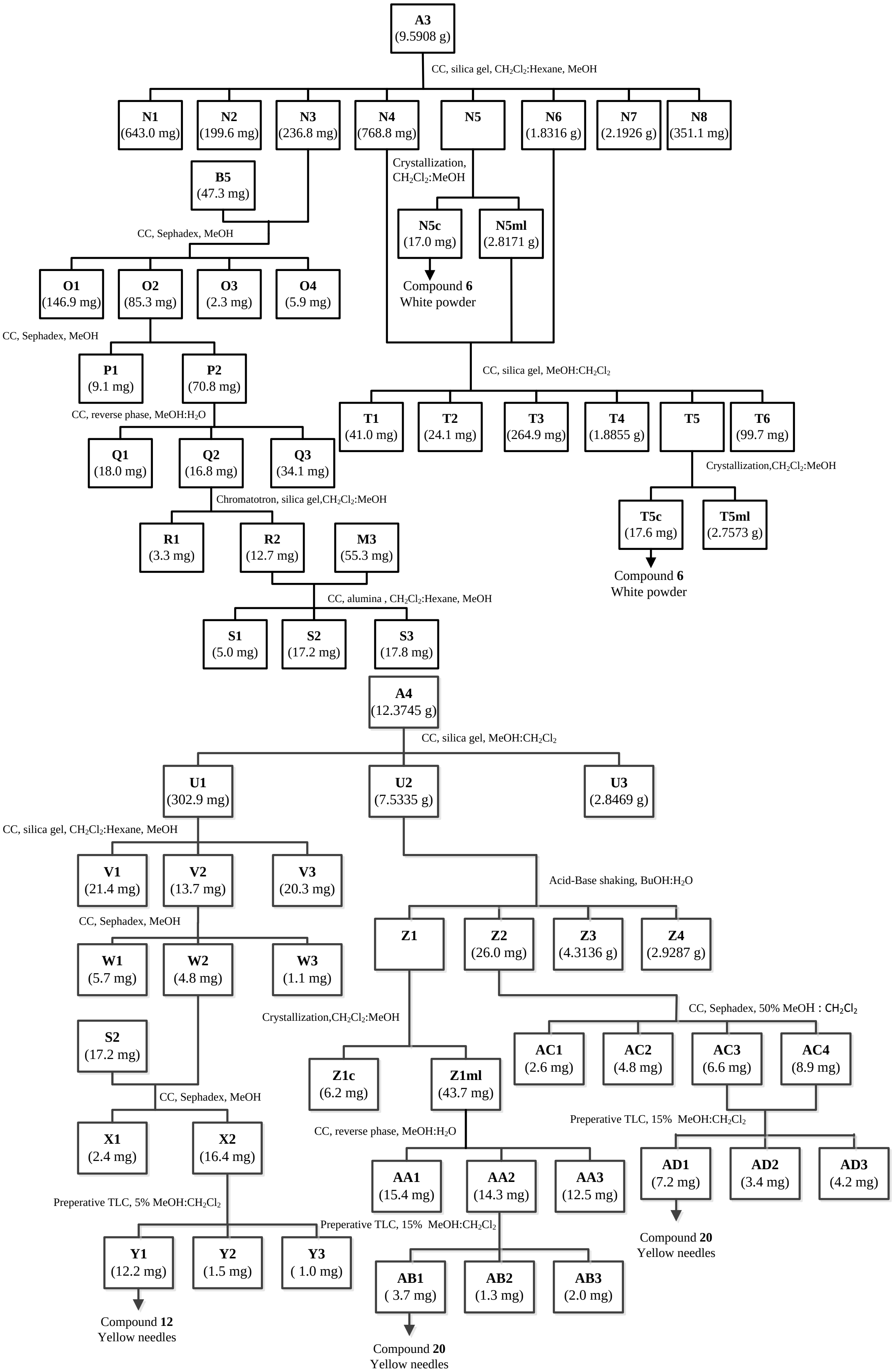



**Figure 18** The isolation of fractions A3 and A4

### 2.2.4 Structure elucidation

The chemical structures of the isolated compounds will be determined by means of spectroscopic methods, including IR (Infrared Spectroscopy), UV (Visible-Ultraviolet Spectroscopy), NMR (Nuclear Magnetic Resonance Spectroscopy) and MS (Mass Spectrometry).

### 2.2.5 Biological activities

The crude extracts were tested for biological activities, cytotoxic, anti-inflammatory and anti-HIV-1 activities. (tested by colaboratory)

- Cytotoxicity assay: The cytotoxic assays were performed by using the colorimetric method (*in vitro* Sulforhodamine B (SRB) assay in 96-well microtiter plates), and ellipticine was used as a positive control. The cell lines used in the experiment are P-388 (murine lymphocytic leukemia), KB (human nasopharyngeal carcinoma), Col-2 (human colon cancer), MCF-7 (human breast cancer), Lu-1 (human lung cancer) and ASK (rat glioma) cell lines.
- Anti-HIV-1 assay: Anti-HIV-1 RT and Syncytium assays were tested by using Nevirapine and AZT as positive controls.
- Antiinflammatory assay: Ethyl phenylpropiolate (EPP)-induced ear edema in rats was used for evaluation of anti-inflammatory activity using phenylbutazone as a positive control.

The fractions from partition and first column chromatography were test for cytotoxic and anti-inflammatory activities.

The isolated compounds were only subjected for cytotoxicity because some compounds could be isolated in very small amount which was not enough for the other activitites.

# CHAPTER III
# RESULTS AND DISCUSSION

## 3.1 Bioassay-guided fractionation

The maceration of the leave and twig powder (1.0 kg) and stem powder (2.0 kg) with distilled methanol yielded the crude methanol extracts of the mixture of leaves and twigs 102.03 g and the crude methanol extracts of stems 72.37 g. The methanol extracts were tested for cytotoxic, anti-inflammatory and anti-HIV-1 activities. The results are shown in tables 1, 2 and 3, respectively.

The methanol extract of leaves and twigs showed potent cytotoxicity aginst P-388 (murine lymphocytic leukemia), MCF-7 (human breast cancer), Lu-1 (human lung cancer) and had moderate activity to KB (human oral nasopharyngeal carcinoma) and Col-2 (human colon cancer) cells. Whereas, the methanol extract of stem exhibited cytotoxic activity against three cancer cell lines, P-388, MCF-7 and Lu-1. The two extracts showed potent anti-inflammatory activity comparing with phenylbutazone used as a positive control. Moreover, the methanol extracts indicated anti-HIV-1 activity in anti-syncytium formation assay. The stem extract was very active in HIV-1-RT assay while the leaves and twigs extract was moderate active.

Therefore, the methanol extracts were further purified by chromatographic techniques and recrystallization in order to find bioactive compounds in the leaves, twigs and stem of *M. sirikitiae.*

**Table 1** Cytotoxic activity of the methanol extracts

| **MeOH extracts** | **Cell Lines** | | | | | |
|---|---|---|---|---|---|---|
| | **P-388** | **KB** | **Col-2** | **MCF-7** | **Lu-1** | **ASK** |
| **Leaves & Twigs** | 0.67 | 5.1 | 12 | 2.58 | 3.92 | - |
| **Stem** | 7 | - | - | 13 | 17 | - |

Cytotoxic assay: $ED_{50} \leq 20$ μg/ml is considered active, - = inactive (>20 μg/ml). P-388: murine lymphocytic leukemia, KB: human oral nasopharyngeal carcinoma, Col-2: human colon cancer, MCF-7: human breast cancer (Michican Cancer Foundation), Lu-1: human lung cancer, ASK: cell line from rat glioma cell.

**Table 2** Anti-inflammatory activity of the methanol extracts

| **MeOH extracts** | **Dose** | **Edema thickness (μm)** | | | | **% Inhibition** | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **mg/ear** | **15 min** | **30 min** | **1 h** | **2 h** | **15 min** | **30 min** | **1 h** | **2 h** |
| **Leaves & Twigs** | 3 | 5±3 | 28±4 | 58±6 | 28±5 | 92 | 80 | 68 | 81 |
| **Stem** | 3 | 5±3 | 42±12 | 68±14 | 43±7 | 92 | 70 | 63 | 70 |
| **Phenylbutazone** | - | 13±5 | 42±6 | 80±9 | 45±6 | 78 | 70 | 56 | 69 |

Value are mean ± SEM (N=6), Significant from control P < 0.001

**Table 3** Anti-HIV-1 activity of the methanol extracts

| MeOH extracts | Syncytium ($^{\Delta Tat/Rev}$MC99+1A2) assay | | | | HIV-1-RT assay |
|---|---|---|---|---|---|
| | $IC_{50}$(µg/mL) | $EC_{50}$(µg/mL) | TI ($IC_{50}/EC_{50}$) | Activity | % inhibition at 200 µg/mL |
| **Leaves & twigs** | 16.43 | <7.8 | 2.11 | A | 64.27 (M) |
| **Stem** | 33.40 | <7.8 | 4.28 | A | 80.27 (VA) |
| **AZT** | > $10^{-8}$ | 3.17 x $10^{-9}$ | > 5.15 | | |

Syncytium assay: $IC_{50}$ = dose of compound that inhibited 50% metabolic activity of uninfected cells. $EC_{50}$= dose of compound that reduced 50% syncytium formation by $^{\Delta}$Tat/RevMC99 virus. A = active; I = inactive, <50% reduction at the $IC_{50}$ indicated, T = Toxic

RT assay: Inhibition (%) at 200 µg/mL; VA = very active (>70% inhibition), M = moderately active (>50-70% inhibition), W = weakly active (30-50% inhibition), I = inactive (<30% inhibition)

Hexane, EtOAc, and BuOH fractions partitioned from the leaves and twigs extract of *M. sirikitiae* were subjected to cytotoxic (Table 4) and anti-inflammatory activities (Table 4). It was found that the hexane and EtOAc fractions could inhibit the growth of all tested cell lines except ASK cells (rat glioma cell). Moreover, the hexane and EtOAc fractions were moderately active in anti-inflammatory via ethyl phenylpropiolate (EPP)-induced ear edema model. Whereas, the BuOH fraction was not toxic to all tested cell lines and had weak anti-inflammatory activity. The fractions A1-A9 separated from the mixed hexane and EtOAc fractions were tested for cytotoxic (Table 4) and anti-inflammatory activities (Table 5). The results indicated that fractions A3-A8 showed strong to moderate cytotoxic activity against to some tested cell lines. Fractions A1-A9 exhibited weak anti-inflammatory activity.

**Table 4** Cytotoxic activity of fractions from the leaves and twigs extract

| Fractions | Cell Lines | | | | | | |
|---|---|---|---|---|---|---|---|
| | P-388 | KB | Col-2 | MCF-7 | Lu-1 | ASK | Hex-293 |
| **Hexane fraction** | 0.11 | 6.02 | 12.1 | 0.35 | 5.88 | 11.18 | 0.66 |
| **EtOAc fraction** | 0.68 | 7.52 | 13.97 | 2.68 | 7.55 | 14.47 | 3.54 |
| **BuOH fraction** | - | - | - | - | - | - | - |
| **A1** | - | - | - | - | - | - | - |
| **A2** | - | - | - | - | - | - | - |
| **A3** | 4.00 | 11.60 | 16.44 | 16.90 | 13.24 | - | 10.37 |
| **A4** | 3.78 | 12.90 | 13.80 | 15.70 | 12.67 | - | 10.55 |
| **A5** | 0.58 | 12.50 | 16.20 | 2.12 | 9.75 | - | 0.68 |
| **A6** | 0.40 | 13.40 | - | 0.74 | 7.00 | - | 1.65 |
| **A7** | 8.56 | 15.30 | - | 16.90 | - | - | 11.89 |

| Fractions | Cell Lines | | | | | | |
|---|---|---|---|---|---|---|---|
| | P-388 | KB | Col-2 | MCF-7 | Lu-1 | ASK | Hex-293 |
| **A8** | 12.30 | 14.20 | 18.80 | 1.22 | 13.53 | 19.3 | 12.82 |
| **A9** | - | - | - | - | - | - | - |

Cytotoxic assay: $ED_{50} \leq 20$ μg/ml is considered active, - = inactive (>20 μg/ml). P-388: murine lymphocytic leukemia, KB: human oral nasopharyngeal carcinoma, Col-2: human colon cancer, MCF-7: human breast cancer (Michican Cancer Foundation), Lu-1: human lung cancer, ASK: rat glioma cell, HEX-293: human embryonic kidney cells.

**Table 6** Anti-inflammatory activity of fractions from the leaves and twigs extract

| Fractions | Dose | Edema thickness (μm) | | | | % Inhibition | | | |
|---|---|---|---|---|---|---|---|---|---|
| | mg/ear | 15 min | 30 min | 1 h | 2 h | 15 min | 30 min | 1 h | 2 h |
| **Hexane** | 3 | 28±3 | 43±3 | 63±3 | 63±3 | 78 | 74 | 63 | 54 |
| **EtOAc** | 3 | 20±0 | 42±2 | 58±2 | 62±2 | 84 | 75 | 66 | 55 |
| **BuOH** | 3 | 22±2 | 38±2 | 145±3 | 98±2 | 84 | 78 | 28 | 38 |
| **A1** | 3 | 103±6 | 147±4 | 153±4 | 140±4 | 28 | 20 | 25 | 8 |
| **A2** | 3 | 128±4 | 163±4 | 170±4 | 143±3 | 10 | 11 | 17 | 5 |
| **A3** | 3 | 70±6 | 133±10 | 167±8 | 130±10 | 49 | 25 | 10 | 8 |
| **A4** | 3 | 77±3 | 137±3 | 153±4 | 138±5 | 44 | 23 | 17 | 2 |
| **A5** | 3 | 60±7 | 97±6 | 162±7 | 132±5 | 56 | 45 | 13 | 7 |
| **A6** | 3 | 50±4 | 108±80 | 128±7 | 117±8 | 63 | 39 | 31 | 18 |
| **A7*** | - | - | - | - | - | - | - | - | - |
| **A8** | 3 | 83±4 | 140±6 | 163±6 | 135±6 | 42 | 24 | 20 | 11 |
| **A9** | 3 | 78±7 | 147±7 | 163±6 | 137±6 | 43 | 17 | 12 | 4 |
| **Phenyl-butazone** | 1 | 35±7 | 78±5 | 82±7 | 63±3 | 76 | 57 | 60 | 58 |

Value are mean ± SEM (N=6), Significant from control $P < 0.001$

* Not determine

Fractions A1-A6 separated from the stem extract of *M. sirikitiae* were tested for cytotoxic (Table 4) and anti-inflammatory activities (Table 5). The results indicated that fraction A2 and A3 showed cytotoxic activity against to some tested cell lines. In addition, A1 showed strong anti-inflammatory activity, whereas the other fractions were moderately active.

**Table 4** Cytotoxic activity of the fractions from the stem extract

| Fractions | Cell Lines | | | | | | |
|---|---|---|---|---|---|---|---|
| | **P-388** | **KB** | **HT-29** | **MCF-7** | **A549** | **ASK** | **Hek-293** |
| **A1** | - | - | - | - | - | - | - |
| **A2** | < 4 | 17.51 | - | 0.93 | 13.58 | 14.96 | < 4 |
| **A3** | 15.56 | - | - | 18.22 | - | - | 15.44 |
| **A4** | - | - | - | - | - | - | - |
| **A5** | - | - | - | - | - | - | - |
| **A6** | - | - | - | - | - | - | - |
| **Ellipticine** | 0.55 | 0.61 | 0.62 | 0.57 | 0.68 | 0.64 | 0.65 |

Cytotoxic assay: $ED_{50} \leq 20$ μg/mL is considered active, - = inactive (> 20 μg/mL). P-388: murine lymphocytic leukemia, KB: human oral nasopharyngeal carcinoma, HT-29: human colon cancer, MCF-7: human breast cancer (Michican Cancer Foundation), A549: human lung cancer, ASK: cell line from rat glioma cell, and Hek-293: human embryonic kidney cell.

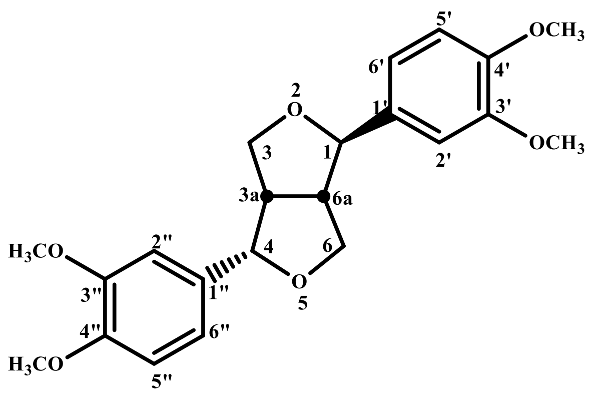
**Table 5** Anti-inflammatory activity of the fractions from the stem extract

| **Fractions** | **Dose mg/ear** | **Edema thickness (μm)** | | | | **% Inhibition** | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **15 min** | **30 min** | **1 h** | **2 h** | **15 min** | **30 min** | **1h** | **2h** |
| **A1** | 3 | 40±5 | 63±10 | 103±12 | 87±7 | 72 | 65 | 50 | 43 |
| **A2** | 3 | 80±6 | 95±7 | 93±5 | 87±4 | 41 | 46 | 50 | 39 |
| **A3** | 3 | 67±8 | 80±6 | 95±7 | 95±7 | 51 | 55 | 49 | 33 |
| **A4** | 3 | 75±7 | 78±7 | 133±4 | 117±6 | 45 | 56 | 28 | 18 |
| **A5** | 3 | 60±5 | 123±3 | 143±3 | 125±5 | 59 | 32 | 23 | 17 |
| **A6** | 3 | 77±6 | 125±13 | 137±10 | 120±10 | 47 | 31 | 27 | 20 |
| **Phenylbuta-zone** | 1 | 35±7 | 78±5 | 82±7 | 63±3 | 76 | 57 | 60 | 58 |

Value are mean ± SEM (N = 6), significant from control P < 0.001

The isolation of MeOH extract of *Mitrephora sirikitiae* leaves and twigs resulted in five lignans, (-)-epieudesmin (**1**), phyllegenin (**2**), magnone A (**3**), forsythialan B (**4**), and 2-(3,4-methylene-dioxyphenyl)-6-(3,5-dimethoxyphenyl)-3,7-dioxabicyclo[3.3.0]octane (**10**), one dihydrobenzofuran lignan, 3′,4-0dirnethylcedrusin (**9**), one flavonoid glycoside, quercetin 3,7-dimethylether 3'-O-$\alpha$-L-rhamnopyranosyl-(1$\rightarrow$2)-$\beta$-glucopyranoside (**5**), one steroidal glycoside, stigma-5-en-3-O-$\beta$-glucopyranoside (**6**), and five alkaloids, liriodenine (**7**), dicentrinone (**8**), N-trans-feruloyltyramine (**11**), oxo-O-methylpukateine (**12**), and epiberberine (**13**). Moreover, chemical study of the stem extract led to the isolation of one lignan, (-)-epieudesmin (**1**), three diterpenoids, kaurenoic acid (**14**), trachyloban-19-oic-acid (**15**), ciliaric acid (**18**), three sterols, β-sitosterol (**16**), stigmasterol (**17**), stigma-5-en-3-O-$\beta$-glucopyranoside (**6**), and three alkaloids, liriodenine (**7**), oxo-O-methylpukateine (**12**), 5-methoxy-4-methyl-1H-1-aza-2,9,10-anthracenetrione (**19**) and stepharanine (**20**). The chemical structures of the isolated compounds **1-20** were determined by means of spectroscopic methods, including IR (Infrared Spectroscopy), UV (Visible-Ultraviolet Spectroscopy), NMR (Nuclear Magnetic Resonance Spectroscopy) and MS (Mass Spectrometry).

#### 3.2.4.1 (-)-Epieudesmin (1)




Physical characteristics: White crystals from $CH_2Cl_2$/MeOH, m.p. 128-130 $^{o}$C

UV $\lambda_{max}^{MeOH}$ nm (log $\varepsilon$): 231 (3.91), 279 (4.38)

FTIR $\nu_{max}$ cm$^{-1}$: 2918, 2915, 1607, 1590, 1463, 1265, 1160, 1142

$[\alpha]_D^{23}$: -123.66 (*c* 1.0, $CHCl_3$)

EIMS *m/z* (% relative intensity): 386 $[M]^+$ (68), 355 $[M\text{-}OCH_3]^+$ (6), 340 $[M\text{-}OCH_3\text{-}CH_3]^+$ (9), 309 $[M\text{-}2OCH_3\text{-}CH_3]^+$ (7), 220 (10), 205 (13), 194 (14), 189 (15), 177 (36), 165 (100), 151 (26)

HR-ESI-MS : *m/z* found: 409.1621 $[M+Na]^+$, calcd. for $C_{22}H_{26}O_6Na$, 409.1627

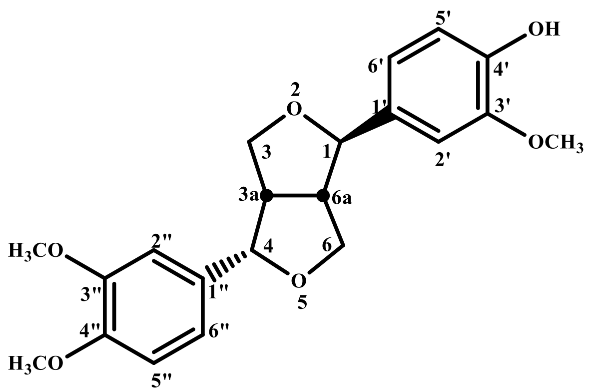
Compound **1** was obtained as white crystals by crystallization from MeOH/$CH_2Cl_2$. The structure of compound **1** was determined to (-)-epieudesmin by comparison of its spectroscopic and physical data with those previously reported by Ahmed AA *et al.* in 2002 [18].

**Table 6** $^{1}$H-NMR and $^{13}$C-NMR spectroscopic data of compound **1**

| **Carbon** | $\delta_C$ **(ppm)** | $\delta_H$ **(ppm) (no. of proton, *mult*., *J* (Hz))** | **HMBC** |
|---|---|---|---|
| 1 | 87.64 (CH) | 4.48 (1H, *d*, 7.2) | C-3, C-3a, C-6, C-6a, C-1′, C-2′, C-6′ |
| 3 | 69.74 ($CH_2$) | 3.34 (1H, *t*, 8.4) | C-1, C-3a, C-4 |
| | | 3.88 (1H, *m*) | |
| 3a | 50.18 (CH) | 3.39 (1H, *m*) | C-3, C-4 |
| 4 | 82.07 (CH) | 4.91 (1H, *d*, 5.7) | |
| 6 | 77.04 ($CH_2$) | 3.88 (1H, *m*) | C-3a, C-6a, C-4, C-1 |
| | | 4.16 (1H, *dd*, 0.8, 9.4) | |
| 6a | 54.50 (CH) | 2.95 (1H, *m*) | C-1″, C-2″, C-6″ |
| 1′ | 133.72 (C) | - | |
| 2′ | 109.23 (CH) | 6.95 (1H, *d*, 1.9) | C-1, C-1′, C-6′ |
| 3′ | 149.28 (C) | - | |
| 4′ | 148.77 (C) | - | |
| 5′ | 110.10 (CH) | 6.87 (1H, *d*, 8.2) | C-1′, C-2′,C-6′ |
| 6′ | 118.47 (CH) | 6.93 (1H, *dd*, 8.2, 1.9) | C-1, C-2′ |
| 1″ | 131.00 (C) | - | |
| 2″ | 109.07 (CH) | 6.97 (1H, *d*, 1.9) | C-4, C-1″, C-6″ |
| 3″ | 148.90 (C) | - | |
| 4″ | 148.07 (C) | - | |
| 5″ | 111.10 (CH) | 6.88 (1H, *d*, 8.5) | C-1″, C-2″, C-6″ |
| 6″ | 117.75 (CH) | 6.90 (1H, *dd*, 8.5, 1.9) | C-4, C-2″ |
| 3′-OMe | 55.93 ($CH_3$) | 3.93 (3H, *s*) | C-3′ |
| 4′-OMe | 55.97 ($CH_3$) | 3.91 (3H, *s*) | C-4′ |
| 3″-OMe | 55.93 ($CH_3$) | 3.94 (3H, *s*) | C-3″ |
| 4″-OMe | 55.95 ($CH_3$) | 3.92 (3H, *s*) | C-4″ |

$^{13}$C-NMR (125 MHz) and $^{1}$H-NMR (500 MHz) were recorded in chloroform-*d.*

### 3.2.4.2 (-)-Phylligenin (2)




Physical characteristics: White crystals from $CH_2Cl_2$/MeOH

UV $\lambda_{max}^{MeOH}$ nm (log ε): 231 (3.95), 280 (4.38)

FTIR $\nu_{max}$ $cm^{-1}$: 3430, 2961, 2939, 2844, 1607, 1589, 1515, 1469, 1460, 1447, 1460, 1417, 1382, 1347, 1278, 1264, 1256, 1236, 1193, 1157, 1143, 1127, 1076, 1023

$[\alpha]_D^{24}$: -128.53 (*c* 1.0, $CHCl_3$)

EIMS *m/z* (% relative intensity): 372 $[M]^+$ (68), 341 $[M\text{-}OCH_3]^+$ (6), 326 $[M\text{-}OCH_3\text{-}CH_3]^+$ (9), 273 (8), 205 (21), 194 (13), 189 (19), 177 (27), 165 (42), 151 (100)

HR-ESI-MS : *m/z* found: 395.1471 $[M+Na]^+$, calcd. for $C_{21}H_{24}O_6Na$, 395.1471

Compound **2** was obtained as white crystals by crystallization from MeOH/$CH_2Cl_2$. The structure of compound **2** was determined to phylligenin by comparison of its spectroscopic and physical data with those previously reported by Rahman MMA *et al.* in 1990 [19].

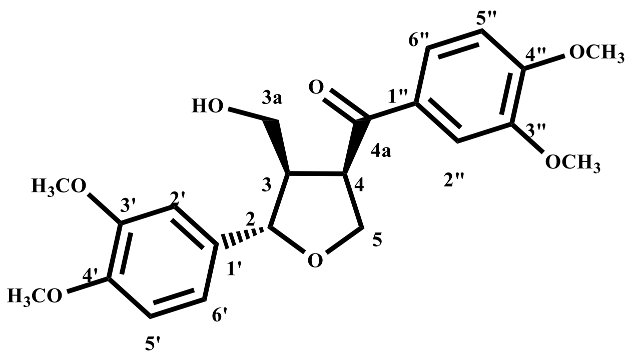
**Table 7** $^1$H-NMR and $^{13}$C-NMR spectroscopic data of compound **2**

| Carbon | $\delta_C$ (ppm) | $\delta_H$ (ppm) (no. of proton, *mult.*, *J* (Hz)) | HMBC |
|---|---|---|---|
| 1 | 82.66 (CH) | 4.92 (1H, *d*, 5.5) | C-3, C-6a, C-1′, C-2′, C-6′ |
| 3 | 71.02 ($CH_2$) | 3.89 (1H, *m*) | C-1, C-3a, C-4, C-6a |
| | | 4.18 (1H, *d*, 9.7) | |
| 3a | 54.52 (CH) | 2.95 (1H, *br dd*, 14.4, 7.2) | C-1, C-3, C-6a, C-1″ |
| 4 | 87.77 (CH) | 4.48 (1H, *d*, 7.1) | C-3, C-3a, C-6, C-6a, C-1″, C-2″, C-6″ |
| 6 | 69.73 ($CH_2$) | 3.39 (1H, *m*) | C-1, C-3a, C-4, C-6a |
| | | 3.89 (1H, *m*) | |

| Carbon | $\delta_C$ (ppm) | $\delta_H$ (ppm) (no. of proton, *mult*., *J* (Hz)) | HMBC |
|---|---|---|---|
| 6a | 50.16 (CH) | 3.39 (1H, *m*) | C-3, C-3a, C-1, C-6 |
| 1′ | 130.95 (C) | - | |
| 2′ | 108.91 (CH) | 6.99 (1H, *br s*) | C-1, C-1′, C-6′ |
| 3′ | 148.82 (C) | - | |
| 4′ | 147.98 (C) | - | |
| 5′ | 111.00 (CH) | 6.93 (1H, *d*, 8.1) | C-1′, C-2′, C-6′ |
| 6′ | 117.72 (CH) | 6.91 (1H, *m*) | C-1, C-2′ |
| 1″ | 133.00 (C) | - | |
| 2″ | 108.56 (CH) | 6.96 (1H, *d,* 1.7) | C-1″, C-3″, C-4″, C-6″ |
| 3″ | 146.77 (C) | - | |
| 4″ | 145.36 (C) | - | |
| 5″ | 114.28 (CH) | 6.93 (1H, *d*, 8.1) | C-1″, C-2″, C-6″ |
| 6″ | 119.22 (CH) | 6.91 (1H, *m*) | C-4, C-2″ |
| 3′-OMe | 55.93 ($CH_3$) | 3.93 (3H, *s*) | C-3′ |
| 4′-OH | - | 5.74 (1H, *s*) | C-3′, C-4′ |
| 3″-OMe | 55.93 ($CH_3$) | 3.99 (3H, *s*) | C-3″ |
| 4″-OMe | 55.95 ($CH_3$) | 3.92 (3H, *s*) | C-4″ |

$^{13}$C-NMR (125 MHz) and $^{1}$H-NMR (500 MHz) were recorded in chloroform-*d.*

#### 3.2.4.3 Magnone A (3)




Physical characteristics: White crystals from $CH_2Cl_2$/MeOH

UV $\lambda_{max}^{MeOH}$ nm (log ε): 230 (4.37), 276 (4.13), 305 (3.93)

FTIR $\nu_{max}$ cm$^{-1}$: 3461, 2912, 2839, 1651, 1592, 1583, 1517, 1467, 1441, 1420, 1334, 1288, 1237, 1203, 1192, 1154, 1104, 1080, 1020

$[\alpha]_D^{25}$: 18.54 (*c* 1.0, MeOH)

HR-ESI-MS : *m/z* found: 425.1584 $[M+Na]^+$, calcd. for $C_{22}H_{26}O_7Na$, 425.1576

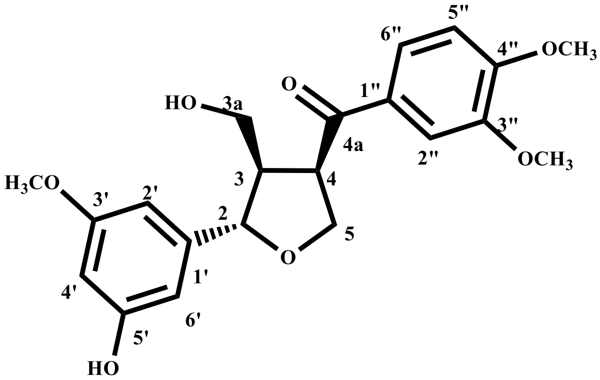
Compound **3** was obtained as white crystals by crystallization from $MeOH/CH_2Cl_2$. The structure of compound **3** was determined to magnone A by comparison of its spectroscopic and physical data with those previously reported by Jung KY *et al.* in 1998 [20].

**Table 8** $^1$H-NMR and $^{13}$C-NMR spectroscopic data of compound **3**

| Carbon | $\delta_C$ (ppm) * | $\delta_H$ (ppm) (no. of proton, *mult.*, *J* (Hz)) | HMBC |
|---|---|---|---|
| 2 | 83.82 (CH) | 4.75 (1H, *d*, 9.0) | C-3, C-3a |
| | | | C-1′, C-2′, C-6′ |
| 3 | 52.14 (CH) | 2.77 (1H, *m*) | - |
| 3a | 61.51 ($CH_2$) | 3.84 (2H, *dd*, 10.9, 4.5) | C-2, C-3, C-4 |
| 4 | 49.72 (CH) | 4.24 (1H, *m*) | C-2, C-3a, C-4a, C-5 |
| 4a | 197.97 (C=O) | - | - |
| 5 | 70.90 ($CH_2$) | 4.24 (1H, *m*) | C-2, C-4, C-4a |
| | | 4.37 (1H, *dd*, 11.2, 10.9) | |
| 1′ | 132.94 (C) | - | |
| 2′ | 109.56 (CH) | 7.09 (1H, *d*, 1.9) | C-1′, C-3′, C-6′ |
| 3′ | 148.95 (C) | - | |
| 4′ | 149.27 (C) | - | |
| 5′ | 110.82 (CH) | 6.89 (1H, *d*, 8.2) | C-1′, C-3′, C-6′ |
| 6′ | 119.36 (CH) | 6.99 (1H, *dd*, 8.2, 1.9) | C-2, C-2′ |
| 1″ | 129.75 (C) | - | |
| 2″ | 119.36 (CH) | 7.63 (1H, *d*, 2.0) | C-4a, C-1″, C-4″ |
| 3″ | 149.27 (C) | - | |
| 4″ | 153.70 (C) | - | |
| 5″ | 110.72 (CH) | 6.98 (1H, *d*, 8.3) | C-1″, C-3″, C-4″, C-6″ |
| 6″ | 123.23 (CH) | 6.91 (1H, *dd*, 8.2, 2.0) | C-4a, C-2″, C-5″ |
| 3′-OMe | 55.95 ($CH_3$) | 3.93 (3H, *s*) | C-3′ |
| 4′-OMe | 56.06 ($CH_3$) | 4.00 (3H, *s*) | C-4′ |
| 3″-OMe | 55.95 ($CH_3$) | 3.97 (3H, *s*) | C-3″ |
| 4″-OMe | 56.81 ($CH_3$) | 4.01 (3H, *s*) | C-4″ |

$^{13}$C-NMR (100 MHz) and $^1$H-NMR (400 MHz) were recorded in chloroform-*d*.

### 3.2.4.4 Forsylthialan B (4)



Physical characteristics: White crystals from $CH_2Cl_2$/MeOH

UV $\lambda_{max}^{MeOH}$ nm (log ε): 230 (4.41), 277 (4.18), 303 (3.99)

FTIR $\nu_{max}$ $cm^{-1}$: 3396, 3259, 2954, 2872, 2841, 1672, 1597, 1587, 1515, 1463, 1439, 1418, 1357, 1347, 1287, 1259, 1158, 1122, 1088, 1060, 1033, 1020, 1005, 968

$[\alpha]_D^{23}$: 14.00 (*c* 0.45, $CHCl_3$)

EIMS *m/z* (% relative intensity): 388 $[M]^+$ (8), 218 (14), 207 (9), 196 (38), 180 (93), 165 (100), 151 (60)

HR-ESI-MS : *m/z* found: 411.1428 $[M+Na]^+$, calcd. for $C_{21}H_{24}O_7Na$, 411.1420

Compound **4** was obtained as white crystals by crystallization from MeOH/$CH_2Cl_2$. The structure of compound **4** was determined to forsylthialan B by comparison of its spectroscopic and physical data with those previously reported by Piao XL *et al.* in 2008 [21].

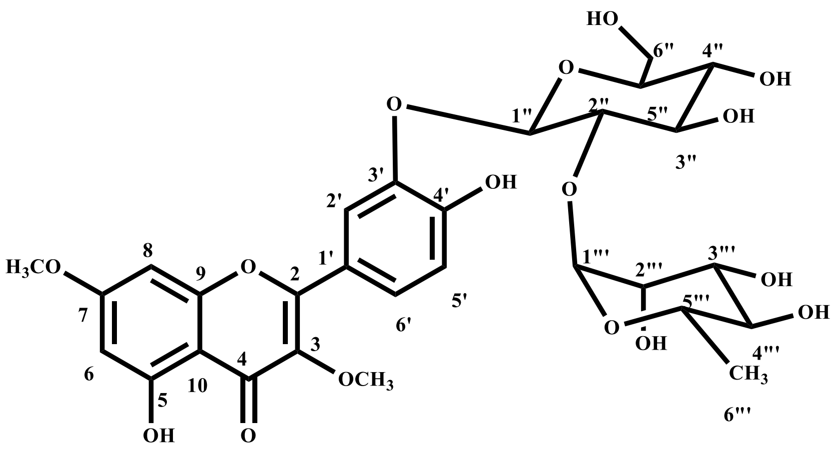
**Table 9** $^1$H-NMR and $^{13}$C-NMR spectroscopic data of compound **4**

| **Carbon** | $\delta_C$ **(ppm) *** | $\delta_H$ **(ppm)** (**no. of proton, *mult.*, *J* (Hz)**) | **HMBC** |
|---|---|---|---|
| 2 | 83.91 (CH) | 4.73 (1H, *d*, 9.1) | C-3, C-3a, C-1′, C-2′, C-6′ |
| 3 | 52.21 (CH) | 2.96 (1H, *m*) | - |
| 3a | 61.44 ($CH_2$) | 3.73 (1H, *dd*, 10.9, 5.6) | C-2, C-3, C-4 |
| | | 3.83 (1H, *dd*, 10.9, 4.4) | |
| 4 | 49.67 (CH) | 4.24 (1H, *m*) | C-2, C-3, C-3a, C-5 |
| 4a | 198.04 (C=O) | - | - |
| 5 | 70.85 ($CH_2$) | 4.24 (1H, *m*) | C-2, C-3 |
| | | 4.37 (1H, *m*) | C-2, C-3, C-4 |
| 1′ | 132.31 (C) | - | - |
| 2′ | 108.92 (CH) | 7.08 (1H, *s*) | C-2, C-1′, C-3′, C-5′, C-6′ |

| Carbon | $\delta_C$ (ppm) * | $\delta_H$ (ppm) (no. of proton, *mult.*, *J* (Hz)) | HMBC |
|---|---|---|---|
| 3′ | 146.88 (C) | - | - |
| 4′ | 114.04 (CH) | 6.93 (1H, *s*) | C-2′, C-3′, C-5′ |
| 5′ | 145.63 (C) | - | - |
| 6′ | 120.17 (CH) | 6.94 (1H, *s*) | C-2, C-1′, C-2′, C-5′ |
| 1″ | 129.77 (C) | - | - |
| 2″ | 110.57 (CH) | 7.63 (1H, *d*, 2.0) | C-3″, C-4″, C-6″ |
| 3″ | 149.26 (C) | - | - |
| 4″ | 153.70 (C) | - | - |
| 5″ | 110.12 (CH) | 6.98 (1H, *d*, 8.4) | C-1″, C-3″, C-4″ |
| 6″ | 123.23 (CH) | 7.67 (1H, *dd*, 8.4, 2.0) | C-2″, C-4″, C-5″ |
| 3′-OMe | 56.02 ($CH_3$) | 3.99 (3H, *s*) | C-3′ |
| 3″-OMe | 56.06 ($CH_3$) | 4.01 (3H, *s*) | C-3″ |
| 4″-OMe | 56.18 ($CH_3$) | 4.02 (3H, *s*) | C-4″ |

$^{13}$C-NMR (100 MHz) and $^{1}$H-NMR (400 MHz) were recorded in chloroform-*d.*

#### 3.2.4.5 Quercetin 3,7-dimethylether 3'-O-*α*-L-rhamnopyranosyl-(1→2)-*β*-glucopyranoside (5)




Physical characteristics: Yellow solid from MeOH

UV $\lambda_{max}^{MeOH}$ nm (log ε): 253 (4.24), 268 (4.24)

FTIR (neat) $\nu_{max}$ cm$^{-1}$: 3449, 3281, 2915, 1659, 1593, 1517, 1496, 1453, 1437, 1377, 1339, 1314, 1289, 1260, 1236, 1211, 1172, 1126, 1075, 1045, 1021

$[\alpha]_D^{26}$: -168.21 (*c* 1.0, Pyridine)

EIMS *m/z* (% relative intensity): 386 $[M-C_{12}H_{21}O_9]^+$ (100), 312 (19), 283 (12), 203 (19)

HR-ESI-MS : *m/z* found: 661.1759 $[M+Na]^+$, calcd. for $C_{29}H_{34}O1_6Na$, 661.1745

Compound **5** was obtained as white needles by crystallization from $MeOH/CH_2Cl_2$. The structure of compound **5** was determined to quercetin 3,7-dimethylether 3'-O-$\alpha$-L-rhamnopyranosyl-(1$\rightarrow$2)-$\beta$-glucopyranoside by comparison of its spectroscopic and physical data with those previously reported by Sinz A *et al.* in 1998 [22].

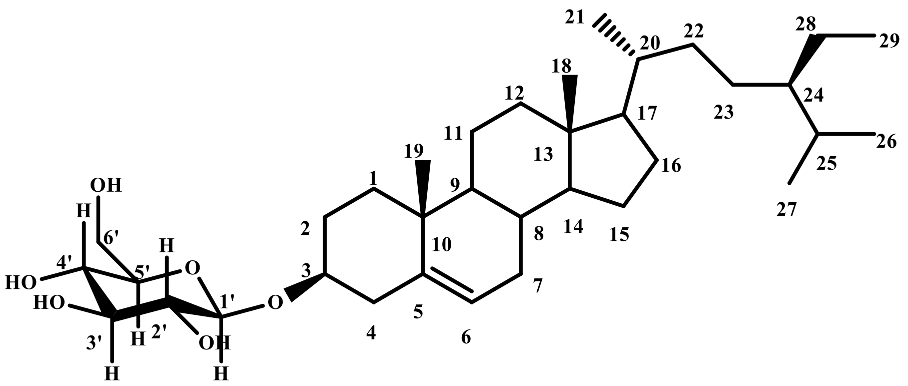
**Table 10** $^1$H-NMR and $^{13}$C-NMR spectroscopic data of compound **5**

| Carbon | $\delta_C$ (ppm) | $\delta_H$ (ppm) (no. of proton, *mult*., *J* (Hz)) | HMBC |
|---|---|---|---|
| 2 | 156.14 (C) | - | - |
| 3 | 138.40 (C) | - | - |
| 4 | 178.50 (C=O) | - | - |
| 5 | 161.30 (C) | - | - |
| 6 | 98.23 (CH) | 6.38 (1H, *d*, 2.2) | C-5, C-7, C-8, C-10 |
| 7 | 165.58 (C) | - | - |
| 8 | 93.00 (CH) | 6.83 (1H, *d*, 2.2) | C-6, C-7, C-9, C-10 |
| 9 | 156.74 (C) | - | - |
| 10 | 105.61 (C) | - | - |
| 1′ | 120.92 (C) | - | - |
| 2′ | 116.82 (CH) | 7.79 (1H, *d*, 2.1) | C-2, C-1′, C-3′, C-4′, C-6′ |
| 3′ | 145.42 (C) | - | - |
| 4′ | 151.12 (C) | - | - |
| 5′ | 116.60 (CH) | 7.02 (1H, *d*, 8.6) | C-1′, C-3′, C-4′ |
| 6′ | 124.10 (CH) | 7.70 (1H, *d*, 8.6, 2.1) | C-2, C-2′, C-4′ |
| 1″ | 99.44 (CH) | 5.10 (1H, *d*, 7.7) | - |
| 2″ | 77.26 (CH) | 3.60 (1H, *dd*, 8.9, 7.7) | C-1″, C-3″, 1‴ |
| 3″ | 77.76 (CH) | 3.49 (1H, *m*) | C-2″ |
| 4″ | 70.25 (CH) | 3.23 (1H, *m*) | - |
| 5″ | 77.47 (CH) | 3.37 (1H, *m*) | - |
| 6″ | 61.08 ($CH_2$) | 3.49 (1H, *m*) | - |
| | | 3.69 (1H, *m*) | - |

| Carbon | $\delta_C$ (ppm) | $\delta_H$ (ppm) (no. of proton, *mult*., *J* (Hz)) | HMBC |
|---|---|---|---|
| 1‴ | 100.92 (CH) | 5.21 (1H, *d*, 1.1) | C-2‴, C-3‴, C-5‴ |
| 2‴ | 70.96 (CH) | 3.74 (1H, *m*) | - |
| 3‴ | 70.96 (CH) | 3.46 (1H, *m*) | - |
| 4‴ | 72.48 (CH) | 3.20 (1H, *m*) | C-3‴, C-5‴, C-6‴ |
| 5‴ | 69.02 (CH) | 3.87 (1H, *m*) | - |
| 6‴ | 18.49 (CH) | 1.10 (1H, *d*, 6.1) | C-4‴, C-5‴ |
| 3-OMe | 60.21 ($CH_3$) | 3.99 (3H, *s*) | C-3 |
| 7-OMe | 56.55 ($CH_3$) | 4.01 (3H, *s*) | C-7 |
| 3″-OH | - | 5.32 (1H, *d*, 5.6) | C-2″ |
| 4″-OH | - | 5.12 (1H, *d*, 5.6) | C-3″ |
| 6″-OH | - | 4.66 (1H, *d*, 4.2) | - |
| 2‴-OH | - | 4.66 (1H, *d*, 4.2) | C-3‴ |
| 3‴-OH | - | 4.41 (1H, *d*, 5.9) | C-2‴ |

$^{13}$C-NMR (100 MHz) and $^{1}$H-NMR (400 MHz) were recorded in DMSO-$d_6$.

### 3.2.4.6 Stigma-5-en-3-O-*β*-glucopyranoside (6)



Physical characteristics: white powder from $CH_2Cl_2$/MeOH, m.p. 280-285 $^{o}$C (dec.)

FTIR (neat) $\nu_{max}$ cm$^{-1}$: 3600-3200 (3382), 2958, 2931, 1461, 1366, 1068, 1019

$[\alpha]_D^{25}$ : -34.54 (*c* 1.0, pyridine)

EIMS *m/z* (% relative intensity): 576 $[M]^+$, 397 (27), 396 (45), 381.39 (26), 255 (64), 145 (100)

HR-ESI-MS: *m/z* found: 599.4288 $[M+Na]^+$ , calcd. for $C_{35}H_{60}O_6Na$, 599.4288

Compound **6** was obtained as a white powder by recrystallization from $MeOH/CH_2Cl_2$. The compound **6** was determined to stigma-5-en-3-O-*β*-glucopyranoside or daucosterol. The structure of compound **6** was also confirmed by comparison of the spectroscopic and physical data with those previously reported by Faizi *et al*. in 2007 [23].

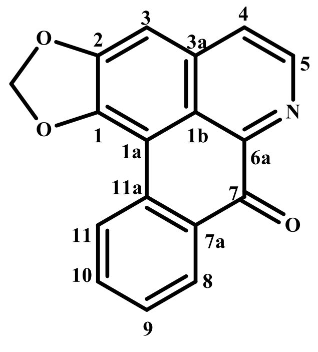
**Table 11** $^{1}$H-NMR and $^{13}$C-NMR spectroscopic data of compound **6**

| Carbon | $\delta_C$ (ppm) | $\delta_H$ (ppm) (no. of proton, mult., J (Hz)) | HMBC |
|---|---|---|---|
| 1 | 37.56 ($CH_2$) | 1.00 (1H, *m*) | C-5, C-19 |
| | | 1.75 (1H, *m*) | C-3, C-5 |
| 2 | 30.33 ($CH_2$) | 1.76 (1H, *m*) | C-3 |
| | | 2.14 (1H, *br d*, 10.7) | - |
| 3 | 78.23 (CH) | 3.98 (1H, *m*) | C-1′ |
| 4 | 39.42 ($CH_2$) | 2.49 (1H, *br t*, 11.1) | - |
| | | 2.76 (1H, *br d*, 11.1) | C-2, C-3, C-5, C-6, C-10 |
| 5 | 141.00 (C) | - | - |
| 6 | 121.97 (CH) | 5.37 (1H, *br s*) | C-4, C-7, C-8, C-10 |
| 7 | 32.25 ($CH_2$) | 1.66 (1H, *m*) | - |
| | | 1.91 (1H, *m*) | - |
| 8 | 32.14 (CH) | 1.39 (1H, *m*) | C-9, C-14 |
| 9 | 50.43 (CH) | 0.90 (1H, *m*) | C-7, C-8, C-11, C-19 |
| 10 | 37.00 (C) | - | - |
| 11 | 21.36 ($CH_2$) | 1.45 (2H, *m*) | - |
| 12 | 40.04 ($CH_2$) | 1.12 (1H, *m*) | C-17 |
| | | 1.98 (1H, *m*) | - |
| 13 | 42.56 (C) | - | - |
| 14 | 56.92 (CH) | 0.98 (1H, *m*) | C-7, C-8, C-9, C-13 |
| 15 | 24.58 ($CH_2$) | 1.03 (1H, *m*) | - |
| | | 1.56 (1H, *m*) | - |
| 16 | 28.60 ($CH_2$) | 1.26 (1H, *m*) | - |
| | | 1.86 (1H, *m*) | - |
| 17 | 56.34 (CH) | 1.12 (1H, *m*) | - |
| 18 | 12.05 ($CH_3$) | 0.68 (3H, *s*) | C-12, C-13, C-17 |
| 19 | 19.49 ($CH_3$) | 0.96 (3H, *s*) | C-1, C-10 |
| 20 | 36.46 (CH) | 1.41 (1H, *m*) | - |

| Carbon | $\delta_C$ (ppm) * | $\delta_H$ (ppm) (no. of proton, *mult.*, *J* (Hz)) | HMBC |
|---|---|---|---|
| 21 | 19.09 ($CH_3$) | 1.01 (3H, *d*, 6.3) | C-17, C-20, C-22 |
| 22 | 34.30 ($CH_2$) | 1.10 (1H, *m*) | C-17 |
| | | 1.41 (1H, *m*) | - |
| 23 | 26.50 ($CH_2$) | 1.26 (2H, *m*) | C-24, C-28 |
| 24 | 46.14 (CH) | 1.01 (1H, *m*) | C-22, C-29, C-26, C-27 |
| 25 | 29.57 (CH) | 1.70 (1H, *m*) | C-24, C-27 |
| 26 | 19.30 ($CH_3$) | 0.88 (3H, *d*, 6.4) | C-24, C-25, C-27 |
| 27 | 20.04 ($CH_3$) | 0.90 (3H, *d*, 6.4) | C-24, C-25, C-26 |
| 28 | 23.48 ($CH_2$) | 1.31 (2H, *m*) | - |
| 29 | 12.23 ($CH_3$) | 0.93 (3H, *t*, 7.5) | C-24, C-28 |
| 1′ | 102.65 (CH) | 5.09 (1H, *d*, 7.7) | C-3, C-3′, C-5′ |
| 2′ | 75.39 (CH) | 4.09 (1H, *br t*, 7.6) | C-3′ |
| 3′ | 78.51 (CH) | 4.32 (1H, *m*) | C-2′, C-4′ |
| 4′ | 71.79 (CH) | 4.32 (1H, *m*) | C-2′, C-3′ |
| 5′ | 78.51 (CH) | 4.01 (1H, *m*) | C-1′ |
| 6′ | 62.92 ($CH_2$) | 4.45 (1H, *dd*, 11.2, 4.7) | - |
| | | 4.60 (1H, *br d*, 11.2) | - |

$^{13}$C-NMR (100 MHz) and $^{1}$H-NMR (400 MHz) were recorded in pyridine-$d_5$.

### 3.2.4.7 Liriodenine (7)



Physical characteristics: yellow needles from $CH_2Cl_2$/MeOH, m.p. 263-265 $^{o}$C (dec.)

UV $\lambda_{max}^{MeOH}$ nm (log ε): 248 (4.47), 268 (4.38), 310 (3.90), 416 (4.06)

FTIR $\nu_{max}$ cm$^{-1}$: 1658, 1600, 1571, 1507, 1469, 1439, 1362, 1306, 1259, 1226, 1205, 1113, 1041, 1012, 955

EIMS *m/z* (% relative intensity) : 275 [M]$^{+}$ (100), 247 (31), 219 (14), 188 (23)

HR-ESI-MS : *m/z* found 298.0488 $[M+Na]^+$, calcd. for $C_{17}H_9NO_3Na$, 298.0482

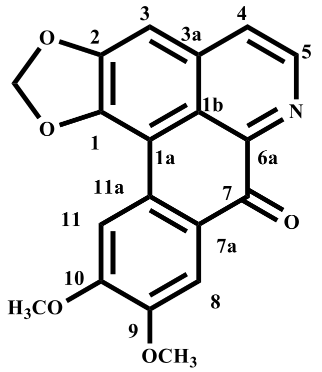
Compound **7** was obtained as yellow needles from MeOH/$CH_2Cl_2$. By comparison of its spectroscopic and physical data with those reported by Zhizhan Zhang *et al.* in 2002 [24] and Chien-Chih Chiu *et al.* in 2012 [25], this compound was identified to be 8*H*-benzo*[g]*-1,3-benzodioxolo[6,5,4-*de*]quinolin-8-one or liriodenine.

**Table 12** $^1$H-NMR and $^{13}$C-NMR spectroscopic data of compound **7**

| **Carbon** | $\delta_C$ **(ppm)** | $\delta_H$ **(ppm) (no. of proton, *mult*., *J* (Hz))** | **HMBC** |
|---|---|---|---|
| 1 | 148.23 (C) | - | - |
| 1a | 107.43 (C) | - | - |
| 1b | 122.91 (C) | - | - |
| 2 | 151.95 (C) | - | - |
| 3 | 103.06 (CH) | 7.04 (1H, *d*, 3.5) | C-1, C-1b, C-2, C-4 |
| 3a | 135.85 (C) | - | - |
| 4 | 124.55 (CH) | 7.69 (1H, *dd*, 7.6, 3.5) | C-1b, C-3, C-3a, C-5 |
| 5 | 143.90 (CH) | 8.69 (1H, *br d*, 5.0) | C-3a, C-3b, C-4, C-6a |
| 6a | 144.31 (C) | - | - |
| 7 | 182.16 (C=O) | - | - |
| 7a | 130.61 (C) | - | - |
| 8 | 128.26 (CH) | 8.40 (1H, *br d*, 7.6) | C-7, C-10, C-11a |
| 9 | 128.40 (CH) | 7.48 (1H, *br t*, 7.6) | C-7a, C-11 |
| 10 | 134.03 (CH) | 7.64 (1H, *br t*, 7.6) | C-8, C-9, C-11a |
| 11 | 127.22 (CH) | 8.44 (1H, *br t*, 7.6) | C-1a, C-7a, C-9 |
| 11a | 132.67 (C) | - | - |
| $OCH_2O$ | 102.67 ($CH_2$) | 6.31 (3H, *s*) | C-1, C-2 |

$^{13}$C-NMR (100 MHz) and $^1$H-NMR (400 MHz) were recorded in a mixed solvent of methanol-$d_4$ and chloroform-*d*

### 3.2.4.8 Dicentrinone (8)



Physical characteristics: Yellow needles from $CH_2Cl_2$/MeOH

UV $\lambda_{max}^{MeOH}$ nm (log ε): 249 (4.18), 270 (4.08), 310 (3.63), 349 (3.69)

FTIR $\nu_{max}$ $cm^{-1}$: 3534, 3350, 2917, 1591, 1575, 1510, 1474, 1452, 1422, 1365, 1343, 1303, 1273, 1252, 1215, 1135, 1091, 1055, 1023, 1000, 965

EIMS *m/z* (% relative intensity) : 335 $[M]^+$ (100), 320 (61), 304 (80), 289 (74), 276 (44), 263 (58), 249 (58), 234 (45), 221 (54), 206 (26)

HR-ESI-MS : *m/z* found 358.0699 $[M+Na]^+$, calcd. for $C_{19}H_{13}NO_5Na$, 358.0691

Compound **8** was obtained as yellow needles from MeOH/$CH_2Cl_2$. By comparison of its spectroscopic and physical data with those reported by Zhou BN *et al.* in 2012 [26], this compound was identified to be dicentrinone.

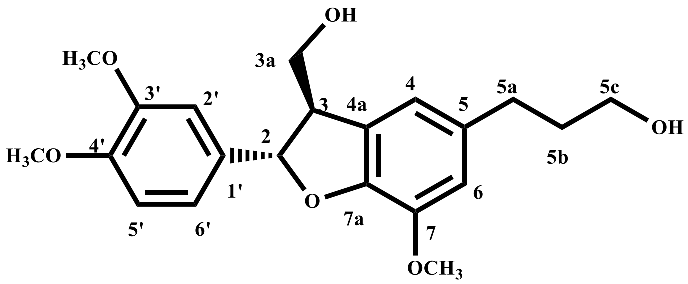
**Table 13** $^1$H-NMR and $^{13}$C-NMR spectroscopic data of compound **8**

| Carbon | $\delta_C$ (ppm) | $\delta_H$ (ppm) (no. of proton, *mult.*, *J* (Hz)) | HMBC |
|---|---|---|---|
| 1 | 147.28 (C) | - | - |
| 1a | 108.08 (C) | - | - |
| 1b | 122.54 (C) | - | - |
| 2 | 151.77 (C) | - | - |
| 3 | 102.74 (CH) | 7.04 (1H, *s*) | C-1, C-1b, C-2, C-4 |
| 3a | 135.74 (C) | - | - |
| 4 | 124.23 (CH) | 7.69 (1H, *d*, 5.3) | C-1b, C-3, C-5 |
| 5 | 144.31 (CH) | 8.69 (1H, *d*, 5.3) | C-3a, C-4, C-6a |
| 6a | 144.95 (C) | - | - |
| 7 | 181.12 (C=O) | - | - |
| 7a | 125.61 (C) | - | - |

| Carbon | $\delta_C$ (ppm) | $\delta_H$ (ppm) (no. of proton, *mult.*, *J* (Hz)) | HMBC |
|---|---|---|---|
| 8 | 109.41 (CH) | 8.40 (1H, *s*) | C-7, C-7a, C-9, C-10, C-11a |
| 9 | 149.45 (C) | - | - |
| 10 | 153.93 (C) | - | - |
| 11 | 108.78 (CH) | 8.44 (1H, *s*) | C-1a, C-7a, C-9, C-10 |
| 11a | 127.81 (C) | - | - |
| $OCH_2O$ | 102.57 ($CH_2$) | 6.31 (3H, *s*) | C-1, C-2 |
| 9-OMe | 56.11 ($CH_3$) | 4.03 (3H, *s*) | C-9 |
| 10-OMe | 56.18 ($CH_3$) | 3.99 (3H, *s*) | C-10 |

$^{13}$C-NMR (100 MHz) and $^{1}$H-NMR (400 MHz) were recorded in a mixed solvent of methanol-$d_4$ and chloroform-*d*

### 3.2.4.9 3′,4-*O*-Dirnethylcedrusin (9)




Physical characteristics: Pale yellow semisolid

UV $\lambda_{max}^{MeOH}$ nm (log ε): 231 (4.19), 281 (3.73)

FTIR $\nu_{max}$ cm$^{-1}$: 3375, 2934, 1604, 1515, 1497, 1451, 1422, 1323, 1259, 1235, 1210, 1137, 1021

$[\alpha]_D^{22}$: 9.88 (c 1.0, MeOH)

EIMS *m/z* (% relative intensity) : 374 $[M]^+$ (4), 356 $[M-H_2O]^+$ (83), 341 $[M-CH_3-H_2O]^+$ (100), 325 $[M-OCH3-H2O]^+$ (39), 309 (15)

HR-ESI-MS : *m/z* found: 397.1624 $[M+Na]^+$, calcd. for $C_{21}H_{26}O_6Na$, 397.1627

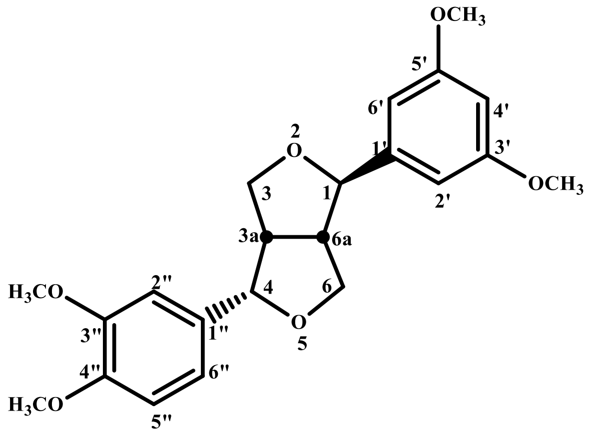
Compound **9** was obtained as pale yellow semisolid. The stucture of compound **9** was determined to 3′,4-*O*-dirnethylcedrusin or 4-*O*-methyldihydro-dehydrodiconiferylalcohol [2-(3′,4′-dimethoxyphenyl)-3-hydroxymethyl-2,3-dihydro-7-methoxy-benzofuran-5-propan-1-ol] by comparison of its spectroscopic and physical data with those previously reported by Pieters L *et al*. in 1993 [27].

**Table 14** $^{1}$H-NMR and $^{13}$C-NMR spectroscopic data of compound **9**

| **Carbon** | $\delta_C$ **(ppm)** | $\delta_H$ **(ppm)** (no. of proton, *mult*., *J* (Hz)) | **HMBC** |
|---|---|---|---|
| 2 | 88.70 (CH) | 5.52 (1H, *d*, 6.1) | C-3, C-3a, C-4, C-7a, C-1′ C-2′, C-6′ |
| 3 | 55.50 (CH) | 3.45 (1H, *br q*, 6.1) | C-2, C-3a, C-4a, C-7a, C-1′ |
| 3a | 65.02 ($CH_2$) | 3.74 (1H, *dd*, 11, 7.3) | C-2, C-3, C-4a |
| | | 3.81 (1H, *dd*, 11, 5.8) | |
| 4 | 117.92 (CH) | 6.71 (1H, *s*) | C-3, C-5a, C-6, C-7a |
| 4a | 129.73 (C) | - | - |
| 5 | 136.99 (C) | - | - |
| 5a | 32.89 ($CH_2$) | 2.61 (2H, *t*, 7.7) | C-4, C-5, C-6, C-5b, C-5c |
| 5b | 35.80 ($CH_2$) | 1.80 (2H, *tt*, 7.7, 6.5) | C-5, C-5a, C-5c |
| 5c | 62.23 ($CH_2$) | 3.56 (2H, *t*, 6.5) | C-5a, C-5b |
| 6 | 114.11 (CH) | 6.72 (1H, *s*) | C-4, C-5a, C-7, C-7a |
| 7 | 145.22 (C) | - | - |
| 7a | 147.50 (C) | - | - |
| 1′ | 136.19 (C) | - | - |
| 2′ | 110.70 (CH) | 6.91 (1H, *br s*) | C-2, C-1′, C-3′, C-4′, C-6′ |
| 3′ | 150.56 (C) | - | - |
| 4′ | 150.25 (C) | - | - |
| 5′ | 112.87 (CH) | 6.88 (1H, *d*, 8.2) | C-1′, C-3′,C-4′, C-6′ |
| 6′ | 119.43 (CH) | 6.82 (1H, *dd*, 8.2, 1.7) | C-2, C-1′, C-2′, C-4′ |
| 7-OMe | 56.74 ($CH_3$) | 3.84 (3H, *s*) | C-3″ |
| 3′-OMe | 56.42 ($CH_3$) | 3.77 (3H, *s*) | C-3′ |
| 4′-OMe | 56.49 ($CH_3$) | 3.79 (3H, *s*) | C-4′ |

$^{13}$C-NMR (100 MHz) and $^{1}$H-NMR (400 MHz) were recorded in methanol-$d_4$.

### 3.2.4.10 2-(3,4-methylene-dioxyphenyl)-6-(3,5-dimethoxyphenyl)-3,7-dioxabicy-clo[3.3.0]octane (10)



Physical characteristics: Pale yellow semisolid

UV $\lambda_{max}^{MeOH}$ nm (log ε): 230 (4.30), 280 (3.91)

FTIR (neat) $\nu_{max}$ $cm^{-1}$: 2930, 2850, 1593, 1512, 1461, 1419, 1258, 1232, 1156, 1136, 1061, 1021

EIMS *m/z* (% relative intensity): 386 $[M]^+$ (5), 384 $[M-2H]^+$ (34), 367 (14), 337 (41), 325 (82), 292 (32), 275 (20), 263 (27), 220 (32), 202 (41), 189 (100), 165 (85)

Compound **10** was obtained as pale yellow semisolid by crystallization from $MeOH/CH_2Cl_2$. The structure of compound **10** was determined to 2-(3,4-methylene-dioxyphenyl)-6-(3,5-dimethoxyphenyl)-3,7-dioxabicy-clo[3.3.0]octane by comparison of its spectroscopic and physical data with those previously reported by Wang WS *et al.* in 2012 [28].

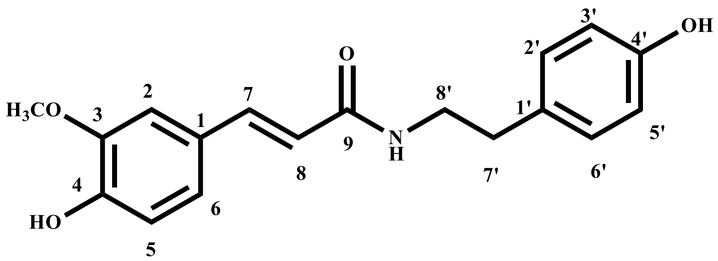
**Table 15** $^{1}$H-NMR and $^{13}$C-NMR spectroscopic data of compound **10**

| Carbon | $\delta_C$ (ppm) * | $\delta_H$ (ppm) (no. of proton, *mult.*, *J* (Hz)) | HMBC |
|---|---|---|---|
| 1 | 84.83 (CH) | 4.64 (1H, *d*, 7.4) | C-3, C-3a, C-1′, C-2′, C-6′ |
| 3 | 62.69 ($CH_2$) | 3.23 (1H, *dd*, 11.6, 5.9) | C-1, C-3a, C-4, C-6a |
| | | 3.30 (1H, *m*) | |
| 3a | 53.57 (CH) | 1.88 (1H, *m*) | C-1, C-6a |
| 4 | 76.36 (CH) | 4.50 (1H, *d*, 8.4) | C-6, C-6a, C-1′, C-2′, C-6′ |
| 6 | 71.43 ($CH_2$) | 3.93 (1H, *dd*, 8.8, 7.6) | C-1, C-4, C-3a |
| | | 4.25 (1H, *dd*, 8.8, 4.4) | C-1, C-3a, C-4, C-6a |
| 6a | 50.77 (CH) | 2.54 (1H, *m*) | C-3a, C-4 |
| 1′ | 136.25 (C) | - | - |
| 2′ | 111.32 (CH) | 6.96 (1H, *s*) | C-1, C-1′, C-3′, C-6′ |

| Carbon | $\delta_C$ (ppm) * | $\delta_H$ (ppm) (no. of proton, *mult.*, *J* (Hz)) | HMBC |
|---|---|---|---|
| 3′ | 149.98 (C) | - | - |
| 4′ | 112.85 (CH) | 6.91 (1H, *br s*) | C-2′, C-3′, C-5′ |
| 5′ | 150.51 (C) | - | - |
| 6′ | 120.02 (CH) | 6.91 (1H, *br s*) | C-1, C-1′, C-2′, C-5′ |
| 1″ | 137.49 (C) | - | - |
| 2″ | 111.73 (CH) | 6.91 (1H, *br s*) | C-4, C-1″, C-3″, C-4″, C-6″ |
| 3″ | 150.46 (C) | - | - |
| 4″ | 149.98 (C) | - | - |
| 5″ | 112.71 (CH) | 6.88 (1H, *d*, 8.2) | C-1″, C-2″, C-3″, C-4″ |
| 6″ | 120.61 (CH) | 6.82 (1H, *dd*, 8.2, 1.7) | C-4, C-2″, C-4″ |
| 3′-OMe | 56.50 ($CH_3$) | 3.81 (3H, *s*) | C-3′ |
| 5′-OMe | 56.50 ($CH_3$) | 3.82 (3H, *s*) | C-5′ |
| 3″-OMe | 56.57 ($CH_3$) | 3.79 (3H, *s*) | C-3″ |
| 4″-OMe | 56.44 ($CH_3$) | 3.79 (3H, *s*) | C-4″ |

$^{13}$C-NMR (100 MHz) and $^{1}$H-NMR (400 MHz) were recorded in methanol-$d_4$.

#### 3.2.4.11 N-*Trans*-feruloyltyramine (11)




Physical characteristics: Pale yellow semisolid

UV $\lambda_{max}^{MeOH}$ nm (log ε): 293 (4.17), 320 (4.24)

FTIR (neat) $\nu_{max}$ cm$^{-1}$: 3269, 2935, 1650, 1587, 1510, 1449, 1427, 1364, 1206, 1154, 1122, 1029, 974

EIMS *m/z* (% relative intensity): 192 $[M-C_8H_9O]^+$ (100), 175 (23)

HR-ESI-MS : *m/z* found: 336.1208 $[M+Na]^+$, calcd. for $C_{18}H_{19}NO_4Na$, 336.1212

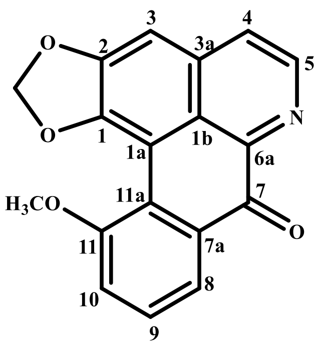
Compound **11** was obtained as pale yellow semisolid. The structure of compound **11** was determined to N-*trans*-feruloyltyramine by comparison of its spectroscopic and physical data with those previously reported by Kim HR *et al.* in 2005 [29].

**Table 16** $^{1}H$-NMR and $^{13}C$-NMR spectroscopic data of compound **11**

| Carbon | $\delta_C$ (ppm) | $\delta_H$ (ppm) (no. of proton, *mult*., *J* (Hz)) | HMBC |
|---|---|---|---|
| 1 | 128.30 (C) | - | - |
| 2 | 123.21 (CH) | 7.04 (1H, *dd*, 8.3, 1.8) | C-3, C-4, C-6, C-8 |
| 3 | 116.47 (CH) | 6.81 (1H, *d*, 8.3) | C-1, C-2, C-4, C-5 |
| 4 | 149.80 (C) | - | - |
| 5 | 149.27 (C) | - | - |
| 6 | 111.60 (CH) | 7.13 (1H, *d*, 1.8) | C-2, C-4, C-5, C-8 |
| 7 | 118.78 (CH) | 6.42 (1H, *d*, 15.7) | |
| 8 | 142.01 (CH) | 7.45 (1H, *d*, 15.7) | C-1, C-2, C-6, C-7, C-9 |
| 9 | 169.17 (C=O) | - | - |
| 1′ | 131.32 (C) | - | - |
| 2′ | 116.27 (CH) | 6.74 (1H, *d*, 8.5) | C-1′, C-3′, C-4′, C-6′ |
| 3′ | 130.72 (CH) | 7.07 (1H, *d*, 8.5) | C-1′, C-2′, C-4′,C-5′, C-7′ |
| 4′ | 156.89 (C) | - | - |
| 5′ | 130.72 (CH) | 7.07 (1H, *d*, 8.5) | C-1′, C-3′, C-4′,C-6′, C-7′ |
| 6′ | 116.27 (CH) | 6.74 (1H, *d*, 8.5) | C-1′, C-2′, C-5′, C-4′ |
| 7′ | 35.78 (CH) | 2.77 (1H, *t*, 7.4) | C-1′, C-8′ |
| 8′ | 42.51 (CH) | 3.48 (1H, *t*, 7.4) | C-9′, C-1′, C-7′ |
| 5-OMe | 56.41 ($CH_3$) | 3.89 (3H, *s*) | C-5 |

$^{13}C$-NMR (100 MHz) and $^{1}H$-NMR (400 MHz) were recorded in methanol-$d_4$.

### 3.2.4.12 *Oxo-O*-methylpukateine (12)




Physical characteristics: Orange needles from $CH_2Cl_2$/MeOH, m.p. 222-224 $^{o}C$ (dec.)

UV $\lambda_{max}^{MeOH}$ nm (log ε): 249 (4.47), 270 (4.41)

FTIR (neat) $\nu_{max}$ cm$^{-1}$: 2916, 1657, 1591, 1572, 1467, 1405, 1364, 1308, 1251, 1233, 1195, 1132, 1109, 1076, 1041, 1014

EIMS *m/z* (% relative intensity): 305 [M$^+$] (55), 275 (100), 248 (45), 220 (42), 190 (63), 163 (38)

HR-ESI-MS : *m/z* found: 328.0582 [M+Na]$^+$, calcd. for $C_{18}H_{11}NO_4Na$, 328.0586

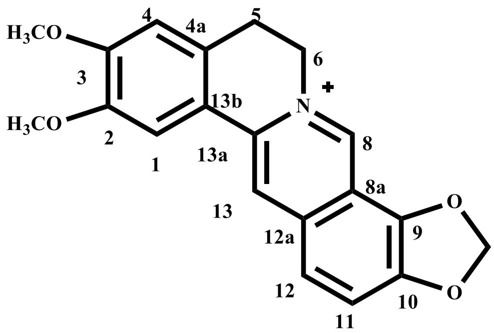
Compound **12** was obtained as orange needles by crystallization from MeOH/$CH_2Cl_2$. By comparison of its spectroscopic and physical data with those reported by LV Ziming *et al.* in 2011 [30], this compound was identified to be 8*H*-benzo[*g*]-1,3-benzodioxolo[6,5,4-*de*]quinolin-8-one,12-methoxy or oxo-*O*-methylpukateine.

**Table 17** $^1$H-NMR and $^{13}$C-NMR spectroscopic data of compound **12**

| Carbon | $\delta_C$ (ppm) | $\delta_H$ (ppm) (no. of proton, *mult.*, *J* (Hz)) | HMBC |
|---|---|---|---|
| 1 | 147.87 (C) | - | - |
| 1a | 108.48 (C) | - | - |
| 1b | 122.07 (C) | - | - |
| 2 | 151.96 (C) | - | - |
| 3 | 103.07 (CH) | 7.17 (1H, *s*) | C-1, C-1b, C-2, C-4 |
| 3a | 135.49 (C) | - | - |
| 4 | 123.62 (CH) | 7.74 (1H, *d*, 5.3) | C-1b, C-3, C-3a, C-5 |
| 5 | 144.20 (CH) | 8.82 (1H, *d*, 5.3) | C-3a, C-4, C-6a |
| 6a | 146.04 (C) | - | - |
| 7 | 181.49 (C=O) | - | - |
| 7a | 135.26 (C) | - | - |
| 8 | 119.75 (CH) | 8.27 (1H, *d*, 8.2) | C-1a, C-10, C-11a |
| 9 | 134.85 (CH) | 7.65 (1H, *t*, 8.2) | C-7a, C-8, C-11 |
| 10 | 112.15 (CH) | 7.10 (1H, *d*, 8.2) | C-8, C-11 |
| 11 | 161.84 (C) | - | - |
| 11a | 120.34 (C) | - | - |
| $OCH_2O$ | 102.41 ($CH_2$) | 6.38 (3H, *s*) | C-1, C-2 |
| 11-OMe | 56.20 ($CH_3$) | 4.07 (3H, *s*) | C-11 |

$^{13}$C-NMR (100 MHz) and $^1$H-NMR (400 MHz) were recorded in a mixed solvent of methanol-$d_4$ and chloroform-*d*.

### 3.2.4.13 Epiberberine (13)




Physical characteristics: Orange needles from MeOH/$CH_2Cl_2$

UV $\lambda_{max}^{MeOH}$ nm (log ε): 243 (4.03), 265 (4.04), 359 (4.08)

FTIR (neat) $\nu_{max}$ $cm^{-1}$: 3351, 2921, 2851, 1602, 1523, 1466, 1344, 1324, 1288, 1232, 1216, 1190, 1134, 1058, 1023

HR-ESI-MS : *m/z* found: 336.1233, calcd. for $C_{20}H_{18}O_4N^+$, 336.1236

Compound **13** was obtained as orange needles by recrystallization from MeOH/$CH_2Cl_2$. By comparison of its spectroscopic data with those reported by Jung HA *et al.* in 2008 [31], this compound was identified to be epiberberine.

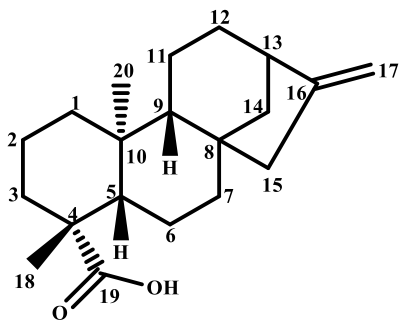
**Table 18** $^{1}$H-NMR and $^{13}$C-NMR spectroscopic data of compound **13**

| Carbon | $\delta_C$ (ppm) | $\delta_H$ (ppm) (no. of proton, *mult*., *J* (Hz)) | HMBC |
|---|---|---|---|
| 1 | 109.05 (CH) | 7.70 (1H, *s*) | C-2, C-3, C-4a, C-13a, C-13b |
| 2 | 149.18 (C) | - | |
| 3 | 151.90 (C) | - | |
| 4 | 111.73 (CH) | 7.10 (1H, *s*) | C-2, C-3, C-5, C-13b |
| 4a | 129.01 (C) | - | |
| 5 | 26.37 ($CH_2$) | 3.23 (1H, *br t*, 6.2) | C-4a, C-6, C-13b |
| 6 | 55.73 ($CH_2$) | 4.90 (1H, *br t*, 6.2) | C-4a, C-5 |
| 8 | 145.06 (CH) | 9.96 (1H, *s*) | C-6, C-8a, C-12a, C-13a |
| 8a | 112.10 (C) | - | |
| 9 | 144.29 (C) | - | |
| 10 | 147.38 (C) | - | |
| 11 | 121.50 (CH) | 8.06 (1H, *d*, 8.7) | C-9, C-10, C-12, C-12a |
| 12 | 122.04 (CH) | 7.86 (1H, *d*, 8.7) | C-8a, C-10, C-12a |

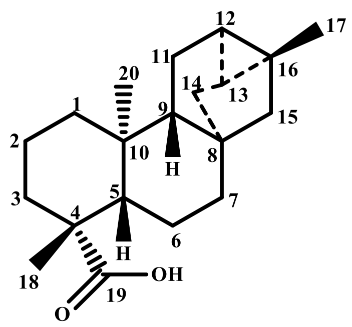
| Carbon | $\delta_C$ (ppm) | $\delta_H$ (ppm) (no. of proton, *mult.*, *J* (Hz)) | HMBC |
|---|---|---|---|
| 12a | 132.89 (C) | - | |
| 13 | 121.14 (CH) | 9.03 (1H, *s*) | C-8a, C-11, C-12, C-13a, C-13b |
| 13a | 137.53 (C) | - | |
| 13b | 119.43 (C) | - | |
| $OCH_2O$ | 104.92 ($CH_2$) | 6.54 (3H, *s*) | C-9, C-10 |
| 2-OMe | 56.59 ($CH_3$) | 3.94 (3H, *s*) | C-2 |
| 3-OMe | 56.31 ($CH_3$) | 3.88 (3H, *s*) | C-3 |

$^{13}$C-NMR (100 MHz) and $^{1}$H-NMR (400 MHz) were recorded in DMSO-$d_6$.

### 3.2.4.14 A mixture of kaurenoic acid (14) and trachyloban-19-oic-acid (15)



A



B

Physical characteristics: colorless crystals from hexane/MeOH

FTIR (neat) $\nu_{max}$cm$^{-1}$: 3012-2667, 1688, 1464, 1268

EIMS *m/z*: 302 (17), 287 (31), 260 (9), 259 (5), 257 (13), 246 (31), 231 (40)

HR-ESI-MS : *m/z* found 325.2133 $[M+Na]^+$, calcd. for $C_{20}H_{30}O_2Na$, 325.2143

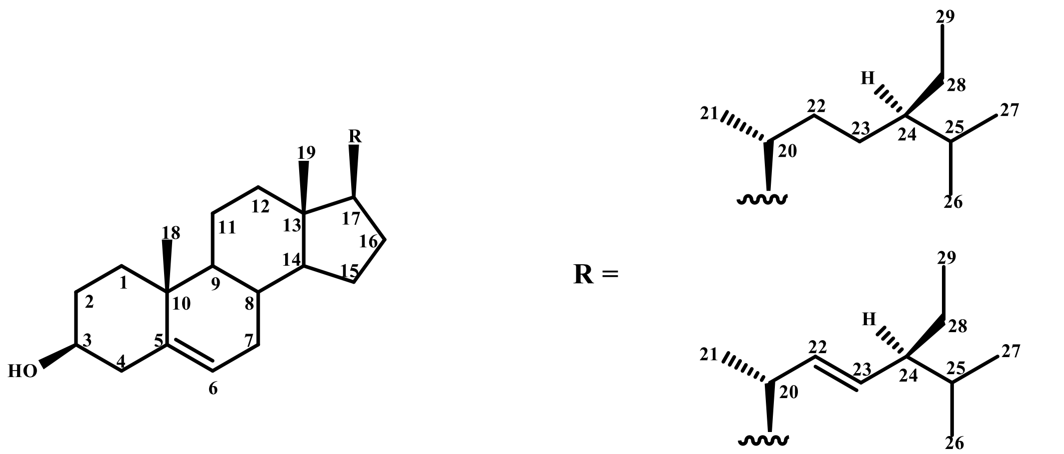
A mixture of compounds **14** and **15** was obtained as colorless crystals by recrystallization from MeOH/$CH_2Cl_2$. Compounds **14** and **15** were determined to (—)-kaur-16-en-19oic acid (kaurenoic acid) and trachyloban-19-oic acid by comparison of their spectroscopic and physical data with those previously reported by Batista *et al.* in 2007 [32] and Ngamrojvanich *et al.* in 2003 [33], respectively.

**Table 19** $^{1}$H-NMR and $^{13}$C-NMR spectroscopic data of compounds **14** and **15**

| Carbon | Compound 14 | | Compound 15 | |
|---|---|---|---|---|
| | $\delta_C$ | $\delta_H$ (mult., $J$ (Hz)) | $\delta_C$ | $\delta_H$ (mult., $J$ (Hz)) |
| 1 | 40.95 | 0.78 (1H, *dd*, 7.8, 3.1) | 39.67 | 0.75 (1H, *dd*, 13.3, 3.8) |
| | | 1.85 (1H, *m*) | | 1.54 (1H, *br s*) |
| 2 | 19.95 | 1.64 (1H, *br dd*, 7.3, 1.8) | 18.92 | 1.35 (1H, *br s*) |
| | | 1.86 (1H, *m*) | | 1.83 (1H, *m*) |
| 3 | 38.06 | 0.96 (1H, *d*, 3.9) | 38.03 | 0.96 (1H, *m*) |
| | | 2.12 (1H, *m*) | | 2.12 (1H, *m*) |
| 4 | 43.89 | - | 44.46 | - |
| 5 | 57.31 | 1.04 (1H, *d*, 6.7) | 57.22 | 0.97 (1H, *dd*, 10.1, 4.0) |
| 6 | 21.95 | 1.73 (2H, *m*) | 22.06 | 1.80 (2H, *m*) |
| 7 | 41.52 | 1.43 (1H, *br d*, 7.2) | 39.44 | 1.31 (1H, *dd*,13.0, 4.9) |
| | | 1.49 (1H, *m*) | | 1.44 (1H, *td*, 13.0, 3.0) |
| 8 | 43.98 | - | 40.97 | - |
| 9 | 55.37 | 1.03 (1H, *d*, 6.7) | 52.99 | 1.06 (1H, *m*) |
| 10 | 39.90 | - | 39.12 | - |
| 11 | 18.66 | 1.58 (2H, *br s*) | 19.95 | 1.64 (1H, *m*), 1.86 (1H, *m*) |
| 12 | 33.35 | 1.51 (1H, *br s*) | 20.76 | 0.55 (1H, *br d*, 7.2) |
| | | 1.58 (1H, *br s*) | | 0.79 (1H, *dd*, 7.7, 3.1) |
| 13 | 44.09 | 2.62 (1H, *s*) | 24.49 | 1.17 (1H, *m*) |
| 14 | 39.93 | 1.10 (1H, *br d*, 6.4) | 33.35 | 2.02 (1H, *m*) |
| | | 1.97 (1H, *br d*, 10.8) | | |
| 15 | 49.20 | 2.03 (2H, *m*) | 50.58 | 1.21 (1H, *d*, 11.2) |
| | | | | 1.37 (1H, *d*, 11.2) |
| 16 | 156.12 | - | 22.61 | - |
| 17 | 103.21 | 4.74 (1H, *s*), 4.80 (1H, *s*) | 20.78 | 1.11 (3H, *s*) |
| 18 | 29.19 | 1.19 (3H, *s*) | 29.09 | 1.22 (3H, *s*) |
| 19 | 184.57 | - | 184.68 | - |
| 20 | 15.81 | 0.93 (3H, *s*) | 12.68 | 0.86 (3H, *s*) |

* $^{13}$C-NMR (100 MHz) and $^{1}$H-NMR (400 MHz) were recorded in chloroform-*d.*

### 3.2.4.15 A mixture of the $\beta$-sitosterol (16) and stigmasterol (17)




Physical characteristics: White needles from $CH_2Cl_2$/MeOH.

FTIR (neat) $\nu_{max}$ $cm^{-1}$: 3600-3200, 3448, 2958, 2937, 2868, 1654, 1049

EIMS *m/z*: 414 $[M]^+$ (7), 412 $[M]^+$ (7)

A mixture of compounds **16** and **17** was obtained as a white powder by recrystallization from MeOH/$CH_2Cl_2$. Compounds **16** and **17** were identified as $\beta$-sitosterol and stigmasterol by comparison of their spectroscopic and physical data with those previously reported by Kojima and co-workers [34].

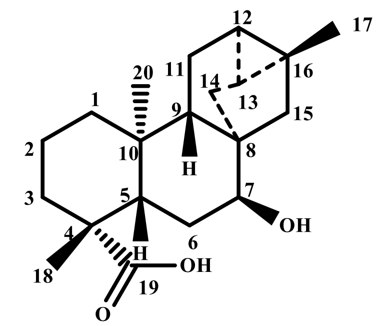
**Table 20** $^1$H-NMR and $^{13}$C-NMR spectroscopic data of a mixture of compounds **16** and **17**

| Carbon | $\delta_C$ (ppm) | | $\delta_H$ (ppm) | |
|---|---|---|---|---|
| | **16** | **17** | **16** | **17** |
| 1 | 37.49 | | | |
| 2 | 31.89 | | | |
| 3 | 72.02 | | 3.50 (1H, m) | 3.50 (1H, m) |
| 4 | 42.54 | | | |
| 5 | 140.99 | | | |
| 6 | 121.91 | | 5.33 (1H, m) | 5.33 (1H, m) |
| 7 | 32.08 | | | |
| 8 | 32.08 | | | |
| 9 | 50.38 | | | |
| 10 | 36.73 | | | |
| 11 | 21.42 | | | |
| 12 | 40.01 | 39.91 | | |
| 13 | 42.54 | 42.45 | | |

| Carbon | $\delta_C$ (ppm) | | $\delta_H$ (ppm) | |
|---|---|---|---|---|
| | **16** | **17** | **16** | **17** |
| 14 | 57.00 | | | |
| 15 | 24.56 | 24.62 | | |
| 16 | 28.45 | 29.11 | | |
| 17 | 56.36 | 56.20 | | |
| 18 | 12.07 | 12.26 | 0.66 (3H, *s*) | 0.67 (3H, *s*) |
| 19 | 19.60 | | 0.98 (3H, *s*) | 0.98 (3H, *s*) |
| 20 | 36.36 | 40.67 | | |
| 21 | 19.00 | 21.48 | 0.90 (3H, *d*, 6.5) | 1.00 (3H, *d*, 6.8) |
| 22 | 34.19 | 138.50 | | 5.13 (1H, *dd*, 15.1, 8.6) |
| 23 | 26.36 | 129.52 | | 4.99 (1H, *dd*, 15.1, 8.8) |
| 24 | 46.09 | 51.45 | | |
| 25 | 29.42 | 32.08 | | |
| 26 | 20.01 | 21.42 | 0.81 (3H, *d*, 6.8) | 0.83 (3H, *d*, 6.7) |
| 27 | 19.26 | | 0.79 (3H, *d*, 7.2) | 0.77 (3H, *d*, 6.5) |
| 28 | 23.31 | 25.60 | | |
| 29 | 12.26 | 12.43 | 0.83 (3H, *t*, 7.5) | 0.78 (3H, *t*, 7.5) |

$^{13}$C-NMR (125 MHz) and $^{1}$H-NMR (500 MHz) were recorded in chloroform-*d.*

### 3.2.4.16 7α-Hydroxytrachyloban-19*β*-oic acid (18)




Physical characteristics: white powder from $CH_2Cl_2$/MeOH, m.p. 132-135 $^{o}$C (dec.)

FTIR $\nu_{max}$ cm$^{-1}$: 3483, 3037-2850, 1691, 1467, 1132, 1053

$[\alpha]_D^{23}$: -193 (c 0.9, pyridine)

EIMS *m/z* (% relative intensity) : 300 $[M-H_2O]^+$ (12), 285 $[M-CH_3-H_2O]^+$ (29), 253 (21), 239 (43), 197 (27), 185 (62), 157 (100)

HR-ESI-MS : *m/z* found: 320.2343 $[M+2H]^+$, calcd. for $C_{20}H_{32}O_3$, 320.2351

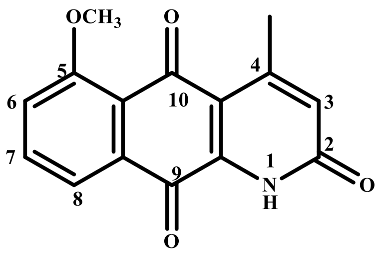
Compound **18** was obtained as white needles by crystallization from MeOH/$CH_2Cl_2$. The stucture of compound **18** was determined to 7$\alpha$-hydroxytrachyloban-19$\beta$-oic acid or ciliaric acid. The structure of compound **18** was also confirmed by comparison of its spectroscopic and physical data with those previously reported by Silvere Ngouela *et al.* in 1998 [35].

**Table 21** $^{1}$H-NMR and $^{13}$C-NMR spectroscopic data of compound **18**

| Carbon | $\delta_C$ (ppm) | $\delta_H$ (ppm) (no. of proton, *mult.*, *J* (Hz)) | HMBC |
|---|---|---|---|
| 1 | 40.06 | 0.96 (1H, *td*, 13.3, 3.8) | C-10, C-2, C-20 |
| | | 1.64 (1H, *d*, 12.8) | C-2 |
| 2 | 19.75 | 1.45 (1H, *br d*, 14.4) | |
| | | 2.26 (1H, *qt*, 13.8, 4.0) | |
| 3 | 38.94 | 1.12 (1H, *td*, 13.4, 4.0) | C-4 |
| | | 2.46 (1H, *d*, 13.2) | C-5, C-10 |
| 4 | 43.62 | - | |
| 5 | 47.94 | 2.18 (1H, *dd*, 10.5, 4.3) | C-4, C-10, C-6, C-18, C-20 |
| 6 | 30.94 | 2.39 (2H, *m*) | C-5, C-10 |
| 7 | 75.17 | 3.84 (1H, *br s*) | C-5, C-14 |
| 8 | 46.11 | - | |
| 9 | 47.10 | 1.90 (1H, *dd*, 11.3, 6.0) | C-15, C-1, C-10 |
| 10 | 39.33 | - | |
| 11 | 19.61 | 1.76 (1H, *dd*, 13.3, 5.0) | C-9, C-10 |
| | | 2.00 (1H, *m*) | C-1, C-10, C-14, C-20 |
| 12 | 21.13 | 0.63 (1H, *d*, 7.7) | C-17 |
| 13 | 24.63 | 0.90 (1H, *dd*, 7.7, 2.7) | C-8, C-17 |
| 14 | 33.04 | 1.28 (1H, *br d*, 11.6) | C-9, C-15 |
| | | 2.08 (1H, *d*, 11.6) | C-13, C-16 |
| 15 | 46.56 | 1.72 (1H, *d*, 11.7) | C-9, C-16, C-12 |
| | | 1.97 (1H, *d*, 11.7) | C-9, C-14, C-13, C-16 |
| 16 | 23.22 | - | |
| 17 | 20.85 | 1.19 (3H, *s*) | C-15, C-14, C-13, C-16, C-12 |
| 18 | 29.24 | 1.39 (3H, *s*) | C-5, C-4, C-3 |
| 19 | 180.47 | - | |
| 20 | 12.99 | 1.19 (3H, *s*) | C-9, C-1, C-10, C-2 |

$^{13}$C-NMR (125 MHz) and $^{1}$H-NMR (500 MHz) were recorded in pyridine-$d_5$.

### 3.2.4.17 5-Methoxy-4-methyl-1*H*-1-*aza*-2,9,10-anthracenetrione (19)




Physical characteristics: yellow powder from $CH_2Cl_2$/MeOH, m.p. 210-212 $^{o}$C (dec.)

UV $\lambda_{max}^{MeOH}$ nm (log ε): 238 (4.00), 255 (4.00), 295 (3.93)

FTIR $\nu_{max}$ cm$^{-1}$: 3000-3500, 3088, 2921, 2851, 1646, 1466, 1449

EIMS m/z: 269 $[M]^{+}$ (47), 254 [M-CH3]+

HR-ESI-MS : m/z found: 292.0582 [M+Na]+, calcd. for $C_{15}H_{11}NO_4Na$, 292.0586

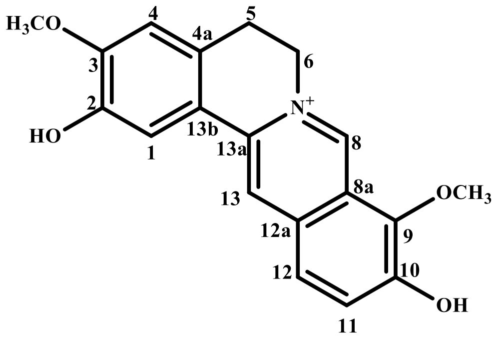
Compound **19** was obtained as a yellow powder. By comparison of its spectroscopic and physical data with those reported by Sheng-Fa Tsai and Shoei-Sheng Lee in 2010 [36], this compound was identified to be 5-methoxy-4-methyl-1*H*-1-aza-2,9,10-anthracenetrione.

**Table 22** $^{1}$H-NMR and $^{13}$C-NMR spectroscopic data of compound **19**

| Carbon | $\delta_C$ (ppm) | $\delta_H$ (ppm) (no. of proton, *mult.*, *J* (Hz)) | HMBC |
|---|---|---|---|
| 2 | 160.94 | - | |
| 3 | 126.88 | 6.63 (1H, *s*) | C-2, C-4a, 4-$CH_3$ |
| 4 | 152.33 | - | |
| 4a | 115.32 | - | |
| 5 | 160.78 | - | |
| 6 | 117.50 | 7.33 (1H, *d*, 8.0) | C-5, C-10a, C-8 |
| 7 | 137.18 | 7.79 (1H, *t*, 8.0) | C-5, C-8a, C-6 |
| 8 | 120.39 | 7.88 (1H, *d*, 8.0) | C-9, C-6 |
| 8a | 135.64 | - | |
| 9 | 181.36 | - | |
| 9a | 140.84 | - | |
| 10 | 176.04 | - | |
| 10a | 120.53 | - | |
| 4-$CH_3$ | 22.82 | 2.68 (3H, *s*) | C-4, C-3, C-4a |
| 5-$OCH_3$ | 56.90 | 4.07 (3H, *s*) | C-5, C-6 |

$^{13}$C-NMR (100 MHz) and $^{1}$H-NMR (400 MHz) were recorded in chloroform-*d*.

### 3.2.4.18 Stepharanine (20)



Physical characteristics: Yellow needles from MeOH/$CH_2Cl_2$

UV $\lambda_{max}^{MeOH}$ nm (log ε): 277 (3.57), 350 (3.50)

FTIR $\nu_{max}$ cm$^{-1}$: 3328, 3092, 2921, 2851, 1607, 1512, 1452, 1369

EIMS *m/z* (% relative intensity): 309 $[M-CH_3]^+$ (72), 248 (100)

HR-ESI-MS : *m/z* found: 324.1229, calcd. for $C_{19}H_{18}O_4N^+$, 324.1232

Compound **20** was obtained as yellow needles by crystallization from MeOH/$CH_2Cl_2$. By comparison of its spectroscopic and physical data with those reported by Ingkaninan *et al.* in 2005 [37], this compound was identified to be stepharanine.

**Table 23** $^1$H-NMR and $^{13}$C-NMR spectroscopic data of compound **20**

| Carbon | $\delta_C$ (ppm) | $\delta_H$ (ppm) (no. of proton, *mult.*, *J* (Hz)) | HMBC |
|---|---|---|---|
| 1 | 113.09 | 7.54 (1H, *s*) | C-2, C-3, C-4a, C-13a |
| 2 | 148.20 | - | |
| 3 | 152.35 | - | |
| 4 | 112.07 | 7.00 (1H, *s*) | C-2, C-3, C-5, C-13b |
| 4a | 128.34 | - | |
| 5 | 27.94 | 3.27 (2H,*br t*, 5.9) | C-4, C-4a, C-13b |
| 6 | 57.47 | 4.87 (2H, *m*) | C-4a |
| 8 | 144.83 | 9.63 (1H, *s*) | C-6, C-9, C-8a, C-12a, C-13a |
| 8a | 124.03 | - | |
| 9 | 143.04 | - | |
| 10 | 151.09 | - | |
| 11 | 132.73 | 7.78 (1H, *d*, 9.0) | C-9, C-12a |
| 12 | 124.67 | 7.88 (1H, *d*, 9.0) | C-8a, C-10 |
| 12a | 135.13 | - | |
| 13 | 121.32 | 8.58 (1H, *s*) | C-8a, C-13a, C-13b |

| Carbon | $\delta_C$ (ppm) | $\delta_H$ (ppm) (no. of proton, *mult*., *J* (Hz)) | HMBC |
|---|---|---|---|
| 13a | 139.36 | - | |
| 13b | 120.77 | - | |
| 3-$OCH_3$ | 56.76 | 3.97 (3H, *s*) | C-3 |
| 9-$OCH_3$ | 62.28 | 4.16 (3H, *s*) | C-9 |

$^{13}$C-NMR (100 MHz) and $^{1}$H-NMR (400 MHz) were recorded in a mixed solvent of methanol-$d_4$ and chloroform-*d*.

Some isolated compounds were subjected for cytotoxic activity against eight tested cell lines, P-388, KB, HT-29, MCF-7, A 549, ASK, and Hek-293. The results are shown in table 24. Two lignans, magnone A (**3**) and 3′,4-*O*-dirnethylcedrusin (**9**) showed strong cytotoxicity against MCF-7 cells with $IC_{50}$ values of 1.77 and 1.41 µg/mL, respectively. Compound **9** was also toxic to A549 cells with $IC_{50}$ value of 3.86 µg/mL. Liriodenine (**7**) and oxo-*O*-methylpukakeine (**12**) exhibited cytotoxic activity against all tested cell lines with $ED_{50}$ values in the range of 2.01 to 3.03 µg/mL. 5-Methoxy-4-methyl-1*H*-1-aza-2,9,10-anthracenetrione (**19**) could inhibit the growth of four tested cell lines, P-338, HT-29, MCF-7, and A 549 with $ED_{50}$ values ranging from 2.24-3.31 µg/mL but did not affect to KB and ASK cell lines. Therefore, compounds **3**, **9** and **19** had specific activity to some tested cell lines, therefore, they have been shown to possess an effective anti-cancer agent.

**Table 24** Cytotoxic activity of the isolated compounds

| Compounds | Cell Lines | | | | | | |
|---|---|---|---|---|---|---|---|
| | **P-388** | **KB** | **HT-29** | **MCF-7** | **A549** | **ASK** | **Hek-293** |
| **1** | 9.95 | 5.58 | - | 13.54 | - | - | - |
| **2** | 12.09 | ND | - | 13.42 | - | - | 15.20 |
| **3** | 3.60 | ND | 11.10 | 1.77 | 15.25 | 17.21 | 9.31 |
| **4** | - | ND | - | 11.11 | - | - | - |
| **5** | ND | ND | ND | ND | ND | ND | ND |
| **6** | - | - | - | - | - | - | - |
| **7** | 2.64 | 3.03 | 2.92 | 2.53 | 2.60 | 2.93 | 2.22 |
| **8** | 18.64 | - | - | 12.37 | - | 16.38 | - |
| **9** | 9.68 | - | 7.38 | 1.41 | 3.86 | 19.30 | 3.17 |
| **10** | 18.66 | | - | 8.47 | - | - | 16.51 |
| **11** | - | - | - | - | - | - | - |

| Compounds | Cell Lines | | | | | | |
|---|---|---|---|---|---|---|---|
| | P-388 | KB | HT-29 | MCF-7 | A549 | ASK | Hek-293 |
| **12** | 2.05 | 2.34 | 2.22 | 2.15 | 2.20 | 2.01 | 2.23 |
| **13** | ND | ND | ND | ND | ND | ND | ND |
| **14+15** | - | - | - | - | - | - | - |
| **16+17** | - | - | - | - | - | - | - |
| **18** | - | - | - | - | - | - | - |
| **19** | 2.57 | 10.2 | 2.73 | 2.24 | 3.31 | 10.48 | 17.71 |
| **20** | - | - | - | - | - | - | - |
| **Ellipticine** | 0.50 | 0.58 | 0.59 | 0.53 | 0.65 | 0.63 | 0.66 |

Cytotoxic assay: $ED_{50} \leq 5$ $\mu$g/mL is considered active, - = inactive (> 20 $\mu$g/mL). P-388: murine lymphocytic leukemia, KB: human oral nasopharyngeal carcinoma, HT-29: human colon cancer, MCF-7: human breast cancer (Michican Cancer Foundation), A549: human lung cancer, ASK: cell line from rat glioma cell, and Hek-293: human embryonic kidney cell; ND= Not determine

# CHAPTER IV
# CONCLUSION

*Mitrephora sirikitiae* Weerasooriya, Chalermglin & R.M.K. Saunders is a plant in the genus *Mitrephora*. The plants in this genus have been reported that they have a variety of chemical constituents (terpenoids, alkaloids, lignans, lignanamides, phenolic compounds, flavonoids and polyacetylenic compounds) and biological activities (antimicrobial, anticancer, antimalarial and antiplatelet activating factor (PAF) receptor binding). *Mitrphora sirikitiae* is a new plant in this genus. Moreover, there is no report on chemical and biological studies of this plant. The preliminary biological screening studies of methanol extract of the stem of *M. sirikitiae* indicated that the extract has cytotoxic, anti-inflammatory, and anti-HIV-1 activities. Therefore, this study focused on the chemical and biological investigation of the mixture of leaves and twigs as well as the stem of *M. sirikitiae*.

The isolation of MeOH extract of *Mitrephora sirikitiae* leaves and twigs by using column chromatography, preparative thin layer chromatography (PTLC) and recrystrallization resulted in five lignans, (-)-epieudesmin (**1**), phyllegenin (**2**), magnone A (**3**), forsythialan B (**4**), and 2-(3,4-methylene-dioxyphenyl)-6-(3,5-dimethoxyphenyl)-3,7-dioxabicyclo[3.3.0]octane (**10**), one dihydrobenzofuran lignan, 3′,4-*O*-dirnethylcedrusin (**9**), one flavonoid glycoside, quercetin 3,7-dimethylether 3'-O-$\alpha$-L-rhamnopyranosyl-(1→2)-$\beta$-glucopyranoside (**5**), one steroidal glycoside, stigma-5-en-3-O-$\beta$-glucopyranoside (**6**), and five alkaloids, liriodenine (**7**), dicentrinone (**8**), N-*trans*-feruloyltyramine (**11**), oxo-O-methylpukateine (**12**), and epiberberine (**13**). Moreover, the methanol extract of the stem was separated by chromatographic techniques (column chromatography, chromatotron, and PTLC), acid-base extraction, and recrystallization to yield one lignan, (-)-epieudesmin (**1**), three diterpenoids, kaurenoic acid (**14**), trachyloban-19-oic-acid (**15**), and ciliaric acid (**18**), three sterols, $\beta$-sitosterol (**16**), stigmasterol (**17**), stigma-5-en-3-O-$\beta$-glucopyranoside (**6**), and three alkaloids, liriodenine (**7**), oxo-O-methylpukateine (**12**), 5-methoxy-4-methyl-1H-1-aza-2,9,10-anthracenetrione (**19**) and stepharanine (**20**). The chemical structures of the isolated compounds **1-20** were determined by means of spectroscopic methods, including IR (Infrared Spectroscopy), UV (Visible-Ultraviolet Spectroscopy), NMR (Nuclear Magnetic Resonance Spectroscopy) and MS (Mass Spectrometry) and by comparison their spectroscopic and physical data with previously reported in the literatures.

The isolated compounds were evaluated for cytotoxic activity against eight tested cell lines, P-388 (murine lymphocytic leukemia), KB (human oral nasopharyngeal carcinoma), HT-29 (human colorectal adenocarcinoma), MCF-7 (human breast cancer), A 549 (human lung adenocarcinoma epithelial cells), Hek 293 (human embryonic kidney 293 cells), and ASK (cell line from rat glioma cells). The results revealed that some lignans and alkaloids exhibited cytotoxicity against some tested cell lines. Two lignans, magnone A (**3**) and 3′,4-*O*-dirnethylcedrusin (**9**) showed strong cytotoxicity against MCF-7 cells with $IC_{50}$ values of 1.77 and 1.41 μg/mL, respectively. Compound **9** was also toxic to A549 cells with $IC_{50}$ value of 3.86 μg/mL. Liriodenine (**7**) and oxo-*O*-methylpukakeine (**12**) exhibited cytotoxic activity against all tested cell lines with $ED_{50}$ values in the range of 2.01 to 3.03 μg/mL. 5-Methoxy-4-methyl-1*H*-1-aza-2,9,10-anthracenetrione (**19**) could inhibit the growth of four tested cell lines, P-338, HT-29, MCF-7, and A 549 with $ED_{50}$ values ranging from 2.24-3.31 μg/mL but did not affect to KB and ASK cell lines. Therefore, magnone A (**3**), 3′,4-*O*-dirnethylcedrusin (**9**) and 5-methoxy-4-methyl-1*H*-1-aza-2,9,10-anthracenetrione (**19**) had specific effect to some cell lines, therefore, they have been shown to possess an effective anti-cancer agent.

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## APPENDIX

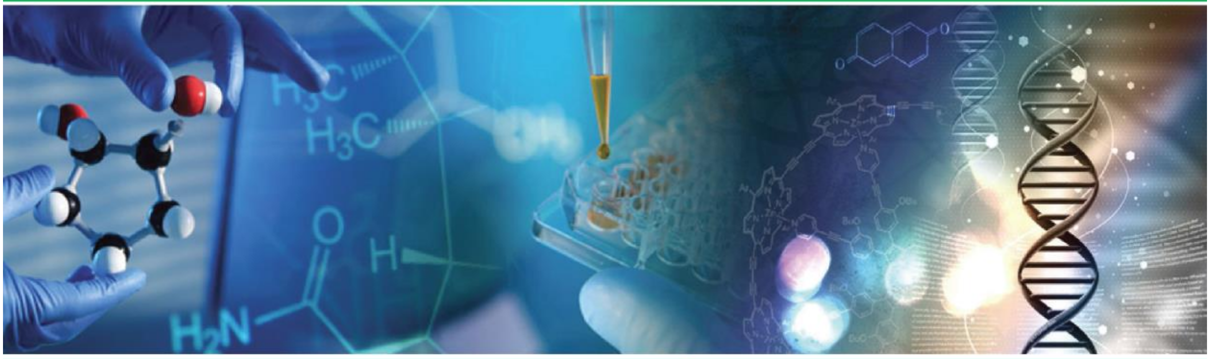
**Output of this research**

1. Investigation of bioactive compounds from the stem of *Mitrephora sirikitiae*, Proceeding in the Pharma Indochina Conference VIII on 4-5$^{th}$ December 2013 at Ho Chi Minh City, Vietnam.
2. Investigation of bioactive compounds from the leaves and twigs of *Mitrephora sirikitiae*, Presentation in the International Congress for Innovation in Chemistry (PERCH-CIC Congress VII) on 5-8$^{th}$ May 2013 at Jomtien Palm Beach Hotel & Resort, Thailand.
3. The genus Mitrephora: Chemistry and biological activities. Precentation in the 2$^{nd}$ Mahidol University-Kitasato University Joint Symposium “Natural Products and Biodiversity” on 9$^{th}$ November 2012 at Kitasato University, Japan.

Proceeding of The Eighth Indochina Conference on Pharmaceutical Sciences

# PHARMA INDOCHINA VIII

## *ASEAN Pharmacy - Integration for Development*



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NHÀ XUẤT BẢN Y HỌC

PO-NP-01

# INVESTIGATION OF BIOACTIVE COMPOUNDS FROM THE STEM OF *MITREPHORA SIRIKITIAE*

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**Abstract**
The biological screening studies of the methanol extract of the stem of *Mitrephora sirikitiae* showed that the extract has anti-inflammatory, anti-HIV-1 and cytotoxic activities. The fractionation of the crude by chromatographic techniques and crystallization led to the isolation of one alkaloid and one diterpenoid, which were determined to be liriodenine and ciliaric acid by means of spectroscopic methods.
**Keywords:** *Mitrephora*, *Mitrephora sirikitiae*, cytotoxic, anti-HIV-1, anti-inflammatory.

## Introduction

*Mitrephora* is a genus in the family Annonaceae. It consists of 48 species of shrubs and small-large trees which are widely distributed in tropical Asia, and north-western Australia. Eight species of *Mitrephora* have previously been recorded from Thailand, including *M. maingayi* Hook. f. & Thomson (Nang-Dang), *M. wangii* Hu (Lumduandoi), *M. tomentosa* Hook. f. & Thomson (Mapuan), *M. vulpine* C. E. C. Fischer (Mapuantai), *M. winitii* Craib (Mahaphrom), *M. alba* Ridl (Phromkoa), *M. keithii* Bidl (Kray) and *M. sirikitiae* Weersooriya, Chalermglin & R. M. K. Saunders (Mahaphrom rachini). Some of them have been used as a tonic medicine in Thailand [1] . Many types of chemical constituents have been reported from the plants in the genus *Mitrephora* such as terpenoids, terpenes, alkaloids, lignans, lignanamides, flavonoids and polyacetylenic acids/esters [2,3]. Moreover, this genus exhibited many biological properties, including antimicrobial [4], anticancer [5], antimalarial [6] and antiplatelet activating factor (PAF) [7].

*Mitrephora sirikitiae* is the new species in genus *Mitrephora*. It was given a royal name because it was found in 2004, the year of 6$^{th}$ cycle of queen Sirikit. In Thailand, this plant is called “Mahaphrom rachini”. It is found at the peak of mountain in Mae-surin waterfall national park, Mae Hong Sorn province. The biological screening studies of the methanol extract of the stems of this plant showed potent anti-inflammatory activity in ethyl phenylpropiolate (EPP)-induced ear edema in rats model, moderate cytotoxic activity against three tested cell lines (P-388: murine lymphocytic leukemia, MCF-7: human breast cancer, and Lu-1: human lung cancer) *in vitro* Sulforhodamine B (SRB) assay and anti-HIV-1 activities in syncytium ($^{\Delta Tat/Rev}$MC99+1A2) assay and HIV-1-RT assay. Therefore, this study focused on the chemical and biological investigation of the stem of *M. sirikitiae*.

In this paper, we report the isolation and characterization of the chemical compounds separated from the crude methanol extracts of the stems of *M. sirikitiae* by chromatographic techniques. The structures of the isolated compounds were elucidated by spectroscopic techniques (NMR, IR, UV and MS).

**Materials and Methods**

***General***

Optical rotations were acquired using JASCO DIP-370 digital polarimeter, 50 mm microcell (1 mL). IR and UV spectra were measured with Nicolet 6700 and SHIMADZU UV-2600, respectively. $^{1}$H-NMR (500 MHz), $^{13}$C-NMR (125 MHz) and 2D-NMR spectra were obtained using Bruker AV 500. ESI-MS and EI-MS data were performed by using Bruker micro TOF spectrometer and Thermo Finnigan Polaris Q, respectively. TLC was carried out on silica gel 60 F254 (Merck, layer thickness 0.2 mm).

***Plant materials***

The stems of *M. sirikitiae* were collected from the peak of mountain in Mae-surin waterfall national park, Mae Hong Sorn Province, Thailand.

***Extraction and isolation***

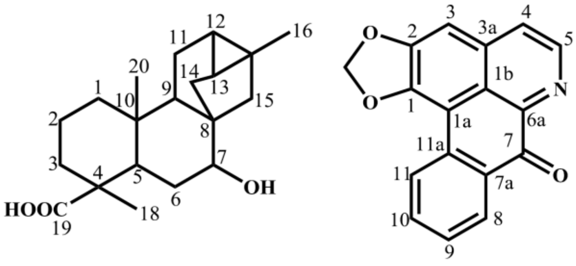
The plant materials were dried at 45-50 °C and ground to fine powder. The powder sample was extracted by maceration with distilled methanol. The solvent was removed under reduced pressure to give crude methanol extract. The crude methanol extract was further separated by quick column chromatography over silica gel (silica gel $P_{60}$ 40 µm-63 µm) eluting with gradient solvents of dichloromethane and methanol to give six fractions (A1-A6) after combination base on TLC characteristic. Fraction A2 was further subjected to column chromatography over silica gel with dichloromethane and methanol to yield fractions B1-B9. Separation of fraction B6 by silica gel column chromatography using dichloromethane and methanol as mobile phase gave four fractions (C1-C4). Fraction C2 was rechromatographed by silica gel column chromatography eluting with dichloromethane and methanol to yield fractions D1-D4. Compounds **1** and **2** were obtained by purification of fractions D1 and D2 by column chromatography over silica gel eluting with dichloromethane-methanol mixture of increasing polarity and crystallization, respectively.

**Results and Discussion**

The investigation of methanolic extract of the stem of *M. sirikitiae* yielded two known compounds **1** and **2** (see the Figure 1).

Compound **1** was recrystallized from methanol as colorless needles. The ESI-MS spectrum showed a quasi-molecular ion peak at *m/z* 317.1505 $[M-H]^{-}$, which established the molecular formula $C_{20}H_{30}O_3$. The IR spectrum showed the absorption bands at 3484 and 1693 $cm^{-1}$ indicating that the presence of hydroxyl group and carboxy carbonyl groups. The $^{13}$C NMR spectrum suggested that **1** has five quaternary, five methine, seven methylene, and three methyl carbons. The characteristic carbon signals at δ 180.5 and δ 75.2 demonstrated carboxy carbonyl and a secondary alcohol. The $^{1}$H NMR spectrum of **1** showed the board triplet δ 3.84 (1H) attributed to the methine of a secondary alcohol, belonging to-$CH_2$-CHOH unit. The structure of **1** was finally identified by long-rang heteronuclear HMBC correlations as ciliaric acid (7α-hydroxytrachyloban-19β-oic acid) [8].

Compound **2** was obtained as yellow needles recrystallized from methanol. The ESI-MS spectrum ($[M+Na]^{+}$ at *m/z* 298.0482) established the molecular formula as $C_{17}H_9NO_3$. The UV absorptions at 228, 245, 270, 281, and 309 nm suggested a highly conjugated system. The IR spectrum showed the presence of carbonyl group (1653 $cm^{-1}$), and methylenedioxy group (1261 and 964 $cm^{-1}$ asymmetric C-O-C stretching). The $^{1}$H NMR spectrum of **2** showed signal of methylenedioxy group at δ 6.36 (2H, s, -$OCH_2O$-). Seven mutually coupled aromatic protons appeared at 7.16 (1H, s), 7.56 (1H, td, $J$ = 8.0, 1.0 Hz), 7.73 (1H, d, $J$ = 8.3, 1.5 Hz), 7.75 (1H, d, $J$ = 5.2 Hz), 8.57 (1H, dd, $J$ = 7.9, 1.4 Hz), 8.63 (1H, d, $J$ = 8.0 Hz), 8.90 (1H, d, $J$ = 5.1 Hz). The $^{13}$C NMR spectrum suggested that **2** has nine quaternary carbons, seven methines, and one methylenes and revealed the presence of a carbonyl group (δ 182.4) and a methylenedioxy group (δ 102.4). Based on the spectroscopic data described above, compound **2** was identified to be liriodenine which was confirmed by comparison of physical and spectroscopic data with those previously reported [9,10].



Ciliaric acid (**1**) Liriodenine (**2**)

**Figure 1:** The structure of the isolated compounds from the methanolic extract of the stem of *M. sirikitae.*

***Ciliaric acid*** **(1)**

The compound was obtained as colorless needles. FTIR $\nu_{max}^{KBr}$ cm$^{-1}$: 3484 (OH), 3130, 1693 (COOH); $[\alpha]_D^{23}$ -193$^o$ (c 0.9, pyridine); $^1$H-NMR (500MHz, Pyr-$d_5$) δ: 0.63 (1H,d, $J$= 7.7 Hz, H-12), 0.90 (1H, dd, $J$ = 2.7, 7.7 Hz, H-13), 1.01 (1H, dt, $J$ = 3.8, 13.3 Hz, H-1a), 1.12 (1H, dt, $J$ = 4.0, 13.4 Hz, H-3a), 1.19 (3H, s, H-17), 1.19 (3H, s, H-20), 1.28 (1H, bd, $J$ = 11.6 Hz, H-14a), 1.39 (3H, s, H-18), 1.45 (1H, m, H-2a), 1.64 (1H, d, $J$ = 12.8 Hz, H-1b), 1.72 (1H,d, $J$= 11.7 Hz, H-15a), 1.86 (2H, bd, $J$ = 14.3 Hz, H-11), 1.89 (1H, m, H-9), 1.97 (1H, d, $J$ = 11.7 Hz, H-15b), 2.08 (1H, d, $J$ = 11.6 Hz, H-14b), 2.18 (1H, dd, $J$ = 4.3, 10.5, H-5), 2.20 (1H, tq, H-2b), 2.42 (2H, m, H-6), 2.47 (1H, bt, H-3b), 3.84 (1H,bt, H-7);$^{13}$C-NMR (125 MHz, Pyr-$d_5$)δ: 13.0 (C-20), 19.6 (C-11), 19.8 (C-2), 20.6 (C-17), 21.1 (C-12), 23.2 (C-16), 24.6 (C-13), 29.3 (C-18), 30.9 (C-6), 33.0 (C-14), 38.9 (C-3), 39.3 (C-10), 40.1 (C-1), 43.6 (C-4), 46.1 (C-8), 46.6 (C-15), 47.1 (C-9), 47.9 (C-5), 75.2 (C-7), 180.5 (C-19); ESI-MS m/z: 317.1505 [M-H]$^-$,calculated for $C_{20}H_{29}O_3$:317.2117.

***Liriodenine*** **(2)**

The compound was obtained as colorless needles. FTIR $\nu_{max}^{KBr}$ cm$^{-1}$: 1653; UV $\lambda_{max}^{MeOH}$ nm (logε): 228 (2.87), 245 (3.10),270 (2.37), 281 (2.71),309 (2.74); $^1$H-NMR (500MHz, $CDCl_3$) δ: 6.36 (2H, s, -$OCH_2O$-), 7.16 (1H, s, H-3), 7.56 (1H,td, $J$ =8.0, 1.0 Hz, H-9), 7.73 (1H, d, $J$ = 8.3, 1.5 Hz, H-10), 7.75 (1H, d, $J$ = 5.2 Hz, H-4), 8.57 (1H, dd, $J$ = 7.9, 1.4 Hz, H-8), 8.63 (1H, d, $J$ = 8.0 Hz, H-11), 8.90 (1H, d, $J$ = 5.1 Hz, H-5); $^{13}$C-NMR (125 MHz, $CDCl_3$) δ: 102.4 (O-CH2-O), 103.2 (C-3), 123.3 (C-1b), 124.1 (C-4), 127.3 (C-11), 128.6 (C-8), 128.8 (C-9), 131.3 (C-7a), 132.9 (C-11a), 133.9 (C-10), 135.7 (C-6a), 144.9 (C-5), 145.4 (C-3a), 147.9 (C-1), 151.7 (C-2), 182.4 (C-7); ESI-MS m/z: 298.0482 [M+Na]$^+$, calculated for $C_{17}H_9NO_3Na$: 298.0480.

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# Investigation of Bioactive Compounds from the Leaves and Twigs of *Mitrephora sirikitiae*

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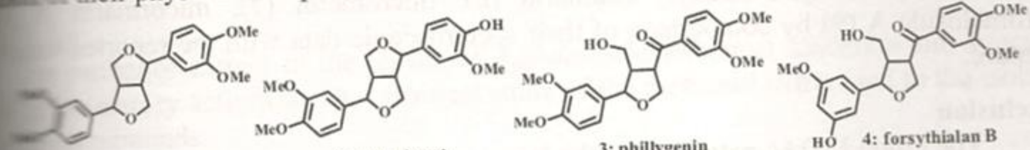
## and Objective

*phora sirikitiae* Weersooriya, Chalermglin & R.M.K. Saunders is an endemic plant of Thailand. A variety of bioactive constituents, alkaloids, terpenoids, steroids, flavonoids, lignanamides, polyacetylenic acid and ester were found in the plants in the same genus. screening of the methanol extracts of the leaves and twigs of *M. sirikitiae* showed to potent cytotoxic, anti-HIV-1 and anti-inflammatory activities. Therefore the research the investigation of bioactive compounds from the methanol extracts of the leaves and this plant.

The methanol extract of the leaves and twigs was separated by chromatographic techniques lization until obtaining pure active compounds. The structure of isolated compounds will by means of spectroscopic method.

The chemical study of the methanol extract of the leaves and twigs of *M. sirikitiae* led to the of four known lignans (1-4). The structures of the isolated compounds were determined by of their physical and spectroscopic data with those reported in the literatures.



1: magnone A 2: epieudesmin 3: phillygenin 4: forsythialan B

The investigation of the methanol extracts of the leaves and twigs of *M. sirikitiae* which moderate to potent cytotoxic, anti-HIV-1 and anti-inflammatory activities led to the isolation lignans, magnone A, epieudesmin, phillygenin and forsythialan B.

*Mitrephora*, *Mitrephora sirikitiae*, cytotoxic, anti-HIV-1, anti-inflammatory

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# 2nd Mahidol University – Kitasato University Joint Symposium
# ~ Natural Products and Biodiversity ~

Date: November 9 (Friday), 11:00–17:05

Place: 211 & 212 Lecture Rooms at Kitasato Institute for Life Sciences, Kitasato University

Organized by Kitasato Institute for Life Sciences, Kitasato University

# The genus *Mitrephora*: Chemistry and biological activities

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The genus *Mitrephora* is in the family Annonaceae and contains about 48 species which are widely found in China, Southern India, Southeast Asia, and Northern Australia.

A variety of chemical constituents, alkaloids, terpenoids, steroids, flavonoids, lignans, lignanamides, polyacetylenic acid and ester were found in the plants in this genus, including *M. maingayi, M. celebica, M. diversifolia, M. glabra, M. thorelii, M. tomentosa* and *M. vulpine*. Moreover, alkaloids, polyacetylenic acids, diterpenes and lignans isolated from *M. glabra*, *M. celebica*, *M. thorelii* and *M. vulpine* have been reported to exhibit cytotoxic, antimicrobial, anti-plasmodial and anti-platelet activating factor activities.

*Mitrephora sirikitiae* Weersooriya, Chalermglin & R.M.K. Saunders is an endemic plant of Northern Thailand. Biological screening of the methanol extracts of the leaves and twigs of *M. sirikitiae* showed moderate to potent cytotoxic, anti-HIV-1 and anti-inflammatory activities.

The chemical study of the methanol extract of the leaves and twigs of *M. sirikitiae* led to the isolation of four known lignans, four known alkaloids, one known flavonoid glycoside and one known steroidal glycoside. The structures of the isolated compounds were determined by comparison of their physical and spectroscopic data with those reported in the literatures.

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