PHYTOCHEMICAL AND BIOACTIVITY STUDIES OF *STROBILANTHES CRISPUS* L.

by

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Chromatography

CC	Column chromatography
GC	Gas chromatography
GC-MS	Gas chromatography-Mass spectrometry
R_f	Retention factor
TLC	Thin layer chromatography
t_R (min)	Retention time (minutes)
TIC	Total ion chromatogram
FID	Flame ionization detector

Instrumental and experimental

IR	Infrared
FID	Flame ionization detector
EI-MS	Electron ionization mass spectrometry
FAB-MS	Fast atom bombardment mass spectrometry
NMR	Nuclear magnetic resonance
COSY	Correlation spectroscopy
DEPT	Distortionless enhancement by polarization transfer
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum correlation
1D, 2D	one- or two- dimensional

Symbols

m/z	mass/charge
eV	electron volt
ppm	part per million
J	coupling constant
Hz	hertz
br	broad
s	singlet
d	doublet
t	triplet
m	multiplet
dd	doublet of doublets
ddd	doublet of doublet of doublets
$[\alpha]_D$	specific optical rotation at the sodium D line
δ_C	carbon-13 chemical shift in parts per million

δ_H	proton chemical shift in parts per million
IC_{50}	concentration of inhibitor required to inhibit a target by 50%

KAJIAN FITOKIMIA DAN BIOAKTIVITI TERHADAP *STROBILANTHES CRISPUS* L.

ABSTRAK

Kajian fitokimia terhadap ekstrak heksana, diklorometana dan metanol daripada daun *Strobilanthes crispus* telah berjaya mengasingkan sembilan sebatian, iaitu 1-heptakosanol (**c-1**), asid lignoserik (**c-2**) dan stigmasterol (**c-3**) daripada ekstrak heksana, campuran empat ester *β*-amirin (**c-4**), tarakseron (**c-5**), tarakserol (**c-6**) dan campuran dua ester tarakserol (**c-7**) daripada ekstrak diklorometana, dan 4-asetil-2,7-dihidroksi-1,4,8-trifeniloktana-3,5-dion (**c-8**) serta stigmasterol 3-*O*-*β*-D-glukopiranosida (**c-9**) daripada ekstrak metanol. Struktur sebatian **c-1** sehingga **c-9** dikenalpasti melalui kaedah spektroskopi dan kromatografi. Pengasingan **c-3** daripada daun *S. crispus* pernah dilaporkan dalam kajian terdahulu, tetapi lapan sebatian lagi merupakan pengasingan pertama kali daripada daun *S. crispus*. Ekstrak diklorometana dan metanol daripada daun serta sebatian yang diasingkan daripada ekstrak tersebut telah diuji untuk aktiviti antibakteria terhadap bakteria Gram-positif: *Bacillus subtilis*, *Staphylococcus aureus*, dan bakteria Gram-negatif: *Klebsiella pneumoniae*, *Escherichia coli* dan *Salmonella typhimurium* dengan menggunakan esei pencairan mikro. Hasil kajian mencadangkan bahawa kebanyakan sampel yang diuji mempunyai aktiviti terhadap *Staphylococcus aureus* dan *Bacillus subtilis* (7.8-62.5 μg mL^{-1}), manakala aktiviti yang sederhana telah diperhatikan terhadap *Salmonella typhimurium* and *Escherichia coli* (31.0-125.0 μg mL^{-1}). Sebatian **c-8** merupakan sebatian yang jarang ditemui dan didapati paling berkesan dalam perencatan pertumbuhan kedua-dua bakteria Gram-positif dan Gram-negatif dengan nilai MIC terendah (antara 7.8 dan 62.5 μg mL^{-1}). Dalam kajian aktiviti

perencatan enzim asetilkolinesterase, sebanyak 85.0% perencatan enzim telah ditunjukkan oleh estrak diklorometana dengan nilai IC_{50} 46.0 µg mL^{-1} pada kepekatan 100.0 µg mL^{-1}. Sebatian yang diasingkan daripada ekstrak tersebut masing-masing menunjukkan aktiviti perencatan asetilkolinesterase pada tahap yang berbeza, dengan **c-8** menunjukkan perencatan enzim yang ketara dengan nilai IC_{50} terendah, iaitu 31.0 µg mL^{-1}, diikuti oleh tarakseron dan campuran dua ester tarakserol, masing-masing ialah 42.0 dan 44.0 µg mL^{-1}. Kajian ini adalah yang pertama melaporkan potensi *S. crispus* terhadap aktiviti perencatan enzim asetilkolinesterase. Keputusan positif yang diperolehi daripada kajian ini telah mencadangkan bahawa tumbuhan ini mempunyai aktiviti antibakteria dan anti-kolinesterase.

PHYTOCHEMICAL AND BIOACTIVITY STUDIES OF *STROBILANTHES CRISPUS* L.

ABSTRACT

Phytochemical investigation of the hexane, dichloromethane and methanol extracts of the leaves of *Strobilanthes crispus* has led to the isolation of nine compounds, comprising 1-heptacosanol (**c-1**), lignoceric acid (**c-2**) and stigmasterol (**c-3**) from the hexane extract, a mixture of four esters of *β*-amyrin (**c-4**), taraxerone (**c-5**), taraxerol (**c-6**) and a mixture of two esters of taraxerol (**c-7**) from the dichloromethane extract, as well as 4-acetyl-2,7-dihydroxy-1,4,8-triphenyloctane-3,5-dione (**c-8**) and stigmasterol 3-*O*-*β*-D-glucopyranoside (**c-9**) from the methanol extract. Structures of compounds **c-1** to **c-9** were elucidated with the aid of chromatographic and spectroscopic techniques. Compound **c-3** has previously been reported from the leaves of *S. crispus*, however, the rest of the compounds were isolated for the first time from this plant. The dichloromethane and methanol leaf extracts, together with the isolated compounds were tested against Gram-positive bacteria: *Bacillus subtilis*, *Staphylococcus aureus*, and Gram-negative bacteria: *Klebsiella pneumoniae*, *Escherichia coli* and *Salmonella typhimurium*, using micro-dilution assay. The majority of the samples tested indicated promising inhibitory activities against *Staphylococcus aureus* and *Bacillus subtilis* (7.8-62.5 μg mL^{-1}), while appreciable activity was observed against *Salmonella typhimurium* and *Escherichia coli* (31.0-125.0 μg mL^{-1}). Compound **c-8**, a rare natural occurring compound, exhibited the strongest inhibitory effect against both Gram-positive and Gram-negative bacteria, with low MIC values (between 7.8 and 62.5 μg mL^{-1}). In the acetylcholinesterase enzyme inhibitory test, the dichloromethane extract showed activity

(85.0%) against acetylcholinesterase with an IC_{50} value of 46.0 µg mL^{-1} at the concentration of 100.0 µg mL^{-1}. The isolates exhibited different levels of acetylcholinesterase inhibitory activities with **c-8** being significantly active in this bioassay with an IC_{50} of 31.0 µg mL^{-1}, followed by taraxerone and the mixture of the two fatty esters of taraxerol, with IC_{50} values of 42.0 and 44.0 µg mL^{-1}, respectively, with reference to galanthamine (control). This study is the first report describing the potential of *S. crispus* in the acetylcholinesterase enzyme inhibitory activity. The results of this study suggested that this plant possesses both antibacterial and anti-cholinesterase activities.

CHAPTER ONE

INTRODUCTION

1.1 Medicinal plants

A recent report shows that approximately 420,000 plant species exist in nature (Vuorela *et al.*, 2004). Medicinal plants are known as plants that are able to produce active constituents to prevent diseases, maintain health or cure ailments. Traditionally, medicinal plants are being explored therapeutically to alleviate ailments in humans for several millennia (Chen *et al.*, 2003; Chattopadhyay *et al.*, 2004; Radad *et al.*, 2006). Numerous studies have been conducted to isolate and identify novel compounds for further investigation of their biological activities (Zheng & Wang, 2001; Cai *et al.*, 2004; Surveswaran *et al.*, 2007). Medicinal plant drug discovery continues to supply crucial leads against assorted targets including cancer, HIV/AIDS, Alzheimer's, malaria and pain (Balunas & Kinghorn, 2005). Among over 15,000 species of higher plants found in Malaysia, 1200 are reported to possess pharmaceutical value, and of which can be classified as medicinal plants (Soepadmo, 1991; Bakar *et al.*, 2006).

1.2 Acanthaceae Family

Acanthaceae family, or Acanthus family, is a taxon of dicotyledonous flowering plants containing almost 346 genera and about 4300 species. Most of them are tropical herbs, shrubs, or twining vines; some are epiphytes. Only a few species are distributed in temperate regions. The four main centers of distributions are Indonesia and Malaysia, Africa, Brazil, and Central America. The representatives of the family can be found

nearly in every habitat, including dense or open forests, scrublands, wet fields, valleys, sea coast, marine areas, and swamps, as an element of mangrove woods (Sasidharan, 2004).

1.2.1 The Genus *Strobilanthes*

Strobilanthes is the second largest genus in the Acanthaceae family after *Justicia* L., comprising approximately 350 species of perennial herbs and subshrubs. It can be found mostly in Asia's tropical region with a few species extending north into Asia's temperate region (Scotland & Vollesen, 2000). It is known as one of the most interesting genera owing to its diversified habits, gregarious nature and scant but elegant flowering. *Strobilanthes* plants typically have opposite leaves which are unequal in size (Liamas, 2003).

1.2.1.1 *Strobilanthes crispus* L. Bremek

Strobilanthes crispus L. Bremek (Figure 1.1) is a bush-like plant that can be found in tropical countries ranging from Madagascar to Indonesia on riverbanks or abandoned fields (Baker & Bakhuizen, 1965). It is popularly known as "daun pecah beling" in Jakarta or "enyoh kilo", "kecibeling" or "kejibeling" in Java and "Hei Mian Jiang Jun" in Chinese. It is a woody spreading shrub that can grow up to 6 feet in height. Javanese would often use this plant as fence hedges due to their height. The leaves are dark green in colour, oblong-lanceolate, a little toothed and elliptical in shape. As the bottom of the leaves is covered with short hairs, the leaves are rough to touch from underside as compared to the top surface. The plant is panicled with cluster of yellow flowers budded

in leafy sheaths. This shrub can be propagated *via* stem cuttings (Sunarto, 1977; Fadzelly *et al.*, 2006).

Figure 1.1 *Strobilanthes crispus* L. Bremek

1.3 Biological properties of *S. crispus*

Throughout history, natural products have afforded us a large number of compounds with antiviral, antibacterial, antimalarial, anti-inflammatory, antioxidant, and anticancer properties. More than 60% of the commercially available anticancer drugs were discovered from natural sources. Infusion of the dried leaves of *S. crispus* has been used in traditional Chinese medicine and folk medicine for their antidiabetic, diuretic, anticancer and blood pressure lowering properties (Perry & Metzger, 1980; Bakar *et al.*, 2006). A poultice of fresh leaves is reported for treatment of wounds and snake bites, and oral administration of the *S. crispus* juice for enhancement of the rate of wound

healing in normal and diabetic rats (Wijayakusuma, 2000; Norfarizan-Hanoon *et al.*, 2009).

The methanolic crude extract of *S. crispus* exhibited potent antibacterial activity against *Bacillus cereus* (Muskhazli *et al.,* 2009). Muslim *et al.* (2010) and Rahmat *et al.* (2006) reported the methanolic extract displayed a promising cytotoxic effect on certain human cancer cell lines such as colon carcinoma cell (HCT 116), non-small cell lung adenocarcinoma cell (NCI-H23) and human breast ductal carcinoma cell (T-47D). Yaacob *et al.* (2010) also reported that a sub-fraction from the dichloromethane extract of *S. crispus* possessed potential as a cancer therapeutic agent which selectively killed breast and prostate cancer cell lines, but not the normal breast epithelial cell line. Not only did the leaves extracts kill the breast cancer cell lines, a significant anti hepatocarcinogenesis effect on rats was also confirmed. It was also found that the hot water extract of the fermented and unfermented leaves has the efficacious ability to reduce blood glucose level in hyperglycemic rats (Fadzelly *et al.*, 2006).

Kusumoto *et al.* (1992) had confirmed that the water extract of *S. crispus* inhibited the proliferation of retrovirus; an agent in viral disease such as acquired immune deficiency syndrome (AIDS) and adult T-cell leukemia. Iqbal *et al.* (2010) reported that a dose-dependent of extracts *S. crispus* showed possible protection against lipid peroxidation and DNA damage induced by Fe-NTA and H_2O_2.

Rahmat *et al.* (2006) reported that there were high antioxidant activities showed by the extracts of *S. crispus*, using the ferric thiocyanate (FTC) and thiobarbituric acid (TBA)

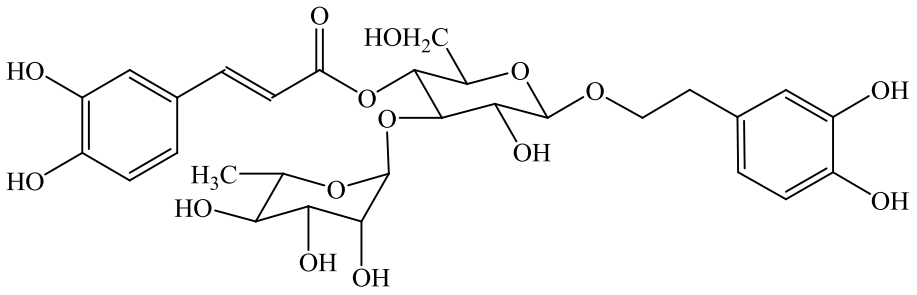
methods. Cosmetics with *S. crispus* as an active ingredient further featured excellent skin brightening properties and also skin pigmentation relieving properties (Sawaki *et al.*, 2002).

1.4 Previous studies on *S. crispus*

Despite being the second largest genus in the Acanthaceae family, only very few species within the genus *Strobilanthes* have been investigated for their phytochemical constituents. Although the literature survey revealed that the leaves of *S. crispus* are potent for treatment of several diseases, only very few reports on its phytochemical investigation are available.

1.4.1 Phytochemical studies on *S. crispus*

The earliest phytochemical investigation on *S. crispus* started in 1987, when Soedira *et al*. (1987) isolated verbacoside **(1)** and seven phenolic acids, namely, caffeic acid **(2)**, ferulic acid **(3)**, gentisic acid **(4)**, *p*-coumaric acid **(5)**, *p*-hydroxybenzoic acid **(6)**, syryngic acid **(7)** and vanillic acid **(8)** from the leaves. Liza *et al*. (2010) reported the presence of eight bioactive flavonoids identified as apigenin **(9)**, (+)-catechin **(10)**, (−)-epicatechin **(11)**, kaempferol **(12)**, luteolin **(13)**, myricetin **(14)**, naringenin **(15)** and rutin **(16)**. *β*-Sitosterol **(17)** and stigmasterol **(18)**, were isolated from the leaf extracts of *S. crispus* (Rahmat *et al.*, 2006). In addition, the leaves were reported to be rich in minerals such as potassium and calcium and contained high levels of vitamins C, B1, B2 which contributed further to its total anti-oxidant activity (Ismail *et al.*, 2000).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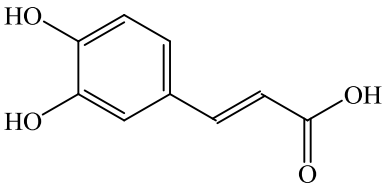
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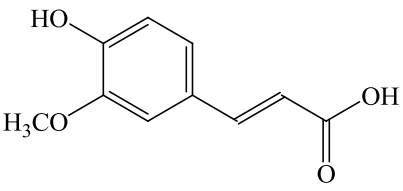
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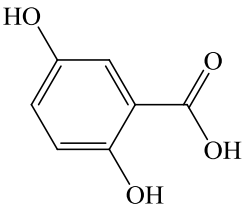
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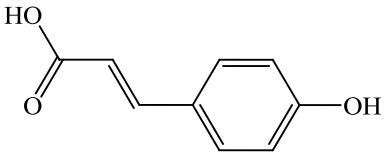
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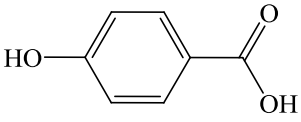
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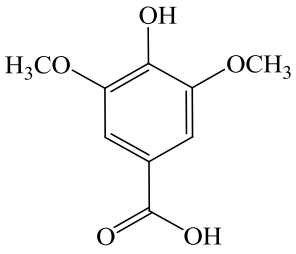
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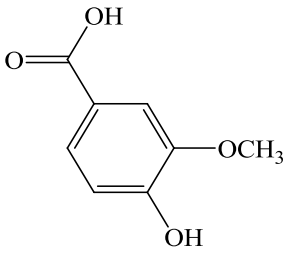
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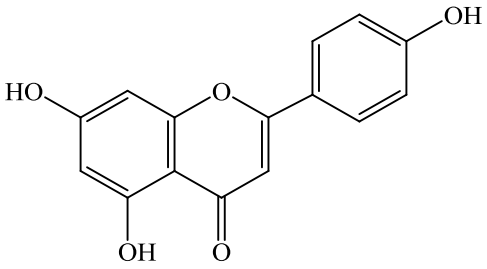
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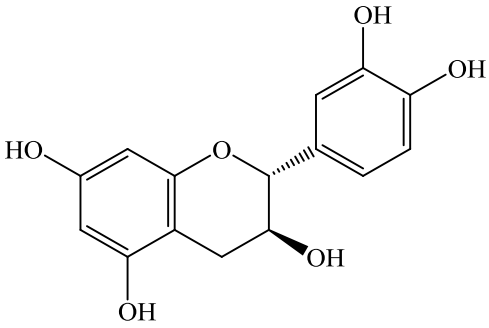
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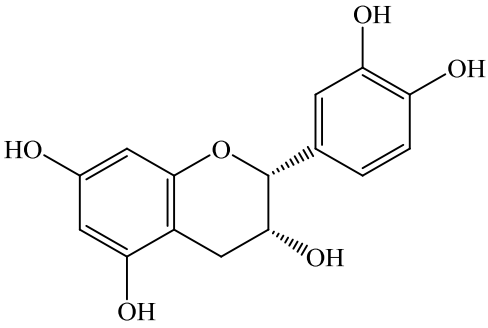
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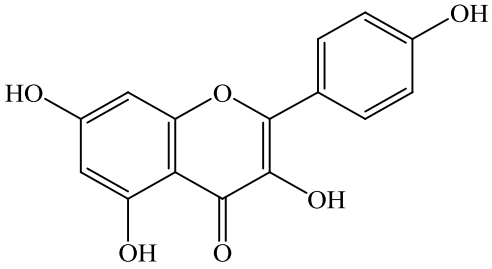
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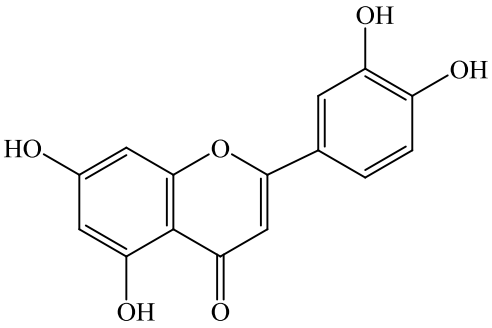
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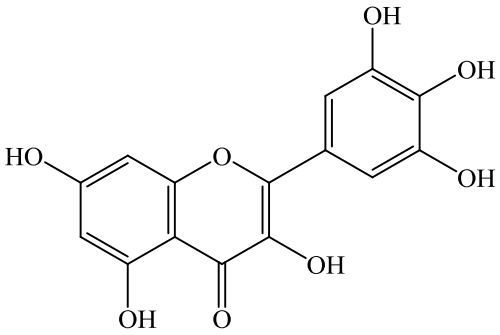
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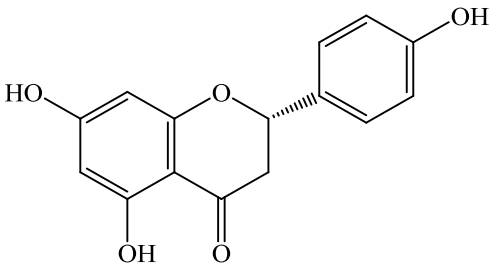
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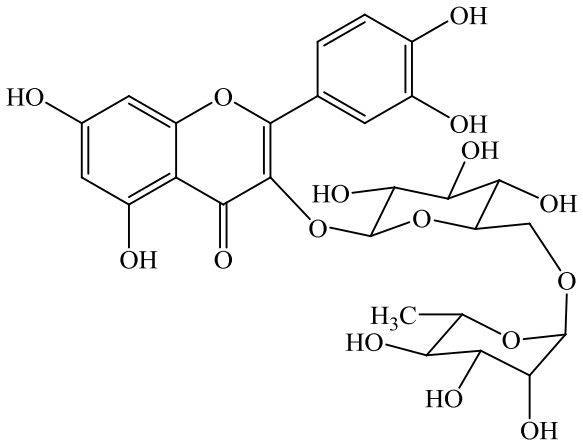
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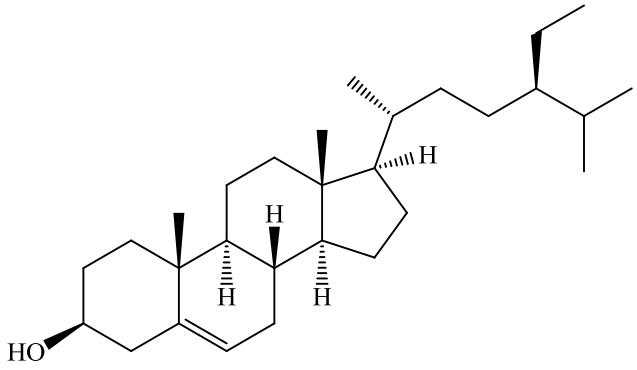
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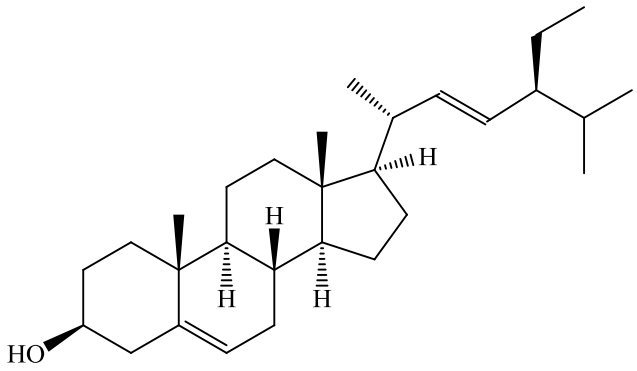

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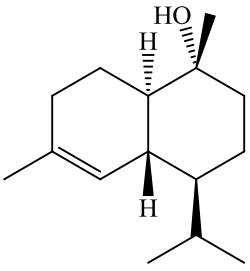
1.4.2 Studies on the volatile constituents of *S. crispus*

Rahmat *et al*. (2006) identified a total of 28 constituents among the volatile constituents of *S. crispus* leaves. They were identified by the GC-MS analysis and the major components were determined to be *α*-cadinol **(19)**, 2,3-dihydrobenzofuran **(20)**, eugenol **(21)**, megastigmatrienone **(22)** and *trans*-phytol **(23)**.



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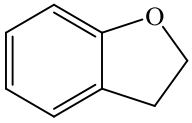

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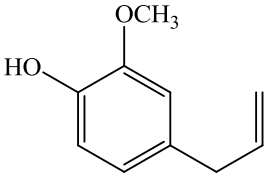

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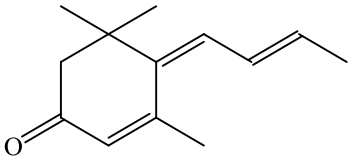

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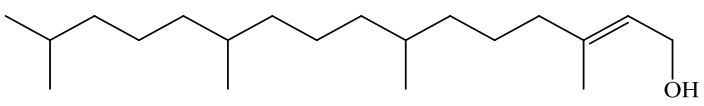

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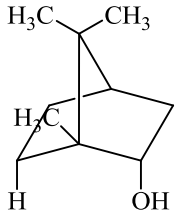
1.5 Studies on other *Strobilanthes* species

1.5.1 *Strobilanthes auriculatus*

A total of twenty-three compounds were identified in the essential oil of *S. auriculatus*, of which the two major components were found to be borneol **(24)** and isoborneol **(25)** (Zutshi, 1970; Weyerstahl *et al.*, 1987; Weyerstahl *et al.*, 1988).



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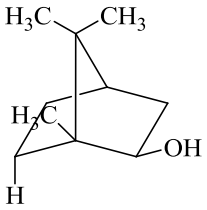
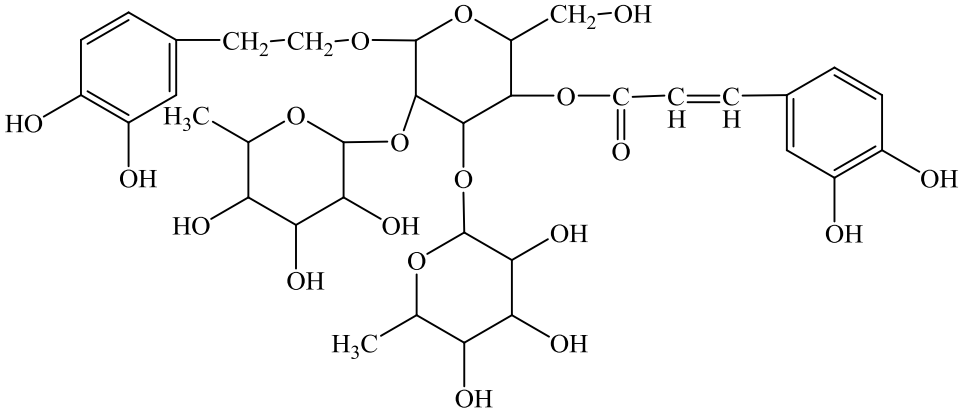


25

1.5.2 *Strobilanthes callosus*

Agarwal & Rangari *et al*. (2001) reported the presence of crassifolioside **(26)**, lupeol **(27)**, stigmasterol 3-*O*-*β*-D-glucopyranoside **(28)**, and few phenylpropanoid glycosides from the chloroform and ethyl acetate extracts of *S. callosus*. Singh *et al.* (2002) reported the presence of taraxerol **(29)** from the benzene extract *S. callosus*.



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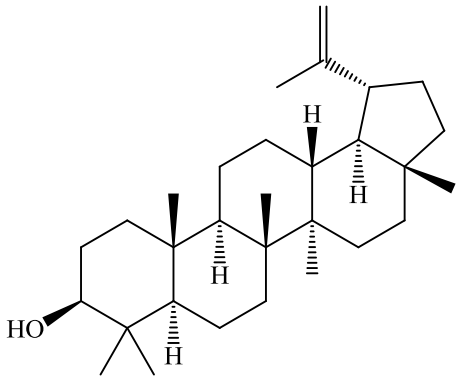

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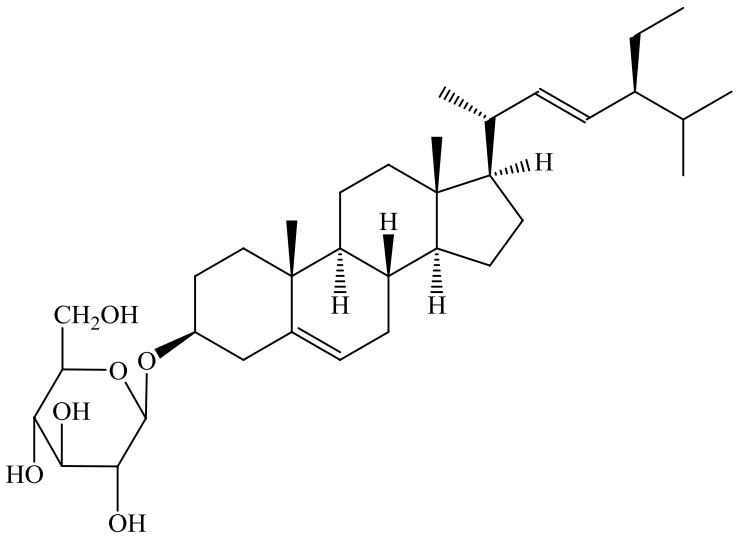

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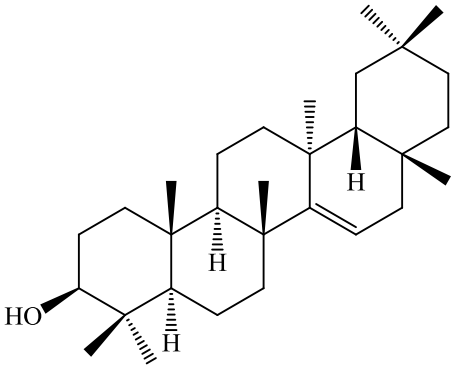

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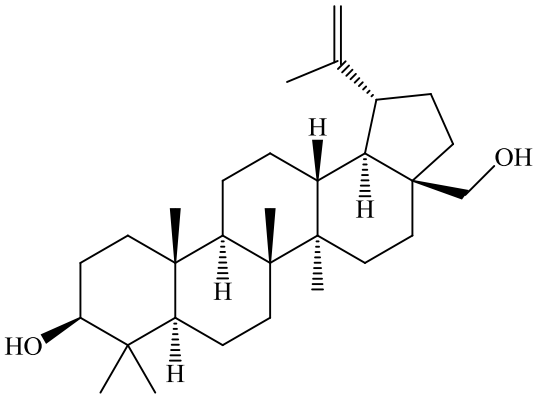
1.5.3 *Strobilanthes ciliatus*

Phytochemical investigations on the extracts of the root and stem of *S. ciliatus* have resulted in the isolation of six compounds, namely betulin **(30)**, lupeol **(27)**, stigmasterol **(18)**, stigmasterol glycoside **(28)**, taraxerol **(29)** and 4-acetyl-2,7-dihydroxy-1,4,8-triphenyloctane-3,5-dione **(31)** (Reneela & Sripathi, 2010).



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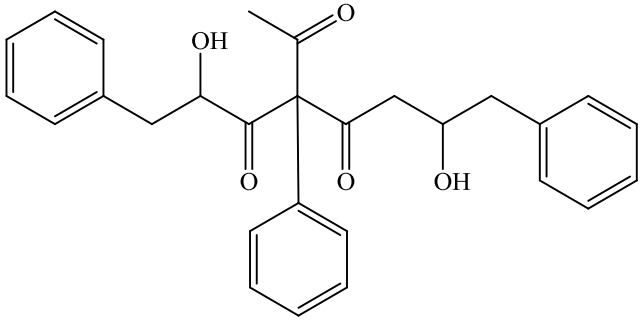
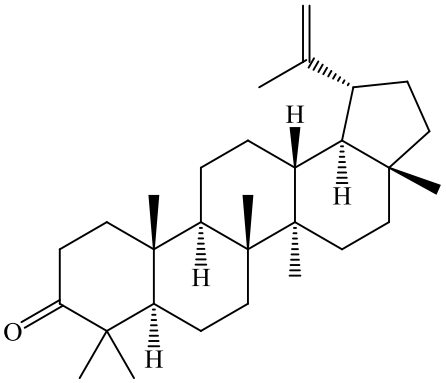


31

1.5.4 *Strobilanthes cusia*

Chen *et al*. (1987) isolated *β*-sitosterol **(17)** and three triterpenoids, namely, betulin **(30)**, lupeol **(27)** and lupenone **(32)** from the roots of *S. cusia*. Isolation by Li *et al*. (1993)

yielded two quinazolinone alkaloids, 4-quinazolinone **(33)**, and 2,4-quinazolinedione **(34)**, and two indole alkaloids, idirubin **(35)** and indigo **(36)**.



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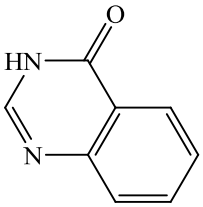

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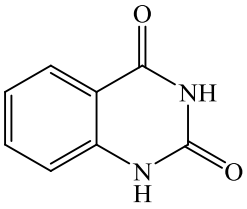

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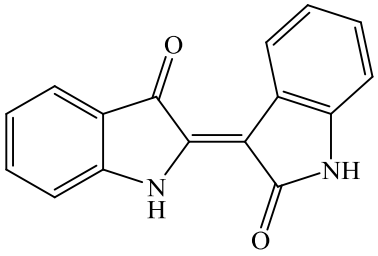

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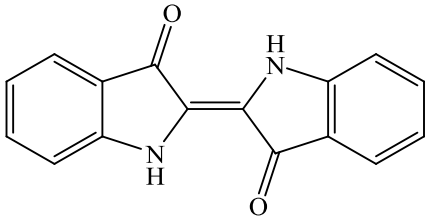
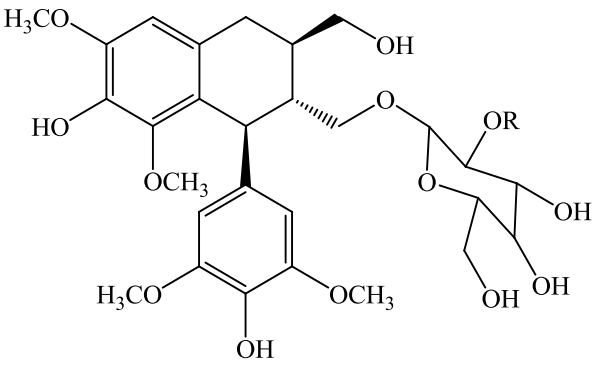


36

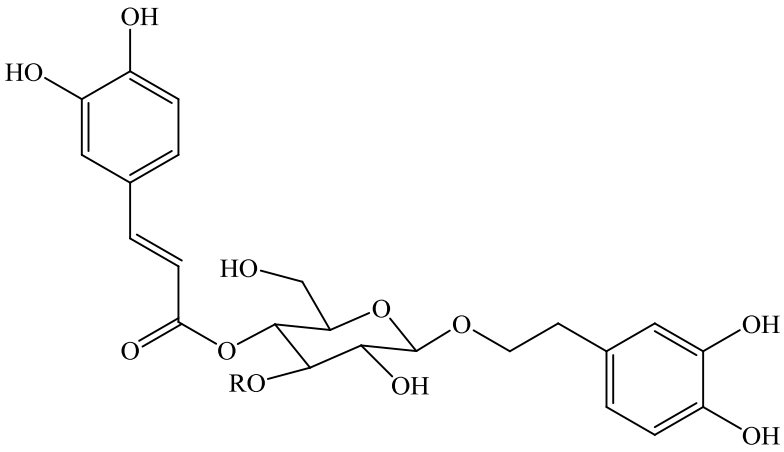
A total of eight compounds were isolated from *S. cusia***,** a new lignan, namely, (+)-lyoniresinol 3*α*-*O*-*β*-D-apiofuranosyl-(1→2)-*β*-D-glucopyranoside **(37)**, (+)-9-*O*-*β*-D-glucopyranosyl lyoniresinol **(38)**, two phenylethanoid glycosides, namely, [2-(3,4-dihydroxyphenylethyl)]-3-*O*-*α*-D-apiofuranosyl-(1→4)-4-*O*-caffeoyl)-*β*-D-glucopyranoside (cusianoside A) **(39)**, and [2-(3,4-dihydroxyphenylethyl)]-3-*O*-*β*-D-xylopyranosyl-(1→3)-4-*O*-caffeoyl)-*β*-D-glucopyranoside (cusianoside B) **(40)**, together

with lupeol **(27)**, acetoside **(41)**, (+)-5,5'-dimethoxy-9-*O*-*β*-D-glucopyranosyl lariciresinol **(42)** and (+)-5,5'-dimethoxy-9-*O*-*β*-D-glucopyranosyl secoisolariciresinol **(43)** (Tomonori *et al.*, 2004).



37 R = D-Apiofuranosyl

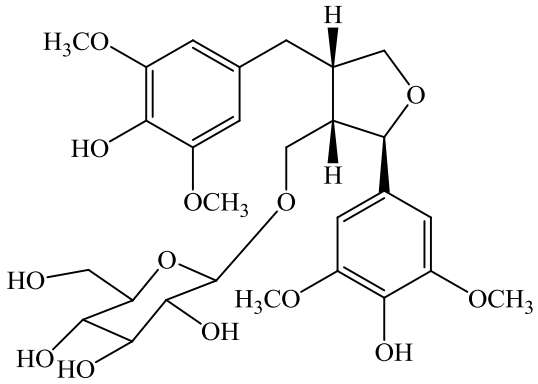
38 R = H



39 R = D-Apiofuranosyl

40 R = D-Xylopyranosyl

41 R = L-Rhamnopyranosyl



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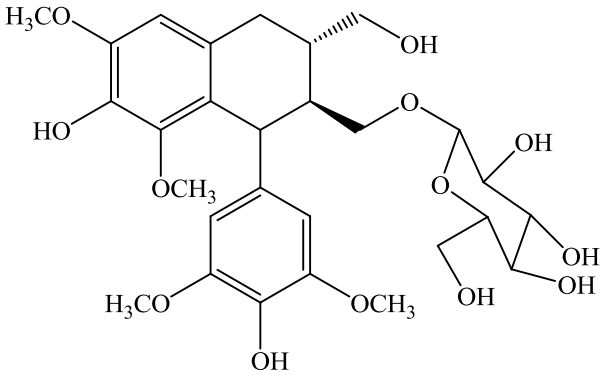
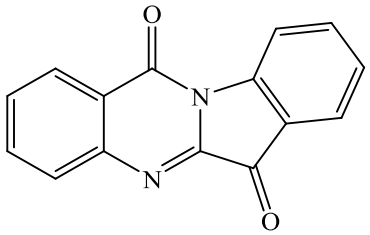


43

Kuntze *et al.* (1979) reported the presence of an alkaloid, namely tryptanthrin **(44)**, from the leaves of *S. cusia*. A previous study conducted by Li *et al*. (2007) confirmed the presence of a seco-pregnane steroid glaucogenin C **(45)** and its monosugar-glycoside cynatratoside A **(46)** in the leaves of *S. cusia*.



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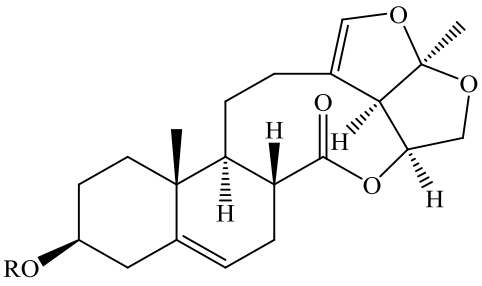
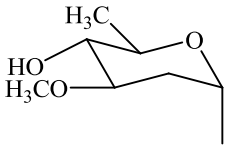
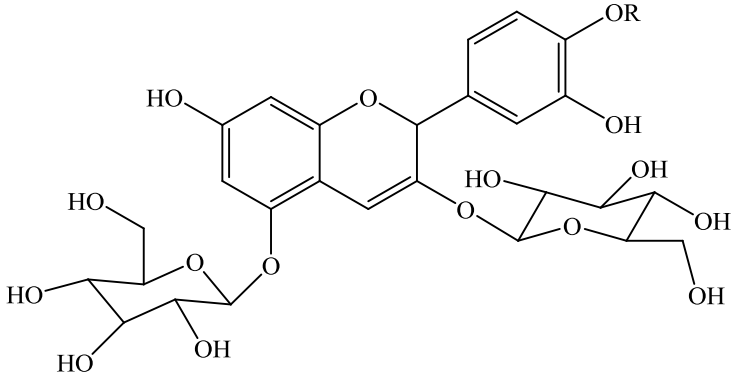


45 R = H

46 R = 

1.5.5 *Strobilanthes dyeriana*

Smith *et al*. (1981) isolated two anthocyanins, namely, cyanidin-3,5-diglucoside **(47)** and peonidin-3,5-diglucoside **(48)**, from the leaves of *S. dyeriana*.



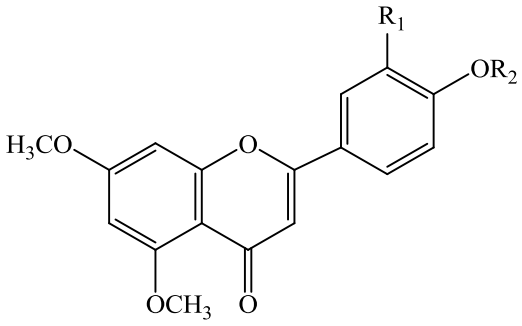
47 R = H

48 R = CH_3

1.5.6 *Strobilanthes formosanus*

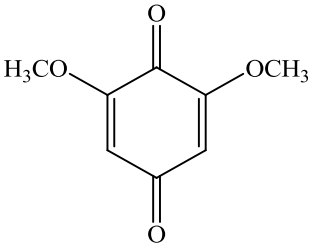
Kao *et al*. (2004) revealed the presence of two new flavone glycosides, 5,7-dimethoxyflavone-4'-*O*-[*β*-D-apiofuranosyl(1→5)-*β*-D-glucopyranoside] **(49)** and 3'-

hydroxy-5,7-dimethoxyflavone-4'-*O*-*β*-D-apiofuranoside **(50)** and together with four known compounds betulin **(30)**, 2,6-dimethoxy-1,4-benzoquinone **(51)**, 4'-hydroxy-5,7-dimethoxyflavone **(52)** and lupeol **(27)** from the stem and roots of *S. formosanus***.**



49 R_1 = H, R_2 = -[*β*-D-apiofuranosyl(1→5)-*β*-D-glucopyranoside]

50 R_1 = OH, R_2 = -*β*-D-apiofuranoside



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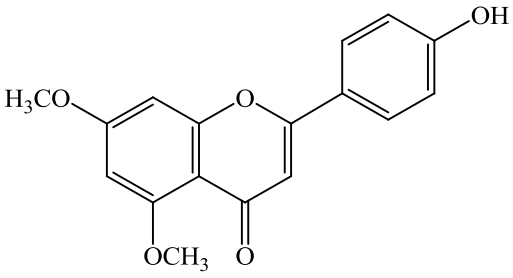

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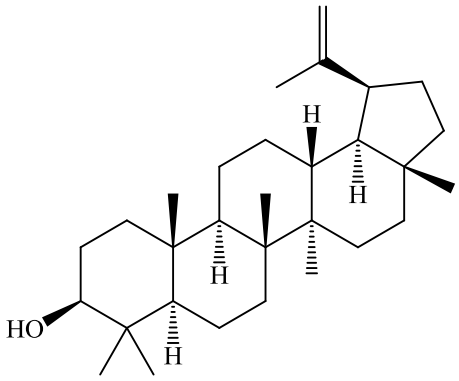
1.5.7 *Strobilanthes ixiocephala*

Agarwal & Rangari *et al*. (2001) isolated a rare triterpenic alcohol 19*β*-lupeol **(53)** from *S. ixiocephala*. Investigation of the essential oil from the flowering tops of *S. ixiocephala* indicated the presence of cadinol **(19)**, *β*-caryophyllene **(54)**, *α*-fenchyl acetate **(55)** and a new sesquiterpene, ixiocephol **(56)** (Agarwal & Rangari, 2003a).



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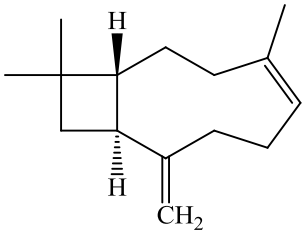

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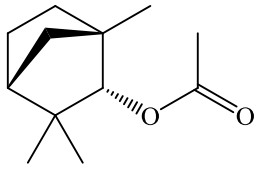

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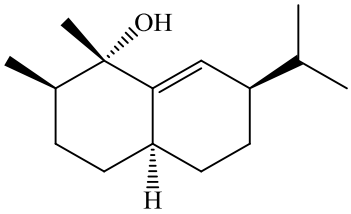

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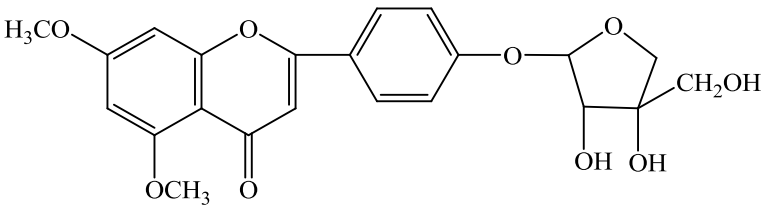
1.5.8 *Strobilanthes japonicas*

A new flavone glycoside, 5,7-dimethoxy-4'-hydroxyflavone-4'-*O*-apioside **(57)**, namely, strobilanthin, together with two known compounds 5,7-dimethoxy-4'-hydroxyflavone **(58)** and stigmasterol **(18)**, were isolated from the extract of *S. japonicas* (Huang *et al.*, 1987).



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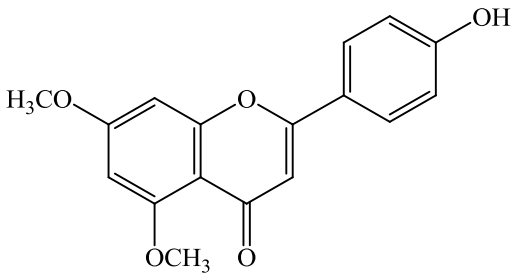

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1.6 Biological properties of other species of the genus *Strobilanthes*

An alkaloid, namely, tryptanthrin **(46)**, isolated from the leaves of *Strobilanthes cusia* was found to possess a marked antifungal properties in treating dermatophytes (Kuntze *et al.*, 1979). Tryptanthrin **(46)** is confirmed to display an inhibitory activity against the growth of *Mycobacterium* tuberculosis (Mitscher & Baker, 1998). The methanol extract of *S. cusia* leaves displayed potent anti-inflammatory and antipyretic effects (Ho *et al.*, 2003). The roots of *S. cusia* are popularly used as a traditional Chinese medicine for treating influenza, epidemic cerebrospinal meningitis, encephalitis B, viral pneumonia and mumps (Tomonori *et al.*, 2004). The dichloromethane and ethyl acetate extracts of *S. cusia* marked a significant cytotoxic activity on cell lines (Nguyen *et al.*, 2006). Steroids such as glaucogenin C **(44)** and cynatratoside A **(45)**, isolated from *S. cusia*, were reported to exhibit antiviral activity (Li *et al.*, 2007).

Lupeol **(27)** and 19α-lupeol **(52)**, isolated from the roots of *Strobilanthus callosus* and *Strobilanthus ixiocephala* exhibit anti-inflammatory and antiarthritic activities (Agarwal & Rangari, 2003b). Both benzene and ethanol extracts and taraxerol **(29)**, isolated from *S. callosus*, demonstrate anti-inflammatory and antimicrobial activities respectively

(Singh *et al.*, 2002). Ngo *et al.* (1995) demonstrated that *Strobilanthes flaccidifolius* juice, when taken orally by normal guinea pigs and rabbits, possessed potent abortive properties.

1.6 Objectives

1. To isolate and identify the chemical constituents of the leaves of *S. crispus*.

2. To characterize the isolated constituents with the aid of different spectroscopic methods, namely, IR, mass spectrometry and nuclear magnetic resonance spectroscopy, including 2D NMR techniques.

3. To evaluate the biological activities of the selected crude extracts and isolated compounds.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Collection of plant material

Strobilanthes cripus was obtained from a commercial supplier and was identified by Mr. V. Shunmugam, a staff of the herbarium of the School of Biological Sciences, Universiti Sains Malaysia, Penang, where a voucher specimen (Voucher No. 11246) has been deposited.

2.2 Extraction procedure

Fresh leaves (3.0 kg) collected from the plant were rinsed with distilled water and were air-dried at room temperature in an open space for 2 weeks. The air-dried leaves (1.0 kg) were powdered and macerated sequentially in hexane, dichloromethane and methanol. Each of the different extractions was performed at room temperature three times (3 × 5 L), 24 h each time. All extracts after filtration were evaporated *in vacuo* using a rotary evaporator to give 10 g (0.33% w/w of fresh leaves), 15 g (0.50% w/w of fresh leaves) and 12 g (0.40% w/w of fresh leaves) of hexane, dichloromethane and methanol extracts, respectively.

2.3 Chromatography

2.3.1 Thin Layer Chromatography

Thin layer chromatography (TLC) were performed on pre-coated TLC plates (20 × 20 cm, coated with 0.2 mm silica gel F_{254} on aluminium sheets, Merck). The spots on TLC plates were visualized with a UV lamp (Vilber Lournet, multiband UV-254/356 nm). Triterpenes and plant sterols were detected with 95% methanolic sulphuric acid by heating the TLC plates with a heat gun after the plates were dipped in the reagent.

2.3.2 Column chromatography

Column chromatography (CC) was carried out using silica gel 60 (230-240 mesh ASTM, Merck, 0.040-0.060 mm) and gradient elution was performed from less polar solvents to more polar solvents (hexane, chloroform, ethyl acetate and methanol). Samples were dissolved in a minimum quantity of an appropriate solvent and taken up in a small quantity of silica gel. Upon drying on a rotary evaporator, a powdered material was produced which was loaded onto the column (Sharp *et al.*, 1989).

2.4 Instrumental

2.4.1 Specific optical rotation measurement

Optical rotations were measured using an ATAGO AP-300 automatic polarimeter (Japan). Sucrose solution (1.0%) was used as a standard and the sodium lamp was set at 589 nm. Samples were dissolved in chloroform and a cell with 100 mm length was used for the measurements.

2.4.2 Melting point determination

Melting points were measured on a Stuart Scientific SMP-1 (United Kingdom) melting point apparatus.

2.4.3 Infrared spectroscopy

Infrared (IR) spectra were recorded on a Perkin-Elmer System 2000 FT-IR spectrometer (England, United Kingdom). Spectra were obtained by the pressed disk technique using potassium bromide (KBr), and scanned in the range 4000-650 cm^{-1}.

2.4.4 Direct-probe mass spectrometry

The electron impact mass spectra (EI-MS), 70 eV and fast atom bombardment mass spectra (FAB-MS) were determined on an Agilent 5975C MSD Mass Spectrometer and a Thermo Finnigan MAT95XL Mass Spectrometer, respectively.

2.4.5 Gas chromatography

GC analysis of the methyl esters from compounds **c-4** and **c-7** were carried out using a Thermo Finnigan instrument, fitted with a Supelcowax 10 fused-silica capillary column (30 m, 0.25 mm ID, 0.25 μm, Supelco Inc., USA) and equipped with a flame ionization detector (FID). The operating conditions were: initial oven temperature, 40°C (held for 10 min), then programmed at 5°C min^{-1} to 250°C, and held for 20 min at 250°C. The carrier gas was N_2 at a flow rate of 2.0 mL min^{-1}. Injector port and detector temperatures were set at 250°C and 275°C, respectively.