PHYTOCHEMICAL AND BIOLOGICAL INVESTIGATION ON THREE SPECIES OF THE GENUS *HOMALOMENA*

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UNIVERSITI SAINS MALAYSIA
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PHYTOCHEMICAL AND BIOLOGICAL INVESTIGATION ON THREE SPECIES OF THE GENUS *HOMALOMENA*

By

ABDUL HAMID

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Doctor of Philosophy

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Abdul Hamid

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Biological activities

COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
COX-3	Cyclooxygenase-3
NSAIDs	Nonsteroidal anti-inflammatory drugs
AChE	Acetylcholinesterase
MIC	Minimum inhibitory concentration
INT	*p*-iodonitrotetrazolium violet
ATCI	Acetylthiocholine iodide

Chromatography

TLC	Thin layer chromatography
PTLC	Preparative thin layer chromatography
CC	Column chromatography
FID	Flame ionization detector
RI	Retention Index
RT	Retention time (minutes)

Mass spectrometry (MS)

EI-MS	electron ionization mass spectrometry
FAB	fast atomic bombardment
DP-MS	Direct Probe- Mass Spectrometry

m/z	mass/charge
eV	electron volt
amu	atomic mass unit

Nuclear magnetic resonance (NMR)

ppm	part per million
J	coupling constant
s	singlet
d	doublet
dd	doublet of doublet
t	triplet
q	quartet
COSY	Correlation Spectroscopy
DEPT	Distortionless enhancement by polarization transfer
HMQC	Heteronuclear Multiple Quantum Correlation
HMBC	Heteronuclear Multiple Bond Correlation
NOESY	Nuclear Overhauser Enhancement Spectroscopy
NOE	Nuclear Overhauser Effect

KAJIAN FITOKIMIA DAN BIOLOGI KE ATAS TIGA SPESIES DARI PADA GENUS *HOMALOMENA*

ABSTRAK

Tiga species daripada genus *Homalomena* iaitu *H. pineodora, H. sagittifolia dan H. coerulescens* telah dipilih untuk penyelidikan ini memandangkan tumbuhan tersebut telah biasa digunakan dalam bidang perubatan tradisional. Minyak pati bahagian daun dan rizom tiga spesies tersebut telah dipencilkan melalui kaedah penghidrosulingan dan dianalisiskan dengan kaedah kapilari GC dan GC-MS dengan menggunakan dua kolum yang mempunyi kekutuban yang berbeza. Komponen-komponen fitokimia yang mudah meruap dipencilkan dan ditulenkan melalui pelbagai sistem pelarut dan kaedah kromatografi dan struktur-struktur sebatian tersebut ditentukan melalui kaedah-kaedah spektroskopi seperti FT-IR, satu dan dua dimensi eksperiman NMR: ^{1}H-NMR, ^{13}C-NMR, DEPT 45, DEPT 90, DEPT 135, ^{1}H-^{1}H-COSY, ^{1}H-^{1}H-NOESY, ^{1}H-^{13}C-HMQC, ^{1}H-^{13}C-HMBC, GC-MS, DP-MS dan LR-MS. Minyak pati, ekstrak dan beberapa daripada sebatian yang telah dipencilkan telah dianalisis untuk aktiviti biologi: antibakteria, aktiviti inhibisi enzim asetilkolinesterase dan aktiviti anti-inflamasi untuk menilai kegunaan tradisionalnya.

Minyak pati daun dan rizom *H. pineodora* mengandungi komponen bukan terpena yang tinggi kandungannya, khususnya, tridek-2-en-4-on (47.5%) dan 2-hidroksi-4-tridekanon (29.5%), dan dipencilkan melalui kaedah GC penyediaan dan struktur sebatian ditentukan melalui kaedah intepretasi spektrum serta dipastikan melalui perbandingan data spektrum dengan piawai asal. Disamping itu, tridek-1-en-4-on dan tridekana-2, 4-dion yang juga dikenal pasti melalui sintesis.

Monoterpena didapati mendominasi profil minyak pati *H. sagittifolia* dan *H. coerulescens*. β-Pinena (16.5 %) dan linalol (34.5 %) merupakan komponen-komponen utama dalam minyak pati rizom *H. sagittifolia* sementara α-pinena (50.1 %) and β-pinena (24.1 %) mencirikan minyak pati daunnya. Dalam minyak pati species *H. coerulescens*, komponen utama dalam minyak pati rizomnya adalah linalol (37.2 %) manakala minyak pati daunnya dicirikan oleh α- pinena (34.3%) and β- pinena (24.9%).

Dari ekstrak daun *H. sagittifolia*, dua kompoun baru iaitu gliserol 1-linoleil-2-linolenil-3-*O*-*β*-D-galaktopiranosida dan katena-poli [(natrim-di-*μ*-*β*-D-glukosa) klorida] telah dipencilkan bersama asid palmitik and *trans*-fitol manakala ekstrak rizom memberikan satu sebatian baru, 1α, 4β, 7β-trihidroksieudesmana bersama tiga kompoun yang pernah dilaporkan iaitu 1β, 4β, 7β-trihidroksieudesmana, 1β, 4β, 7α- trihidroksieudesmana dan oplopanon.

Semua minyak pati mempunyai aktiviti antibakteria yang tinggi serta aktiviti anti-inflamatori dan inhibisi yang baik terhadap enzim antiasetilkolinesterase. Ekstrak daun hanya didapati mempunyai aktiviti sederhana terhadap kajian biologi manakala ekstrak rizom menunjukkan aktiviti yang rendah. Sebatian fitokimia yang dipencilkan daripada ekstrak rizom menunjukkan aktiviti yang baik terhadap aktiviti antibakteria dan aktiviti inhibisi enzim asetilkolinesterase tetapi tidak menunjukkan sebarang aktiviti anti-inflamatori, sementara sebatian yang dipencilkan dari ekstrak daun menunjukkan potensi yang baik terhadap kesemua kajian biologi.

PHYTOCHEMICAL AND BIOLOGICAL INVESTIGATION ON THREE SPECIES OF THE GENUS *HOMALOMENA*

ABSTRACT

Three species from the genus *Homalomena*, namely, *H. pineodora, H. sagittifolia and H. coerulescens* were chosen for this particular research since they have long been used in traditional medicine. The essential oils of the leaves and rhizomes of the three species were isolated by hydrodistillation and analyzed by capillary GC and GC-MS, utilizing two columns of different polarities. Isolation and purification of non-volatile phytochemicals were carried out using various solvent systems and chromatographic methods and their structures were determined by using spectroscopic methods such as FT-IR, one and two dimensional NMR experiments: ^{1}H-NMR, ^{13}C-NMR, DEPT 45, DEPT 90, DEPT 135, ^{1}H-^{1}H-COSY, ^{1}H-^{1}H-NOESY, ^{1}H-^{13}C-HMQC, ^{1}H-^{13}C-HMBC, GC-MS, DP-MS and LR-MS**.** The essential oils, crude extracts and some of the isolated compounds were assayed for biological activity (antibacterial, acetylcholinesterase enzyme inhibitory activity and anti-inflammatory activity) to evaluate their reported traditional uses.

The leaf and rhizome oils of *H. pineodora* contain high levels of non-terpenoid compounds, particularly, (*E*)-tridec-2-en-4-one (47.5%) and 2-hydroxy-4-tridecanone (29.5%), and these were isolated by using preparative GC and their structures were established by means of spectral interpretation and confirmed by comparing their spectral data with those of authentic standards. In addition, two constituents, tridec-1-en-4-one and tridecane-2, 4-dione of *H. pineodora* were also identified by synthesis.

The essential oils of *H. sagittifolia and H. coerulescens* were dominated by monoterpenoids. β-Pinene (16.5 %) and linalol (34.5 %) were the major constituents

of the rhizome oil, while α-pinene (50.1 %) and β-pinene (24.1 %) were predominant in leaf oil of *H. sagittifolia.* In *H. coerulescens,* the main chemical component in the rhizome oil was linalool (37.2 %), while in the leaf oil α- pinene (34.3%) and β- pinene (24.9%) were predominant.

From the leaf extract of *H. sagittifolia*, two compounds, glycerol 1-linoleyl-2-linolenyl-3-*O*-*β*-D-galactopyranoside and catena-poly [(sodium-di-*μ*-*β*-D-glucose) chloride] were isolated together with palmitic acid and *trans*-phytol, while the rhizome extract gave a new compound, 1α, 4β, 7β-trihydroxyeudesmane, together with three known compounds, namely, 1β, 4β, 7β-trihydroxyeudesmane, 1β, 4β, 7α-trihydroxyeudesmane and oplopanone.

All essential oils exhibit remarkable antibacterial and acetylcholinesterase enzyme inhibitory and anti-inflammatory activities. The leaf extract showed moderate activity for all biological tests while the rhizome extract showed very low activity. The non-volatile photochemicals isolated from the rhizome extract showed remarkable anti-bacterial activity and acetylcholinesterase enzyme inhibitory activity but showed no anti-inflammatory activity while isolated compounds from the leaves extract showed remarkable potential for all the biological tests.

CHAPTER ONE

INTRODUCTION

1.1 The Role of Natural Product Chemistry in Drug Discovery

Natural product chemistry has been playing the vital role as one of the productive sources for the development of drugs. Over 100 new products have been derived from natural sources in clinical development, particularly as anti-cancer and anti-infective drugs (Harvey, 2008). Natural product-derived drugs were ranked in the top 35 worldwide selling ethical drugs in the years 2000, 2001, 2002 and there were 15 new natural product-derived drugs launched between 2000 and 2003 (Butler, 2004). Recently, many companies have focused their interests on natural product research due to the failure of alternative drug discovery methods to deliver in key therapeutic areas such as immunosuppression, anti-infectives, and metabolic diseases. Natural products have also played a pivotal role in several antibiotic drug discoveries with most antibacterial drugs being derived from natural products (Butler & Buss, 2006). Natural products continue as a source for innovation in drug discovery by playing a significant role in the discovery and understanding of cellular pathways that are an essential component in the drug discovery process. In many cases, natural products provide compounds as clinical drugs or as biochemical tools that demonstrate the role of specific pathways in diseases and the potential of finding drugs (Gullo *et al.*, 2006). Moreover, it has been reported by the World Health Forum that about 80% of the population in developing countries rely chiefly on herbal medicines for their health care needs (Akerele, 1993).

1.2 Medicinal and aromatic plants of Malaysia

One thousand two hundred species of plants in Peninsular Malaysia and two thousand species in Sabah and Sarawak are reported to have medicinal value having been used for generations in various traditional health care systems (Philip *et al*., 2009). Researchers are increasingly turning their attention to natural products, looking for new leads to develop better drugs against cancer, as well as viral and microbial infections (Philip *et al*., 2009). Some of these plants that are used as traditional medicine are also used as common spices for food. Lately, the demand for natural aromatic resources is increasing in the international essential oil market. Essential oils which are obtained from the bark, leaves, flowers and fruits are natural sources for fragrances, flavors, spices and medicine.

1.3 The genus *Homalomena*

The genus *Homalomena* belongs to the Araceae family. This genus has played an important role in economical aspects by providing medicinal resources (Burkill, 1966). The medicinal properties with proven effects on certain diseases indicate that species within this genus are potentially valuable in medicinal industries (Sulaiman & Mansor, 2002).

1.3.1 *Homalomena* species

Homalomena is a genus of more than 120 species of terrestrial or lithophytic, seldom rheophytic, clumping, rhizomatous, very rarely climbing, mostly aromatic herbs distributed in the Neo- and Asian tropics. The genus is overwhelmingly Asian in distribution with the greatest number of species occurring in archipelagic Malaysia (Sulaiman & Boyce, 2005). These species are usually very variable. The larger

species are about 30-60 cm tall, having short stems and dark green in colour. The smaller species are about 20 cm tall.

1.3.2 *Homalomena pineodora*

Homalomena pineodora Sulaiman & Boyce (Fig.3.1) is a new species found in Peninsular Malaysia. It grows along the riverside in clumps on an inundated site under the forest canopy. The specific epithet is from the Latin *pinea* and *odora* meaning pine-scented in allusion to the very strong smell of pinus produced by crushing the plant tissue. This is a small herb, which can grow up to 25 cm tall. The leaves are D-shaped in cross-section and dorsally shallowly grooved. The spathe exterior is glossy bright green, the upper half deeper green, the lower half somewhat yellow-white, at anthesis the spathe margin is recurved, hyaline and slightly brownish along the edge and the spathe interior glossy greenish white (Sulaiman & Boyce, 2005).

Fig.1.1 *Homalomena pineodora* found in Selama, Perak.

1.3.3 *Homalomena coerulescens*

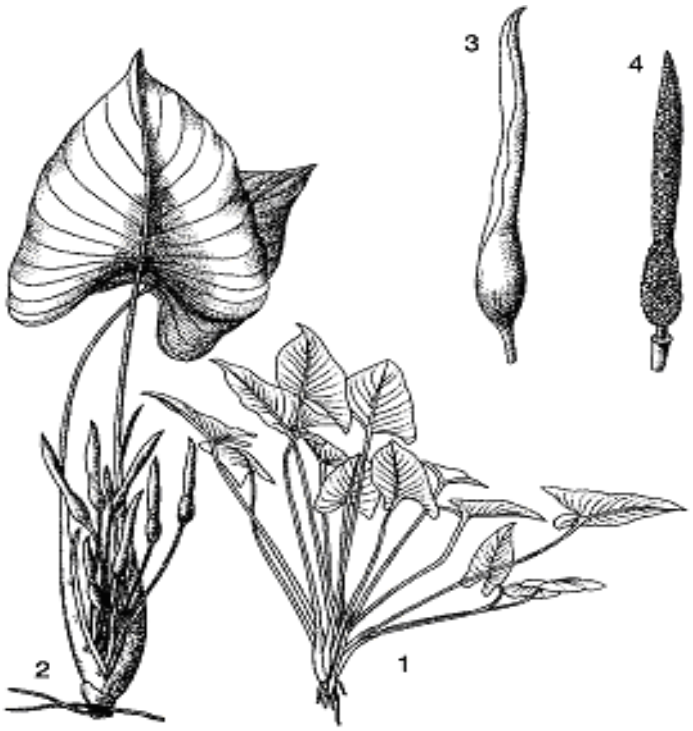
Homalomena coerulescens Jungh. is found throughout Peninsular Thailand, Peninsular Malaysia, Singapore, Sumatra and northern Borneo. It is a fairly large herb similar to *H. sagittifolia*, and can grow up to 60 cm tall (Fig.1.2). The stem is erect, stout and up to 5 cm in diameter, while the vegetative parts are strongly aromatic. Leaves are ovate-arrowhead-shaped with spreading lobes, up to 30 cm long. The spathe is up to 10 cm long and constricted in the upper part, greenish-white or white, often tinged pink at the apex while the spadix is slightly shorter than the spathe. Unlike *H. sagittifolia*, this particular species produces yellow flowers.

Fig.1.2 *Homalomena coerulescens* found in the forests of Selama, Perak.

1.3.4 *Homalomena sagittifolia*

Its distribution is throughout Peninsular Thailand, Peninsular Malaysia, Singapore, Sumatra and northern Borneo. This is a fairly large herb, which can grow up to 60 cm tall. The stem is erect, stout and up to 5 cm in diameter, while the vegetative parts are strongly aromatic. The leaves are ovate-arrowhead-shaped with spreading lobes, up to 30 cm long. The spathe is up to 10 cm long and constricted in the upper part, greenish-white or white, often tinged pink at the apex while the spadix is slightly shorter than the spathe and produces greenish flowers. (Fig.1.3A and 1.3B).

Fig.1.3A *Homalomena sagittifolia* found in the forests of Selama, Perak.



Homalomena sagittifolia Jungh. ex Schott – 1, plant habit; 2, part of flowering plant; 3, inflorescence with spathe; 4, spadix.

Fig. 1.3B The parts of *Homalomena sagittifolia* (Ong, 2003).

1.4 Folklores of *Homalomena* species

The folklores regarding *Homalomena* species are rich enough to be studied. Species of *Homalomena* have been used to treat skin ailments, miscarriage, syphilis, sores, and wounds in Indonesia. Chinese use *Homalomena* species for numbness and rheumatism. Malaysians use for fever, hoarseness, leg sores, lumbago, parturition, sore feet and stomach distention. Philippinos use to cure cough, fever, pain, rheumatism and snakebite (Duke, 1985). The rhizomes of *H. occulta* have been used as folkloric medicine in China for the treatment of rheumatoid arthritis and numbness of the lower extremities through relieving rheumatic conditions and strengthening of the tendons and bones (Yong *et al*., 2008; Sung *et al*., 1992). The volatile oils have been claimed to have anti-inflammatory and antibiotic activities (Hu *et al.*, 2009).

In Malaysia, leaves of *H. coerulescens* are used as a poultice to cure leg sores. The pounded rhizomes are also applied to sore feet and a decoction of the rhizomes and leaves is given for fever. The pounded shoots of *H. sagittifolia* is used to cure distended stomachs (Hanum & Hamzah, 1999; Andersen *et al*., 2003., Burkill, 1966). The decoction of the roots and leaves of *H. griffithii* (Schott) Hook. f. is given to expedite childbirth. In Indonesia, the leaves of *H. alba* Hassk. are used to treat skin diseases. A decoction of *H. javanica* V.A.V.R. has been drunk after miscarriage and also to treat syphilis. In Philippines, the juice of roots or stems of *H. palawanensis* Engl. applied to the bite of water snake to relieve pain (Duke, 1985). Decoction of rhizomes and leaves of *H. sagittifolia* is drunk to cure fever and pounded rhizomes are applied on distended stomach as poultice (Hanum & Hamzah, 1999).

1.5. Background of the pathogenic diseases

1.5.1 Infectious diseases

Infectious diseases are the leading cause of death worldwide (Bandow *et al*., 2003). This situation is very common in tropical countries where insect-borne diseases are common and in undeveloped areas where standards of hygiene and nutrition are low. The morbidity due to any particular parasite varies enormously in different parts of the world owing to geographical and environmental factors which influence the host-parasite relationship. The main groups of the organisms involved are metazoan, fungi, bacteria and viruses. Infection due to metazoa and protozoa are encountered mainly in tropical areas. Thus malaria, amoebic dysentery, sleeping sickness, kalazar and helminthes infections are common diseases in tropical Africa, Central and South America and large parts of Asia. Some bacterial infections may be acute such as

diphtheria and tetanus; others are chronic such as tuberculosis, syphilis and leprosy (Bandow *et al*., 2003; Eldeen *et al*., 2010).

1.5.2 Inflammation

The use of medicinal substances to relieve pain, fever and inflammation dates back to ancient Egypt where a decoction of dried leaves of the myrtle was applied to the back and abdomen of patients (Eldeen *et al*., 2010). Inflammation is a disorder involving localized increases in the number of leukocytes and a variety of complex mediator molecules. The inhibitions of numerous rate-limiting processes could be important in the successful treatment of inflammatory disorder (Mantri & Witiak, 1994). Prostaglandins are ubiquitious substances that initiate and modulate cell and tissue responses involved in physiological process such as platelet aggregation and inflammation (Smith & Dewitt, 1995). Platelet aggregation is characteristic of inflammation (Cheeke *et al.*, 2006). Synthesis of prostaglandins is a key factor in the inflammation process. The primary enzyme responsible for prostaglandin synthesis is cyclooxygenase which occurs in three isoforms, COX-1, COX-2 and COX-3. Nonselective NSAIDs inhibit both COX-1 and COX-2 isoenzymes, Coxibs inhibit only COX-2. Hence, by inhibiting COX-2, the primary noxious mediator can be blocked (Sinatra, 2002). COX-1 is responsible for the production of prostanoids that maintain mucosal blood flow, promote mucose secretion, inhibit neutrophil adherence and maintain renal blood flow. COX-2 is effectively absent in healthy tissues and is induced in migratory and other cells by pro-inflammatory agents such as inflammation (Eldeen *et al*., 2010).

1.5.3 Acetylcholinesterase

Acetylcholine is an organic molecule liberated at nerve endings as a neurotransmitter. It is produced by the synthetic enzyme choline acetyltransferase that uses acetyl co-enzyme A and choline as substrates for the formation of acetylcholine in specific cells known as cholinergic neurons. The principle role of acetylcholinesterase (AChE) is termination of impulse transmission at cholinergic synapses by rapid hydrolysis of acetylcholine (Bar-on *et al*., 2002). Owing to its essential biological role in the mediation of vertebrate and invertebrate nervous transmission, acetylcholinesterase has been medicinally targeted in treatments for Alzheimer's disease, myasthenia gravis, glaucoma, pesticides, and snake venom toxins (Eldeen *et al*., 2010; Ion *et al*., 2010). Acetylcholinesterase inhibitor drugs are effective in delaying neurocognitive decline in people with mild to moderate severity of Alzheimer's disease. However, their effect is only to alleviate symptoms and they do not achieve any permanent improvement (Lewis & Elvin, 2003). The use of anti-inflammatory agents has also been suggested to delay the progress of Alzheimer's disease. Several studies have shown that patient treated with non-steroidal anti-inflammatory drugs may have a reduced risk of developing Alzheimer's disease (Eldeen *et al.*, 2010).

1.6 Previous studies on *Homalomena* species

Sung *et al.* (1992) isolated oplopanone (**1**), oplodiol (**2**), homalomenol (**3**), homalomenol A (**4**), homalomenol B (**5**), 1β, 4β, 7α-trihydroxyeudesmane (**6**) and bullatantriol (**7**) from the roots of *H. aromatica*.

Singh *et al.* (1999) analyzed the chemical composition of the essential oil from the rhizomes of *H. aromatica* Schott by GC and GC/MS. The main components were found to be linalool (62.1%) and terpinen-4-ol (17.2 %), respectively.

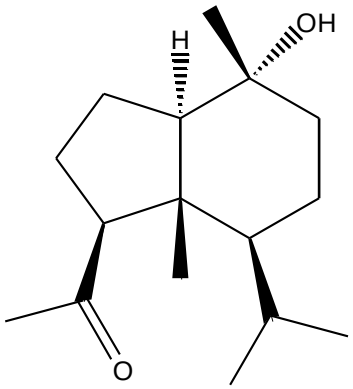
Elbandy *et al*. (2004) studied the constituents of the rhizomes of *H. occulta.* Air dried and finely powdered rhizomes were extracted successively in a Soxhlet apparatus with cyclohexane, dichloromethane, ethyl acetate and methanol. The methanol extract was evaporated under reduced pressure and the residue dissolved in water and extracted with H_2O–sat.BuOH and then evaporated to dryness to obtain a crude extract which was subjected to chromatography on Sephadex LH-20 using methanol as eluent. These workers managed to identify seven chemical constituents, six of them new: hederagenin saponin (**8**), *E, N*-(*p*-coumaroyl) serotonin (**9**), *Z, N*-(*p*-coumaroyl) serotonin (**10**), gingerglycolipid C (**11**), diacylphosphatidylcholines (**12**) and (**13**).

Wong *et al.* (2006) investigated the essential oils of *H. sagittifolia.* The major components found were linalool (61.9%) in the rhizome oil and α-pinene (22.2%) and β-pinene (17.2%) in the leaf oil. From the leaf oil, neointermedeol (**14**) was isolated by preparative GC.

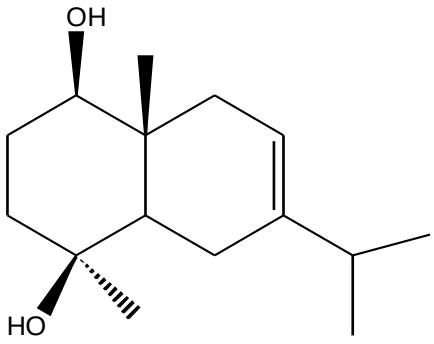
Wang *et al*. (2007) isolated three new eudesmane-type sesquiterpenoids, namely, 1β, 4β, 7β-trihydroxyeudesmane (**15**) 1β, 4β, 6β, 11-tetrahydroxyeudesmane (**16**) and homalomentetraol (**17**) from the aerial parts of *H. occulta*, and known constituents including mucrolidin (**18**), acetylbullantantriol (**19**), maristerminol (**20**), (**2**), (**3**), (**6**) and (**7**).

Hu *et al.* (2008) investigated the chemical constituents of the rhizomes of *H. occulta*, resulting in isolation and identification of four new and four known compounds. The four new constituents were homalomenol C (**21**), homalomenol E (**22**), homalomenol F (**23**), 1α, 4α, 6β-trihydroxyeudesmane (**24**), 1β, 4β, 6α-trihydroxyeudesmane (**25**), and the known compounds were (**1),** (**2**), (**6**) and (**7**).

Hu *et al.* (2009) isolated a new sesquiterpenoid 6α, 7α, 10α-trihydroxyisodaucane (**26**), together with two known sesquiterpenoids, (**1**) and (**2**), from the chloroform extract of the rhizomes of *H. occulta.*



(**1**)



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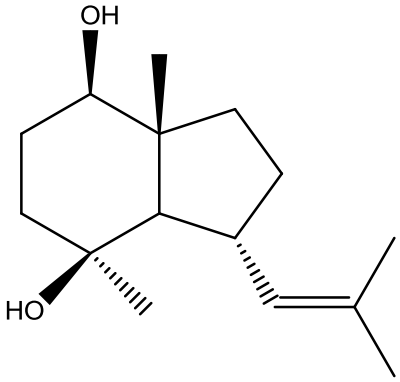

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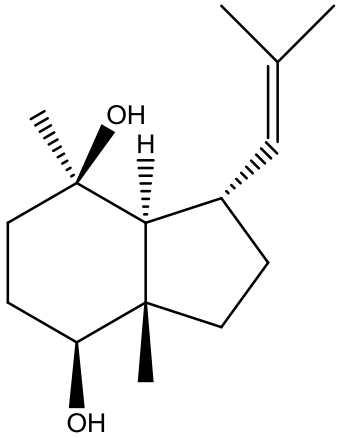
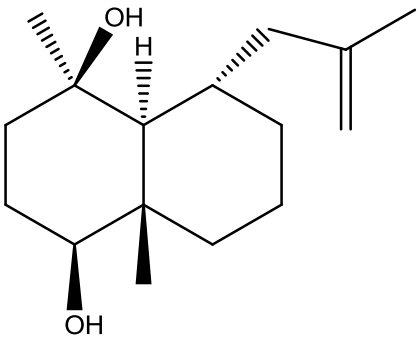


(**4**)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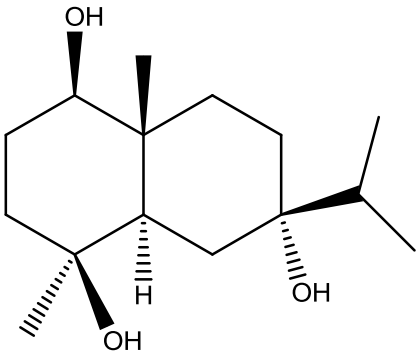
(**5**)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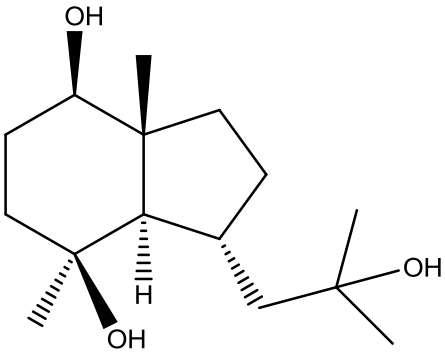
(**6**)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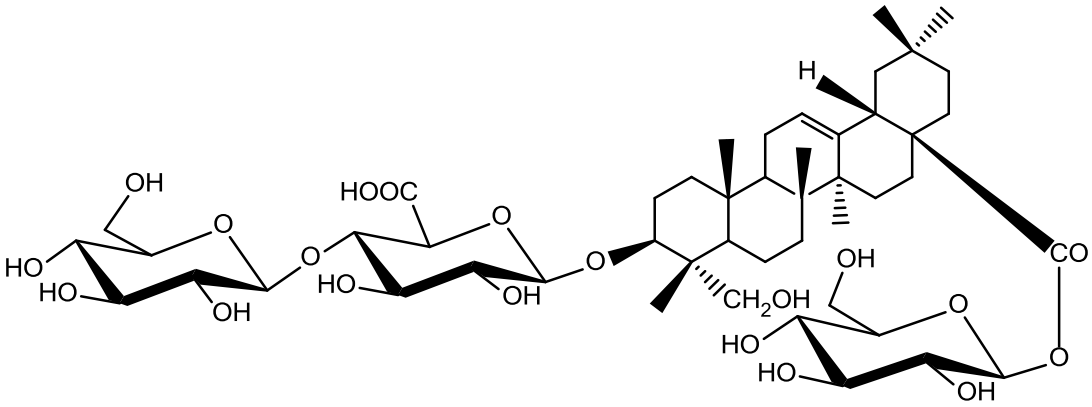
(**7**)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HO
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O
HOOC
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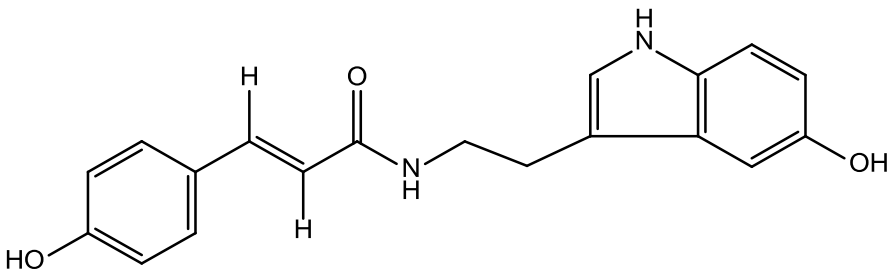
CO
OH
CH2OH
O
HO
HO
OH
O

(**8**)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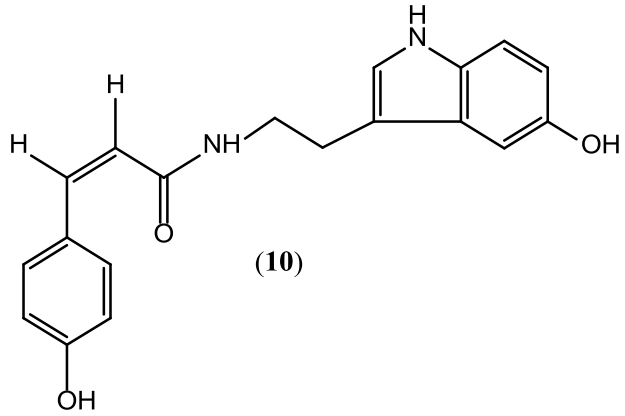
(**9**)



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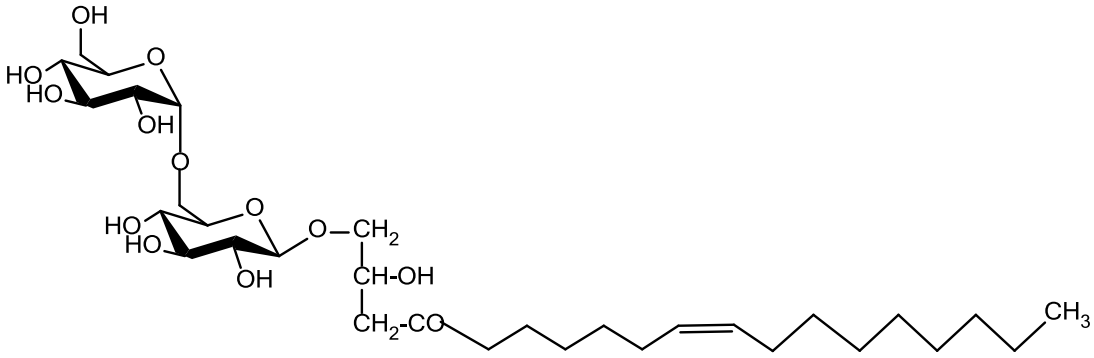

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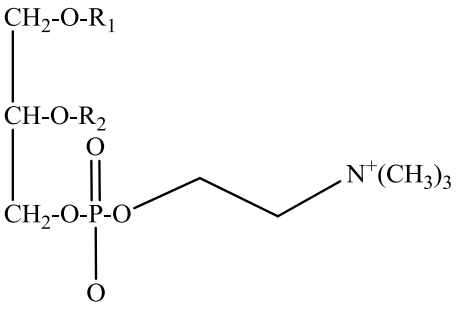
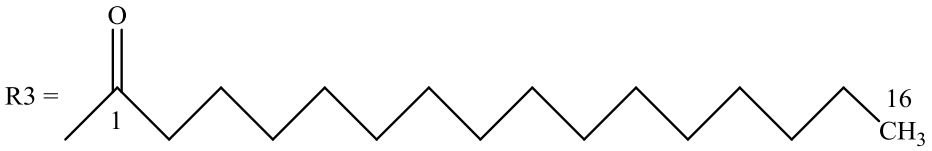
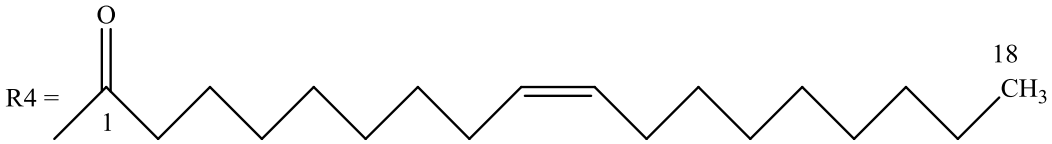
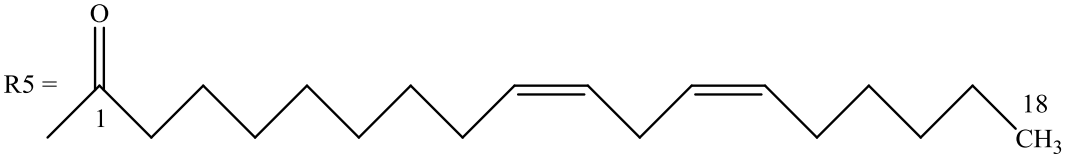


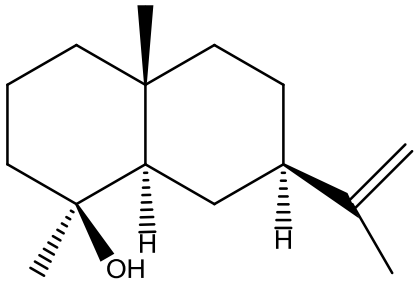
(12) R_1= R3, R_2= R4 or R_1= R4, R_2= R3

(13) R_1= R3, R_2= R5 or R_1= R5, R_2= R3









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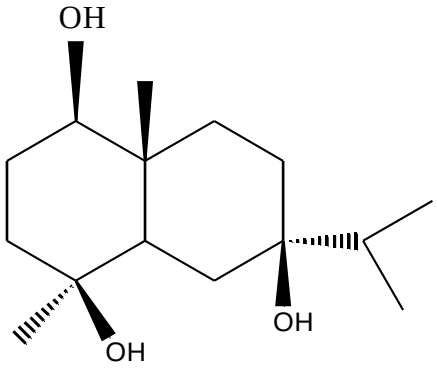

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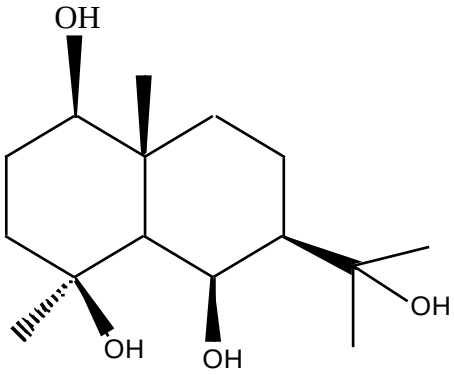

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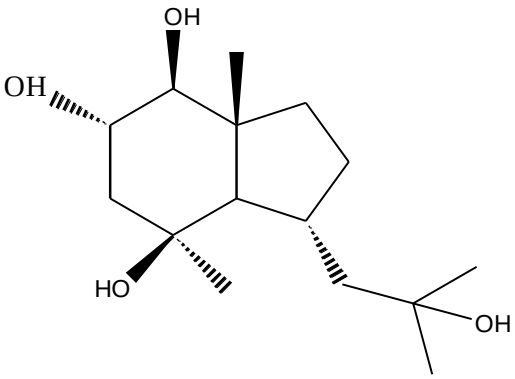

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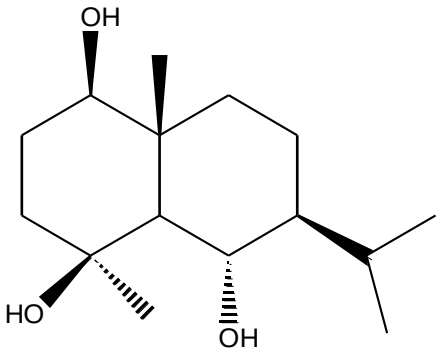

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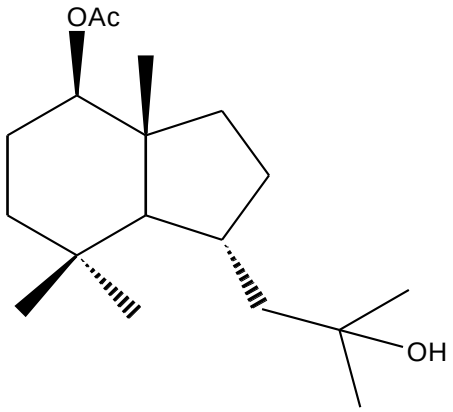

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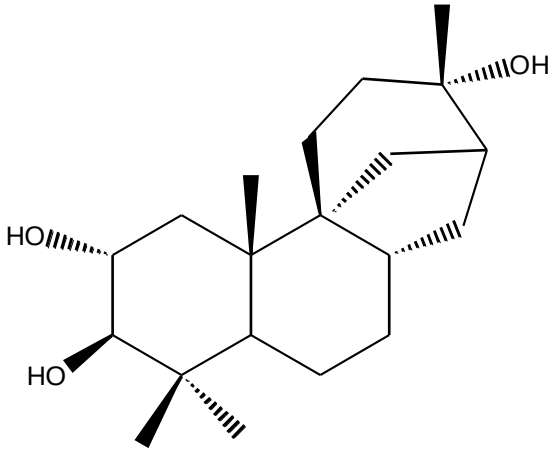

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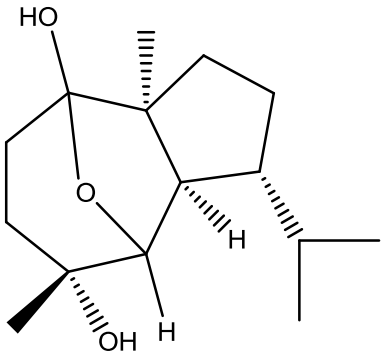

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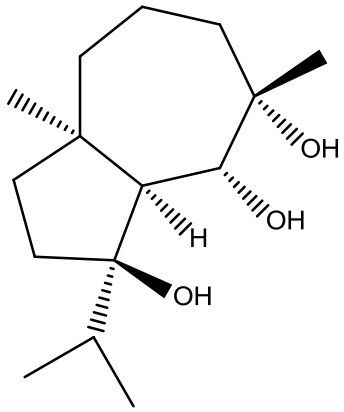

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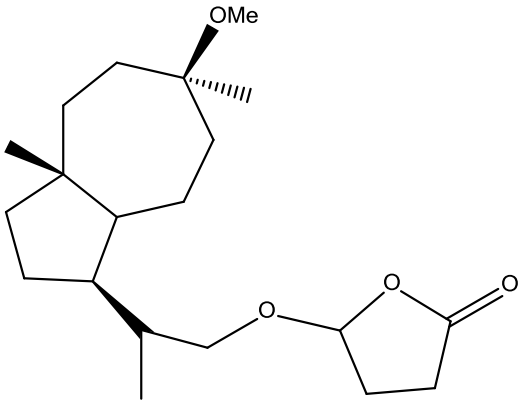

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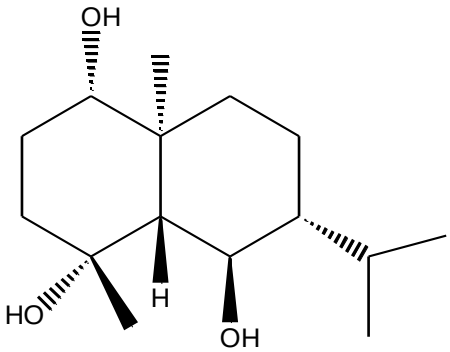

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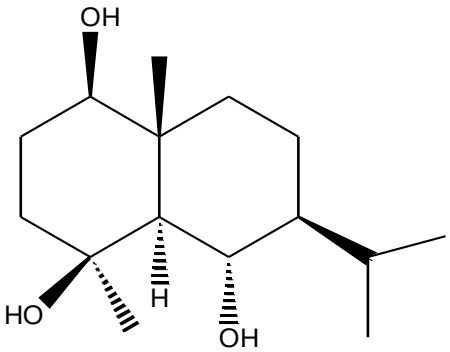

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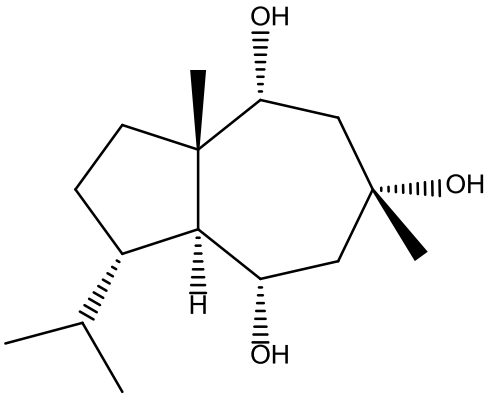

(**26)**

1.7 Objectives of the present research

The objectives of the present research are:

1. To identify the chemical constituents present in the essential oils of the leaves and rhizomes of *H. pineodora, H. sagittifolia* and *H. coerulescens,* using capillary GC and GC-MS techniques.

2. To carry out a comparative study of the chemical compositions of the essential oils of the aforementioned three species as a taxonomic guide for species identification.

3. To isolate and elucidate the structures of the non-volatile phytochemicals from the species *H. sagittifolia*, using various spectroscopic techniques.

4. To evaluate antibacterial, acetylcholinesterase enzyme inhibitory and antiinflammatory activities of the crude extracts, essential oils and isolated phytochemicals in relation to the reported folklores.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Collection of plant material

H. sagittifolia was collected in July 2006 from Selama, Perak, *H. coerulescens* was collected in August 2006 from the aroid garden of School of Biological Sciences, Universiti Sains Malaysia and *H. pineodora* was collected in February 2007 from Selama, Perak. All these species were identified by Mr. Baharuddin Sulaiman, School of Biological Sciences, Universiti Sains Malaysia, and voucher specimens of *H. sagittifolia* (USM 1012), *H. coerulescens* (USM 10506) and *H. pineodora* (USM 10811) have been deposited in the herbarium of Universiti Sains Malaysia.

2. 2. Chemicals and reagents

1. Acetaldehyde (Sigma-Aldrich, USA)
2. Acetone , AR grade (Systerm, Malaysia)
3. Acetylthiocholine iodide (ATCI) (Sigma–Aldrich, USA)
4. Aluminum chloride, $AlCl_3.6H_2O$ (Merck, Germany)
5. Aqueous hydrogen peroxide (30%), AR grade (Systerm, Malaysia)
6. Arachidonic acid (Sigma–Aldrich, USA)
7. Benzophenone (ACROS Organics, USA)
8. Boric acid (Sigma-Aldrich, USA)
9. pH7 buffer (Sigma-Aldrich, USA)
10. Butyllithium 1.6 M in hexane (ACROS Organics, USA)
11. Calcium hydride (Riedel-de Haen, Germany)
12. Chloroform, AR grade (Systerm, Malaysia)

13. Chloroform-d, with 0.03% TMS (v/v), 99.8 atom %D, stabilized with silver foil (ACROS Organics, USA)
14. Chromium trioxide (CrO_3) (Riedel-de Haen, Germany)
15. Decanal (ACROS Organic, USA)
16. Diethyl ether, AR grade (Brightchem S/B, Malaysia)
17. Diisopropylamine (DIPA), (Merck, Germany)
18. 5, 5-Dithiobis-2-nitrobenzoic acid (DTNB) (Sigma–Aldrich, USA)
19. Ethyl acetate, AR grade (Systerm, Malaysia)
20. Ethanol 99 % , AR grade (Brightchem S/B, Malaysia)
21. Ferric chloride, $FeCl_3.6H_2O$ (Merck, Germany)
22. Galanthamine (Sigma–Aldrich, USA)
23. Hydrochloric acid 37% (Fisher Chemicals, UK)
24. Indomethacin (Sigma–Aldrich, USA)
25. *p*-Iodonitrotetrazolium violet (INT) (Sigma–Aldrich, USA)
26. Isopropyl alcohol (Systerm, Malaysia)
27. Lipase Type XI (Sigma-Aldrich, USA)
28. Magnesium sulphate (Systerm, Malaysia)
29. Methanol, AR grade (Systerm, Malaysia)
30. Mueller-Hinton (MH) broth (Oxoid, England)
31. Pentane, AR grade (Merck, Germany)
32. Petroleum ether 60-80 ºC, AR grade (Lab-Scan, Thailand)
33. PTLC pre-coated glass plates (20 cm x 20 cm x 0.5mm) (Merck, Germany)
34. Pyridine (Fluka, Germany)
35. Pyridine-d_5 (Sigma-Aldrich, USA)
36. Sephadex LH-20 (Sigma-Aldrich, USA)

37. Silica gel 60 for column chromatography, (0.040-0.063 mm ; 230-400 mesh ASTM) (Merck, Germany)
38. Sulphuric acid 95-98 % (Systerm, Malaysia)
39. TLC aluminum sheets, precoated silica gel 60 F_{254}, (20 cm x 20 cm) (Merck, Germany)
40. Sodium metal (Sigma-Aldrich, USA)
41. Sodium sulphate (Sigma-Aldrich, USA)
42. Sodium chloride (Fisher Scientific, UK)
43. Sodium carbonate (Fisher Scientific, UK)
44. Sodium hydroxide (Fine Chemicals, Germany)
45. Sodium tetra borate (Sigma-Aldrich, USA)
46. Tetracycline (Hovid, Malaysia)
47. Triethylamine (Merck, Germany)
48. Triton X-100 (Sigma-Aldrich, USA)
49. 2-Undecanone (Sigma-Aldrich, USA)

2.3 Extraction and analysis of essential oils

2. 3.1 Extraction of essential oils

The essential oils of fresh leaves and rhizomes of *H. sagittifolia*, *H. coerulesces* and *H. pineodora* were isolated separately by hydrodistillation utilizing an all glass-apparatus similar to that described in the British Pharmacopoeia (1993) (Fig. 2.1). The plant material was rinsed first with tap water then with distilled water to remove soil and dirt. A known amount (60-190 g) was placed in a 1.0 L or 0.5 L round bottom flask which was filled with distilled water (300-600 mL) to a level such that the plant material was entirely immersed (Table 2.1).

Table 2.1 Weight of plant material and volume of distilled water used for hydrodistillation.

Species	Plant part	Weight (g)	Volume of distilled water (mL)
H. sagittifolia	Leaves	160	600
	Rhizomes	80	300
H. coerulescens	Leaves	180	600
	Rhizomes	60	300
H. pineodora	Leaves	190	600
	Rhizomes	80	300

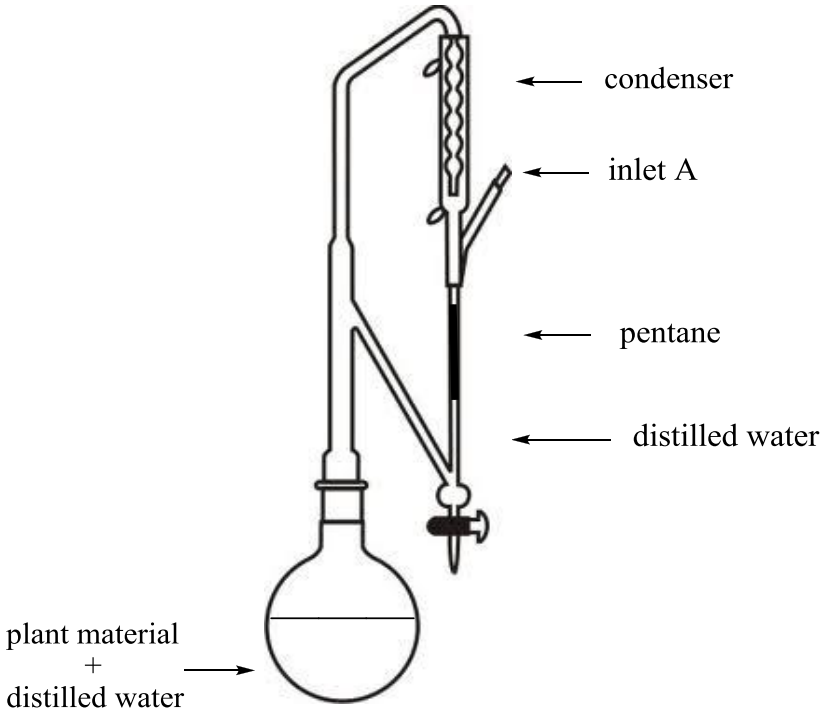

Fig 2.1 Hydrodistillation apparatus used to isolate essential oils.

The entire "V" area of the apparatus (Fig 2.1) was filled with distilled water, following which a small volume (5-6 mL) of freshly distilled pentane was added through inlet A. The inlet A was loosely covered with aluminum foil to minimize the loss of essential oil and pentane during the hydrodistillation. Pre-cooled water was circulated through the condenser during the entire distillation process. From time to

time, a small volume of pentane was added via inlet A to ensure that the volume of pentane was maintained at about 4-5 mL.

After five hours of hydrodistillation, the glass apparatus was left to cool and the essential oil in pentane was collected in a 10 mL vial and was concentrated by a gentle stream of purified nitrogen gas to an appropriate volume (ca. 0.5 mL) for GC and GC-MS analyses.

2.3.2 Analysis of the essential oils

The isolated essential oils were analyzed by capillary gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS).

2.3.2.1 Gas chromatography (GC)

Gas chromatographic analyses were performed employing a Hitachi G-3000 equipped with a flame ionization detector (FID). Throughout the analysis, a non-polar (SPB-1) capillary column (30 m x 0.25 mm id, film thickness 0.25 µm; Supelco, USA), and a polar (Supelcowax 10) capillary column (30 m x 0.25 mm id, film thickness 0.25 µm; Supelco, USA) were used. For both columns, similar operational conditions were applied: initial oven temperature, 50 ºC for 5 min, then increased to 250 ºC at a rate of 5 ºC per minute and held for 10 minutes. The injector and detector temperatures were kept at 250 ºC. The carrier gas was nitrogen with a flow rate of 1.0 mL min^{-1}. The split ratio was 50:1, injection volume 0.4 µL. Peak areas were recorded with a Hitachi D2500 Chromato-Integrator without making correction to detector response. Under identical operation condition, a mixture of *n*-alkanes ranging from C_7-C_{32} was chromatographed on each of the columns for the calculation of the retention indices (RI) of the oil constituents.

2.3.2.2 Retention index calculation

Retention indices were first derived by Kovats as a means of relative retention data suitable for reference in isothermal gas chromatography (Kovats, 1965; Dool & Kratz, 1963). Based on a series of *n*-alkanes, he defined retention indices on a logarithmic scale of adjusted retention times confined by the *n*-alkanes eluting before and after the compound of interest (Massold, 2007).

$$RI = 100\, i \left[\frac{t - t_{(n)}}{t_{(n+i)} - t_{(n)}} \right] + 100n \qquad (1)$$

Under the condition that $t_{(n)} < t < t_{(n+i)}$

t = retention time of the component of interest

$t_{(n)}$ = retention time of the alkane with n carbon atoms which elutes just before the component of interest

$t_{(n+i)}$ = retention time of the alkane with (n+i) carbon atoms which elutes just after the component of interest

i = difference in the number of carbon atoms between the two alkanes.

n = the number of carbon atoms in the alkane

Immediately after an essential oil was chromatographed, a mixture of *n*-alkanes ranging from C_7-C_{30} was injected into the GC column under identical operational conditions. The precise retention index was obtained when it was calculated based on two neighboring alkanes (i.e. i = 1), then the equation (1) is simplified to:

$$RI = 100 \left[\frac{t - t_{(n)}}{t_{(n+1)} - t_{(n)}} \right] + 100n \qquad (2)$$

The above equation (**2**) was used to calculate the *RI* values in this present work.

2.3.2.3 Gas Chromatography–Mass Spectrometry (GC-MS)

Gas chromatography-mass spectrometry (GC-MS) is a widely used method for the identification of volatile organic compounds in complex mixtures. Identification is typically carried out by matching the measured spectra with the spectra in a reference library (Babushok *et al*., 2007). In the present study, the GC-MS analyses were carried out using either a ThermoFinnigan GC 2000 coupled to a Trace MS and equipped with the NIST and MAIN library software, or a Hewlett Packard 5989A GC-MS equipped with a Wiley library software. The same capillary columns and GC operating conditions were employed as described in section **2.3.2.1**, the only difference was the use of helium carrier gas in place of nitrogen. Significant operating parameters: ionization voltage, 70 eV; ion source temperature, 200 ºC; scan mass range, 40-350 a.m.u.

2.3.2.4 Preparative GC

Preparative GC was used to isolate two volatile chemical constituents **(5** and **9**, Table 3.2) from the leaf oil of *H. pineodora* utilizing a Hitachi G-3000 equipped with a stainless steel column (3 m x 5.3 mm i.d.) packed with 5% Carbowax 20 M on 100/120 mesh Supelcoport (Supelco, USA) (Sun *et al.,* 1995). The column was programmed at 5 ºC min^{-1} from 150 to 220 ºC. The injector and detector temperatures were kept at 250 ºC. The carrier gas was nitrogen with a flow rate of 20.0 mL min^{-1}. The split ratio was 1:10 (1 part to FID). The compound was collected in an ice-cooled Pyrex tube (3 mm x 15 cm long).

2.3.2.5 Identification of essential oil components

The identification of the components was based on comparison with retention index values reported in the literature, comparing mass spectral matching from Wiley and NIST library of GC-MS, isolating and synthesizing four constituents to confirm the component identity of *H. pineodora*.

2.4 Isolation of volatile compounds

Two volatile compounds, namely, (*E*)-tridec-2-en-4-one and 2-hydroxy-4-tridecanone (Constituents **5** and **9**, Table 3.2) were isolated from the leaf oils of *H. pineodora* using preparative GC (Section **2.3.2.4**) injecting the volume of 0.6 μL.

2.5 Synthesis of volatile compounds

In order to confirm the identity of the oil constituents of *H. pineodora*, four volatile compounds, 2-hydroxy-4-tridecanone, tridec-2-en-4-one, tridecane-2, 4-dione, and tridec-1-en-4-one were synthesized.

2. 5. 1 Preparation of dry solvents and reagents

2.5.1.1 Preparation of dry diethyl ether and tetrahydrofuran (THF)

Metallic sodium (4.0 g, 0.17 mol) kept under paraffin oil was wiped dry with tissue paper and cut into small pieces which were quickly rinsed with a minimum amount of hexane to remove the paraffin oil and then placed into the 1 mm die of sodium press using forceps. The sodium wire was directly extruded into a round bottom flask which contains diethyl ether (300 mL) or tetrahydrofuran (300 mL). Effervescence took place as hydrogen gas was evolved. When no bubbles evolved benzophenone (3.0 g, 0.016 mol) was added to the flask and reflux was carried out