PHYTOCHEMICAL AND BIOACTIVITY STUDIES OF *MIMUSOPS ELENGI* L.

by

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LIST OF ABBREVIATIONS

Chromatography

CC	Column chromatography
GC	Gas chromatography
GC-MS	Gas chromatography-Mass spectrometry
Rf	Retention factor
TLC	Thin layer chromatography
RI	Retention index

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
NOESY	Nuclear overhauser enhancement spectroscopy
COX	Cyclooxygenase

Symbols

m/z	mass/charge
eV	electron volt
ppm	part per million
J	coupling constant
br	broad

s	singlet
d	doublet
t	triplet
m	multiplet
dd	doublet of doublets
ddd	doublet of doublet of doublets

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KAJIAN FITOKIMIA DAN BIOAKTIVITI TERHADAP *MIMUSOPS ELENGI* L.

ABSTRAK

Kajian fitokimia terhadap ekstrak kloroform daripada daun dan kulit *Mimusops elengi* telah berjaya mengasingkan spinasterol **[15]**, asid ursolik **[18]** dan asid 3*β*,6*β*, 19*α*,23-tetrahidroksiurs-12-en-28-oik **[67]** daripada daun, dan tarakserol **[16]** serta spinasterol 3-*O*-*β*-D-glukopiranosida **[39]** daripada kulit. Kajian terdahulu menunjukkan pengasingan sebatian **[15]** daripada kulit batang pokok dan biji benih pokok, sebatian **[18]** daripada kulit batang pokok and mesokarp, manakala sebatian **[39]** diasingkan daripada biji benih tumbuhan. Tetapi ini merupakan kajian pertama yang melaporkan pengasingan **[15]** dan **[18]** daripada daun, manakala **[39]** daripada kulit pokok. Sebatian **[67]** adalah tidak pernah dikenalpasti daripada *M. elengi* dan famili Sapotaceae. Analisis terhadap komponen mudah meruap daripada bunga melalui kaedah GC dan GC/MS menunjukkan kehadiran 50 sebatian dan didapati bahawa 2-feniletanol (29.8%), (*E*)-2-heksenal (11.8%) dan benzil alkohol (10.4%) merupakan sebatian terbanyak. Ekstrak kloroform daripada daun dan kulit, sebatian terasing dan minyak meruap bunga diuji untuk aktiviti antibakteria dengan menggunakan bakteria Gram-positif dan Gram-negatif, manakala aktiviti bengkak diuji menggunakan ujian siklooksigenase. Kebanyakan sampel yang diuji menunjukkan aktiviti yang baik terhadap *Staphylococcus aureus* (9.7-78 μg/mL) manakala aktiviti yang sederhana ditunjukkan terhadap bakteria Gram-negatif (78-156 μg/mL). Perencatan COX yang tinggi didapati daripada ekstrak daun dan **[15]**, perencatan selektif COX-2 didapati pada **[18]** dan **[67]**, manakala perencatan selektif COX-1 ditunjukkan pada ekstrak kulit **[39]** dan minyak bunga. Kajian ini adalah yang pertama menjelaskan potensi *M. elengi* terhadap aktiviti bengkak. Keputusan

kajian ini menyokong penggunaan tumbuhan ini dalam Ayurvedic sebagai agen mentah antibakteria dan aktiviti bengkak.

PHYTOCHEMICAL AND BIOACTIVITY STUDIES OF *MIMUSOPS ELENGI* L.

ABSTRACT

Phytochemical investigation of the chloroform extracts of the leaves and bark of *Mimusops elengi* led to the isolation of spinasterol **[15]**, ursolic acid **[18]** and 3*β*,6*β*,19*α*,23-tetrahydroxyurs-12-en-28-oic acid **[67]** from the leaves, as well as taraxerol **[16]** and spinasterol 3-*O*-*β*-D-glucopyranoside **[39]** from the bark. Compound **[15]** has previously been reported from the bark and seeds, compound **[18]** has been reported from the bark and mesocarp, and compound **[39]** has been reported from seeds of the plant. However, this is the first report of **[15]** and **[18]** from the leaves and **[39]** from the bark. Compound **[67]** has not, hitherto, been identified in *M. elengi* and the family Sapotaceae. Analysis of the volatile constituents of the flowers by capillary GC and GC/MS resulted in the identification of 50 compounds, the most abundant of which were 2-phenylethanol (29.8%), (*E*)-2-hexenal (11.8%) and benzyl alcohol (10.4%). The chloroform leaf and bark extracts, isolated compounds and flower volatile oil were tested for antibacterial activity using Gram-positive and Gram-negative bacteria, and anti-inflammatory activity using the cyclooxygenase assay. The majority of the samples tested indicated good activity against *Staphylococcus aureus* (9.7-78 μg/mL), while appreciable activity was observed against Gram-negative bacteria (78-156 μg/mL). Strong COX inhibition was observed for the leaf extract and **[15]**, selective COX-2 inhibition was observed for **[18]** and **[67]**, and selective COX-1 inhibition was observed for the bark extract, **[39]** and the flower oil. This study is the first report describing the anti-inflammatory potential of *M. elengi*. The results of this study support the Ayurvedic uses of the plant as crude antibacterial and anti-inflammatory agents.

CHAPTER ONE

INTRODUCTION

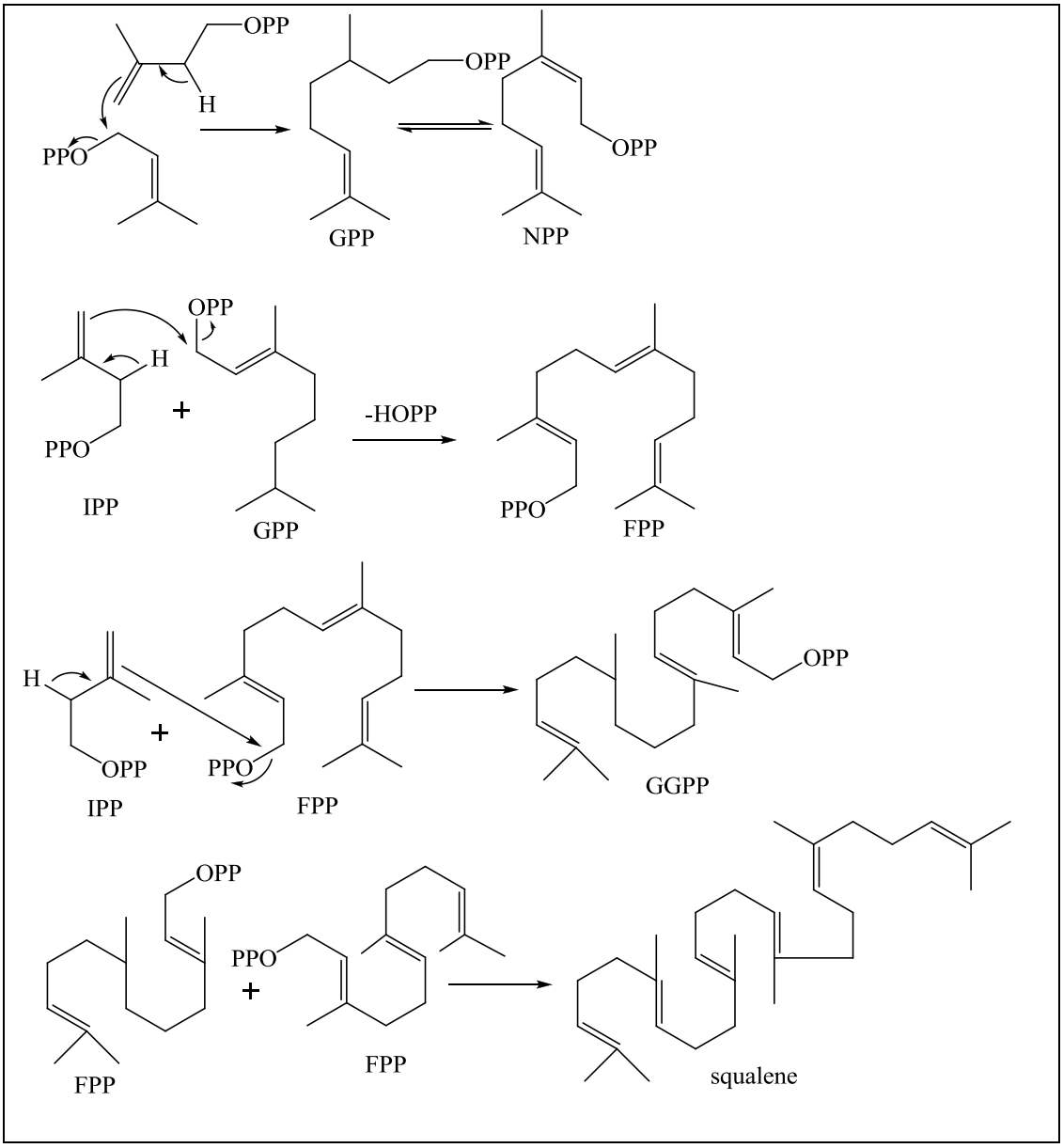
1.1 Natural products

Natural products are structurally diverse libraries of chemical constituents produced by all living organisms for their survival against predators and environment. Natural products and their derivatives are major sources of drug discovery in almost all therapeutic areas (Balunas and Kinghorn, 2005). Despite the research provided by other drug discovery methods, natural products are an inimitable source of drugs and pharmacological agents (Newman *et al.*, 2000). A report by the world health organization suggested that approximately 80% of the world's population uses traditional medicines (Min *et al.*, 1998). Research on natural products presently focuses on phytochemical investigations on plants of biomedical importance in folk medicine.

1.2 Biosynthesis of terpenes

Terpenes are secondary metabolites commonly found in almost all plants; they are derived from isoprenoid units. These isoprene units condense to form monoterpenes, sesquiterpenes, diterpenes, triterpenes and tetraterpenes. The two active forms of the isoprene unit are isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) (Manitto, 1981). DMAPP condenses with IPP in the presence of dimethylallyl transferase to give geranyl pyrophosphate (GPP) and neryl pyrophosphate (NPP); GPP is the precursor of monoterpenes. GPP and IPP further condense to yield farnesyl pyrophosphate (FPP), which is the key intermediate of sesquiterpene synthesis (Jacob *et al.*, 1983; Koyama *et al.*, 1980; Poulter *et al.*,

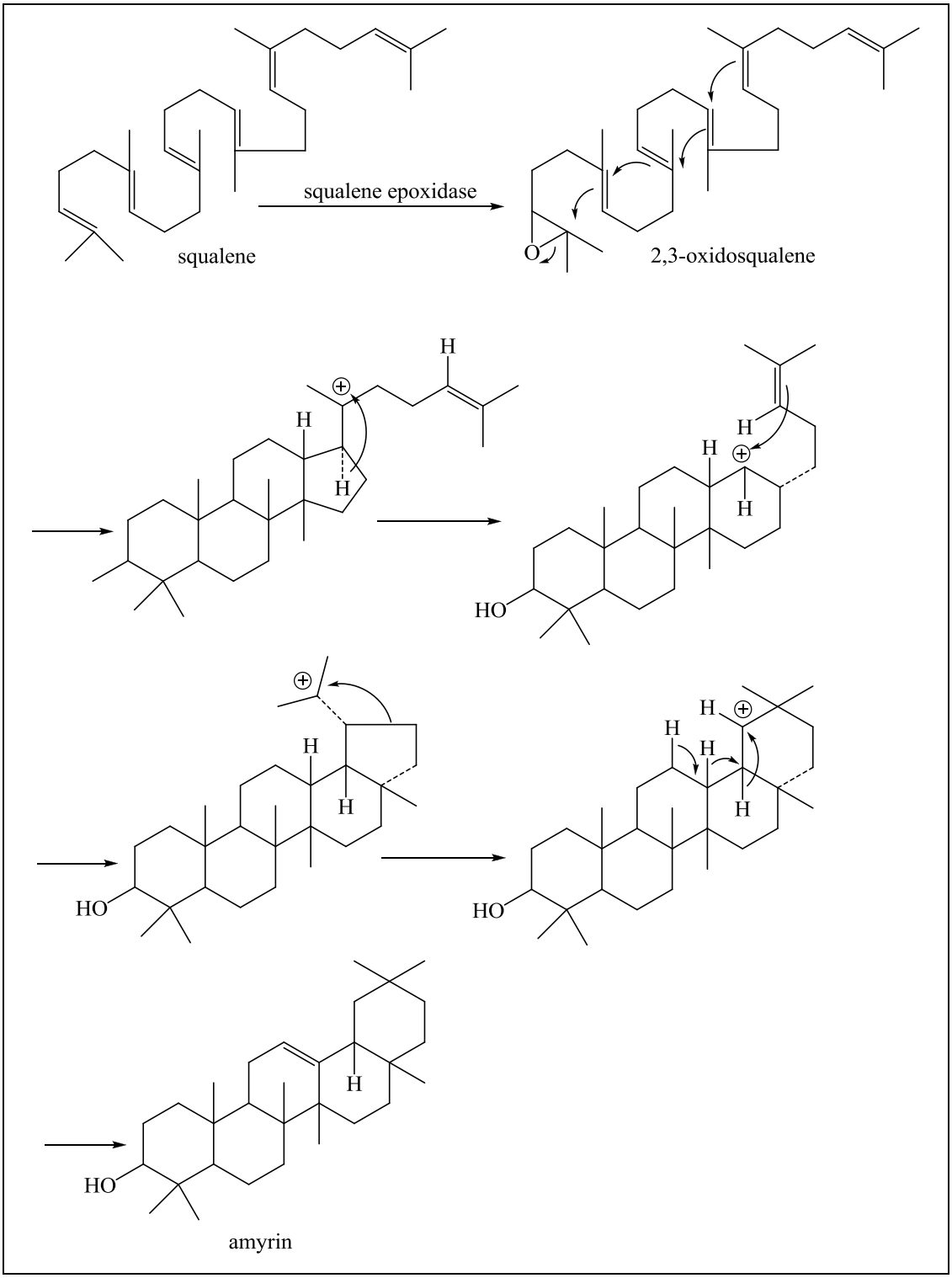
1979). The addition of another isopentyl unit to FPP gives geranylgeranyl pyrophosphate (GGPP) which is cyclized to give diterpenes. Two FPP dimerize in a tail to tail manner to give squalene which is the precursor for various triterpenes (Scheme 1.1) (Rodríguez-Concepción and Boronat, 2002).



Scheme 1.1 Condensation of isopentene units to acyclic terpenoid

Biosynthesis of pentacyclic triterpenes involves a series of Wagner-Meerwein rearrangements during the formation of D and E ring systems from the cyclization

intermediate of 2,3-oxidosqualene. The mechanism of D and E ring formation in case of β-amyrin synthesis involves backbone rearrangement and hydride shifts (Scheme 2.1) (Rees *et al.*, 1968; Seo *et al.*, 1981; Xu *et al.*, 2004).



Scheme 1.2 Cyclization of squalene

1.3 Sapotaceae

This family comprises shrubs and trees that characteristically possess a milky sap. It consists of 35-75 genera, approximately 800 species distributed pantropically with a few species in temperate regions, while seven genera are native to the Malay Peninsula (Hsuan, 1969; Woodland, 1997). The largest genera are *Palanchonella*, *Palaquium* and *Pouteria* with 100, 115 and 150 species, respectively. In Peninsular Malaysia the family is a source of timber; in Central America, *Manilakara zapota* provides chicle (the elastic component of chewing gum). Edible fruits such as the sapodilla plum and star apple are provided by *Achras zapota* and *Chrysophyllum cainiti*, respectively. The Upper Cretaceous and more recent deposits have indicated fossil pollens of this family (Woodland, 1997).

1.3.1 *Mimusops*

The genus *Mimusops* is native to tropical parts of Africa and Asia. Parts of wood, flowers and fruits of this genus have also been found in ancient Egyptian tombs. Members of this genus possess chartaceous or coriaceous leaves, solitary or fascicled flowers and 1-4 seeded oblong pulpy fruits (Friis *et al.*, 1986; Hutchings *et al.*, 1996; Ridley and Hutchinson, 1967; Shahwar and Raza, 2009).

1.3.1.1 *Mimusops elengi* L.

Mimusops elengi (Figure 1.1) is an evergreen tree of Indian or Burmese origin. It is 30 feet tall (Ridley and Hutchinson, 1967). The trunk divides into several limbs forming a thick scattering round crown. The greyish brown bark is deeply ridged and fissured. The leaves are arranged axially or alternately and are spaced along the twigs. The leaf is oblong elliptic, tipped, possesses wavy edges and a dull green

tenuous leathery stalk half to one inch long, while the blade is 2-6 inches long and 1-3 inches wide. The flowers are half an inch wide, fragrant, shortly stalked and are usually found in auxiliary clusters of 2-6 flowers. Their white petals are arranged in a star like ring which appear to be dropping down (Corner, 1988). The oblong, pointed, smooth, fleshy fruit is one inch long (Ridley and Hutchinson, 1967), which upon ripening turns orange red from green. It possesses eight persistent sepals at the base and contains one hard blackish brown seed (Corner, 1988). *M. elengi* is cultivated throughout the tropics (Corner, 1988) and is presumably wild in Pahang, Malaysia (Ridley and Hutchinson, 1967).

Figure 1.1 *Mimusops elengi* L.

1.4 Medicinal properties of *M. elengi*

Diverse medicinal properties have been reported for different parts of the plant. The leaves are used for treating cholagouge, stomache and act as antihelmintic. The juice of the leaves is also applicable to wounds. The leaves and stem are cooked with pork to yield a tonic and the leaves are further presumed to treat snake bite. The leaves are also known to possess antioxidant and anti-inflammatory properties (Ambasta *et al.*, 1986; Chopra *et al.*, 1956; Dastur, 1962; Saha *et al.*, 2008).

The bark is styptic and astringent thus its decoction is handy in catarrh of the bladder and urethra. As a febrifuge it is given in fevers and used to increase female fertility. The decoction also treats diseases of the gums, sore mouth and is also utilized as an antidiuretic, antitoxin and a premature-ejaculation suppressor (Dastur, 1962; Paranjepe, 2001; Sharma *et al.*, 2000). The bark and the leaf extracts are known to possess potent antibacterial and antifungal activities (Ali *et al.*, 2008; Shahwar and Raza, 2009).

A paste of the root prepared with vinegar is applied to swellings on the face, and a paste prepared with water is used as a dressing for pustular skin eruptions (Dastur, 1962). The aqueous flower distillate is valued as a perfume. The flowers are used for treating heart disease, while wounds and ulcers are also bathed with an astringent flower lotion. The dried powdered flowers are used as a snuff for relief from cephalagia. The flower extract of *M. elengi* administrated in anesthetized dogs was found to exhibit diuretic action (Dastur, 1962; Kanjanapothi and Tejasen, 1971; Paranjepe, 2001; Sharma *et al.*, 2000).

Mastication of the unripe fruit is practical for strengthening and fixing loose teeth, while ripe fruit pulps are helpful in chronic dysentery. The fruit is used externally for headache and its lotion can be applied to wounds and sores. Preserves and pickles are also produced from the fruit. The fruit possesses significant antioxidant capacity and radical scavenging effect, the green fruit showing greater antioxidant capacity and phenolic content as compared to the orange-ripe fruit (Boonyuen *et al.,* 2009; Chaovanamethakul *et al.,* 2007; Kumar and Arora, 2007).

The blend of the seeds in oil is supposedly practical in obstinate constipation, particularly in children (Ambasta *et al.*, 1986; Chopra *et al.*, 1956; Dastur, 1962), while saponins extracted from the roots, leaves, fruits, seeds, and bark showed a moderate degree of spasmolytic activity (Banerji *et al.*, 1982). The plant is also reported to possess antibacterial, anti-diabetic and antifungal properties (Ganu and Jadhav, 2010; Rao *et al.*, 2009; Mohamed *et al.*, 1996; Satish *et al.*, 2008).

1.5 Previous studies on *M. elengi*

A literature survey indicated that all the plant parts of *M. elengi* have been investigated previously. However, the seeds were focused extensively and triterpenes were the most commonly found compounds.

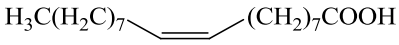
1.5.1 Phytochemical studies on *M. elengi*

The earliest investigations began in 1910 when Kesava-Menon isolated a volatile oil from the flowers which on extraction with ether extract yielded 18.47% of a yellowish brown oil (Kesava-Menon, 1910). Later, Rau and Simnsen isolated behenic acid **(1)**, palmitic acid **(2)**, stearic acid **(3)** and oleic acid **(4)** from the seed

oil (Rau and Simnsen, 1922). The petroleum ether extract of the seeds also yielded linoleic acid **(5)**, arachidic acid **(6)**, lauric acid **(7)**, myristic acid **(8)** and *β*-sitosterol **(9)** apart from the previously reported oleic acid **(4)**, palmitic acid **(2)** and stearic acid **(3)** (Sinha, 1962).

$CH_3(CH_2)_nCOOH$

(1) n = 20
(2) n = 14
(3) n = 16
(6) n = 18
(7) n = 10
(8) n = 12



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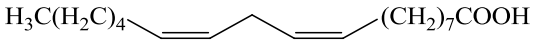
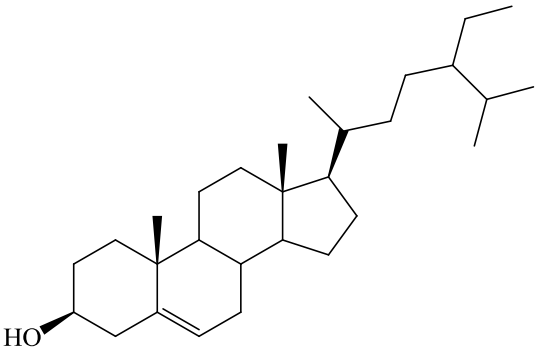


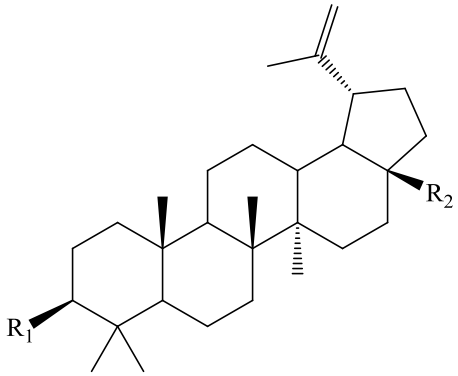
(5)



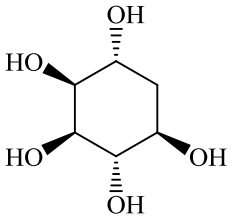
(9)

Misra and Mitra investigated all the parts of the plant and the following compounds were identified: betulinic acid **(10)**, lupeol **(11)**, quercitol **(13)**, *β*-sitosterol glucoside **(14)**, spinasterol **(15)**, taraxerol **(16)**, taraxerone **(17)** and ursolic acid **(18)** from the bark, hederagenin **(19)**, lupeol **(11)** and α-spinasterol **(15)** from the heart-wood, hentriacontane **(20)**, *β*-carotene **(21)** and quercitol **(13)** from the leaves, lupeol acetate **(12)**, and taraxerol **(16)** from the root, quercitol **(13)** and ursolic acid **(18)** from the mesocarp, quercetin **(22)**, dihydroquercetin **(23)**, *β*-sitosterol glucoside **(14)** and quercitol **(13)** from the testa, *β*-sitosterol glucoside **(14)**, quercitol **(13)** and α-

spinasterol **(15)** from the kernel (Misra and Mitra, 1967a; Misra and Mitra, 1967b; Misra and Mitra, 1968).

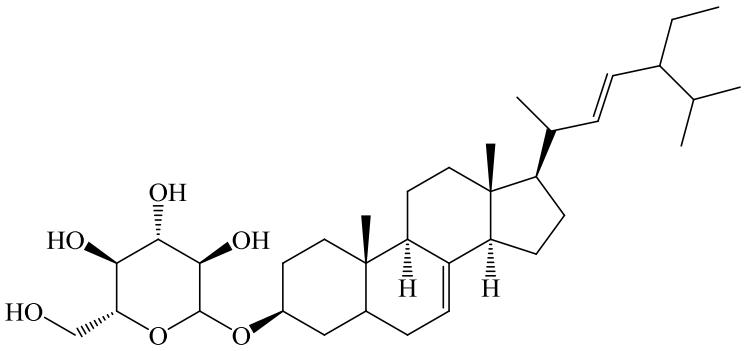


(10) R_1 = OH, R_2 = COOH
(11) R_1 = OH, R_2 = CH_3

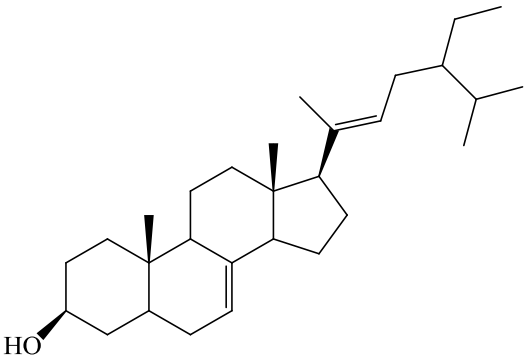


(13)

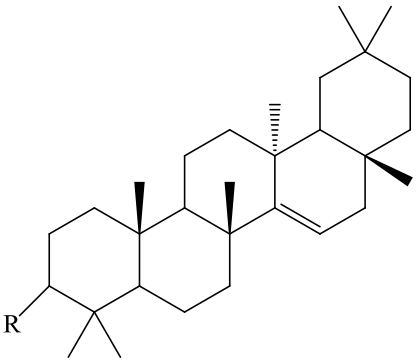
(12) R_1 = $OCCH_3$ (O above C), R_2 = CH_3



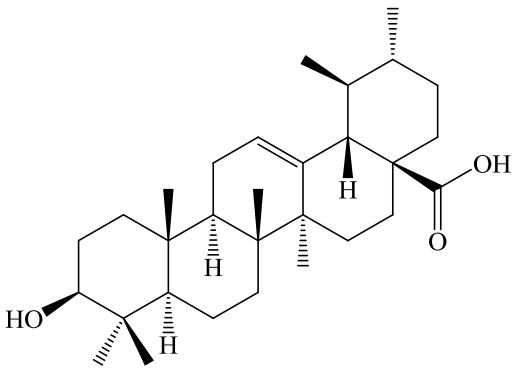
(14)



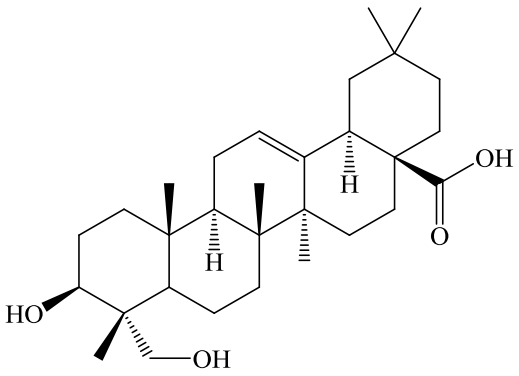
(15)



(16) R = *β*-OH
(17) R = -O



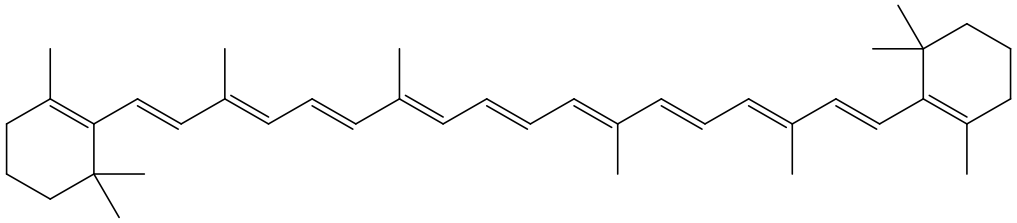
(18)



(19)

$CH_3(CH_2)_{29}CH_3$

(20)



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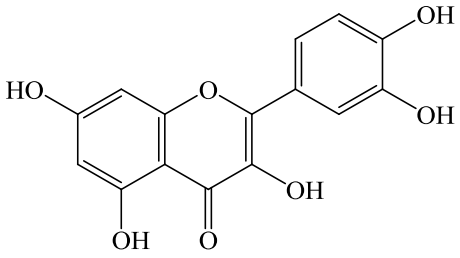

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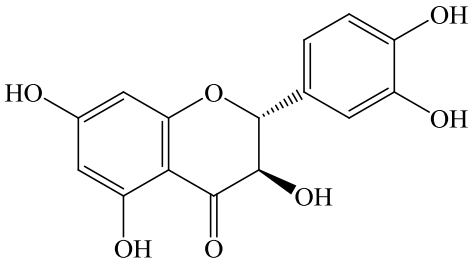
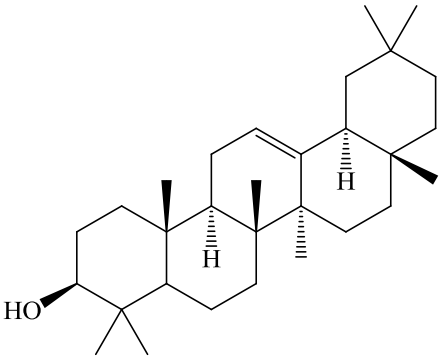


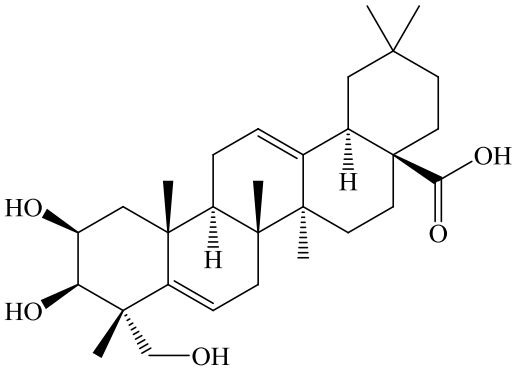
(23)

A mixture of saponins was isolated from the bark which on hydrolysis yielded *β*-amyrin **(24)** and bassic acid **(25)** (Varshney and Logani, 1969). Subramanian and Nair indicated the presence of myricetin 3-*O*-L-rhamnoside **(26)** and myricetin 3-*O*-*β*-D-galactoside **(27)** in the ethanol extract of the plant (Subramanian and Nair, 1973). D-mannitol **(28)**, *β*-sitosterol **(9)** and *β*-sitosterol-D-glucoside **(14)** were reported from the flowers. However, their identification was based on melting point, thin-layer chromatography and IR spectroscopy (Gupta *et al.*, 1976). A new steroidal

saponin 5*β*-stigmast-9(11)-en-3-*O*-*β*-D-glucopyranosyl (1→5)-*O*-*β*-D xylofuranoside **(29)** was isolated from the roots (Saxena and Shrivastava, 1988).



(24)



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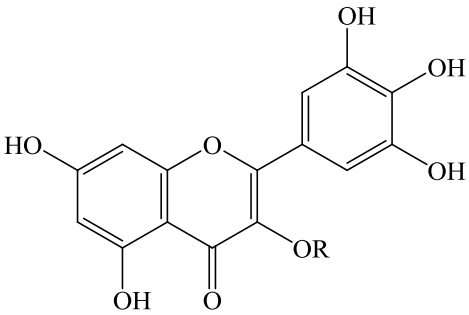
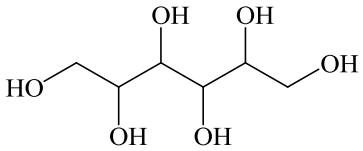


(26) R = *α*-L-rhamnosyl
(27) R = *β*-D-galactosyl



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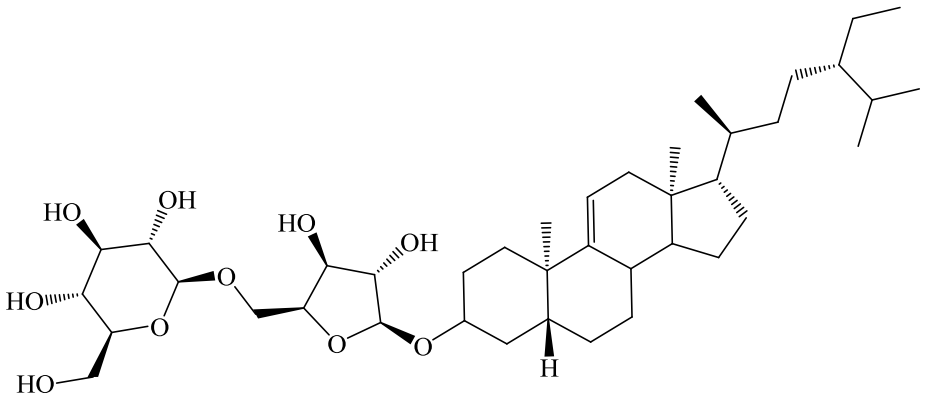
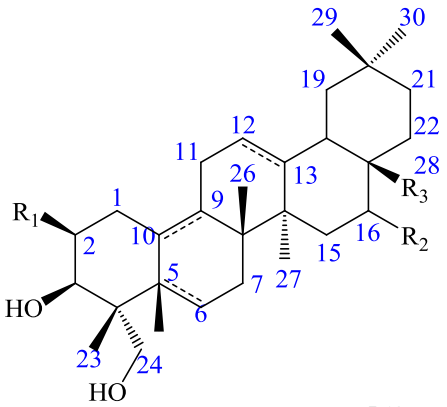


(29)

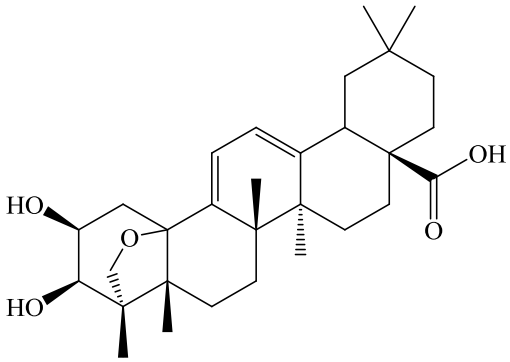
Mandal and Maity reported behenic acid **(1)**, linoleic acid **(5)**, oleic acid **(4)**, palmitic acid **(2)** and stearic acid **(3)** from the seed kernels (Mandal and Maity, 1991). Later, Sen *et al*. isolated from the seeds new pentacyclic triterpenes, mimugenone **(30)**,

mimusopgenone **(31)**, mimusopic acid **(32)** and mimusopsic acid **(33)** (Sen *et al.*, 1993; Sen *et al.*, 1995). Sahu isolated from the seeds two saponins, mimusopside A **(34)** and mimusopside B **(35)**, in addition to dihydroquercetin **(23)**, Mi-glycoside **(36)** and α-spinasterol glucoside **(37)** (Sahu, 1996; Sahu, 1995).

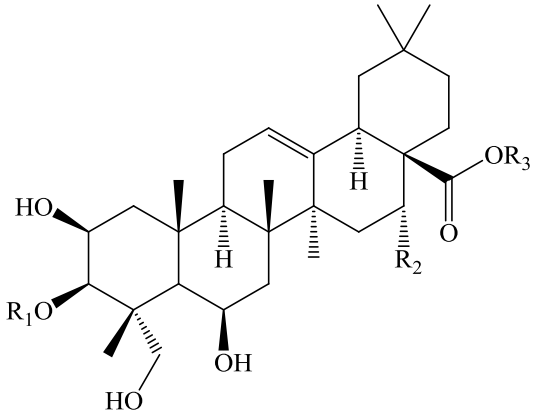
Jahan *et al*. isolated (24*R*)-5α-stigmasta-7,22(*E*)-dien-3*α*-ol (C-3 epimer of chondrillasterol) **(38)**, spinasterol glucopyranoside **(39)**, spinasterol galactopyranoside **(40)** and 3*β*-hydroxy-lup-20(29)-ene-23, 28-dioic acid **(41)**, in addition to *β*-amyrin **(24)**, lupeol **(11)**, taraxerol **(16)** and ursolic acid **(18)** from the plant (Jahan *et al.*, 1995a; Jahan *et al.*, 1995b; Jahan *et al.*, 1996).



(30) R_1 = H, R_2 = O, R_3 = CH_3 $\Delta^{5,12}$
(31) R_1 = OH, R_2 = O, R_3 = H $\Delta^{5,12}$
(32) R_1 = OH, R_2 = H, R_3 = COOH $\Delta^{10,12}$



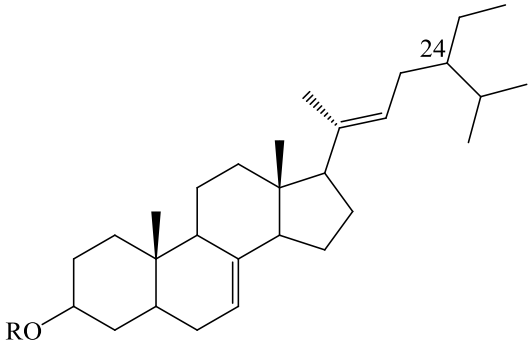
(33)



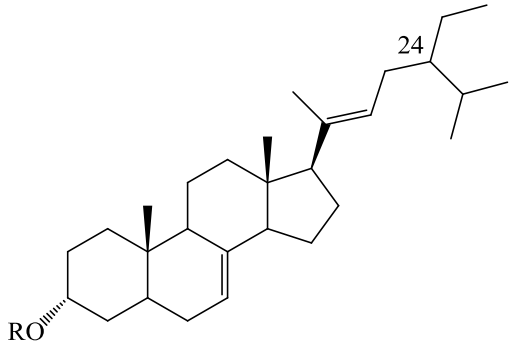
(34) R_1 = *β*-D-glucopyranosyl, R_2 = H, R_3 = *α*-L-rhamnopyranosyl (1→2)-*α*-L-arabinopyranosyl

(35) R_1 = *β*-D-glucopyranosyl, R_2 = OH, R_3 = α-L-rhamnopyranosyl (1→2)-α-L-arabinopyranosyl

(36) R_1 = *β*-D-glucopyranosyl, R_2, R_3 = H



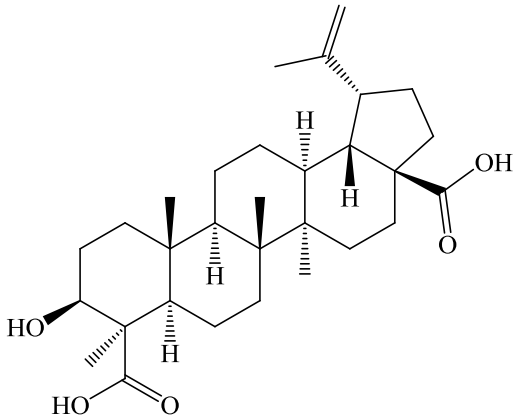
(37) R = *β*-D-glucosyl



(38) R = H, (24*R*)

(39) R = *β*-D-glucopyranosyl, (24*R*)

(40) R = *β*-D-galactopyranosyl, (24*R*)

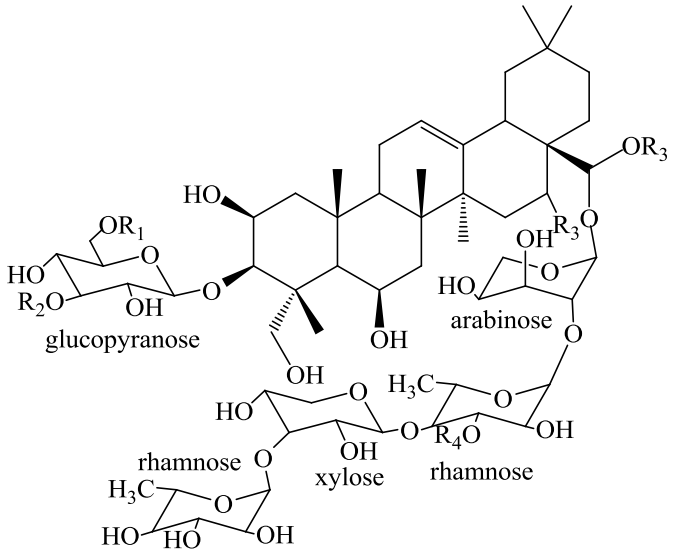


(41)

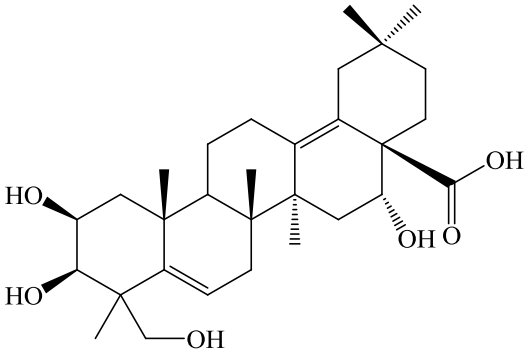
In addition to Mi-saponin A **(42)** and 16α-hydroxy Mi-saponin A **(43)**, Sahu *et al*. identified two novel triterpenoid saponins, mimusopin **(44)** and mimusopsin **(45)**, and two novel minor triterpenoid saponins mimusin **(46)** and elengin **(47)** (Sahu *et*

al., 1995; Sahu *et al.*, 1997; Sahu *et al.*, 1998). Later, a new triterpene, mimusic acid **(48)**, and the known mimusopic acid **(33)** were also reported (Sahu *et al.*, 1999).

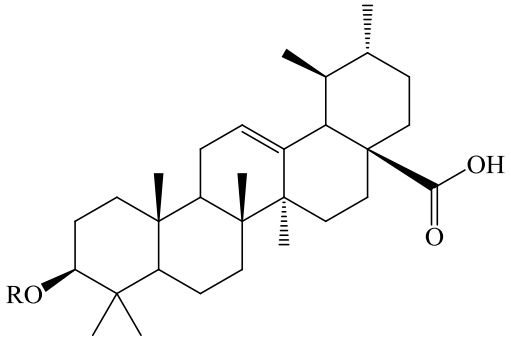
Two new triterpenes, 3*β*-(*p*-hydroxy-*cis*-cinnamoyloxy)-urs-12-en-28-oic acid **(49)** and 3*β*, 19*β*, 23-trihydroxy-urs-12-ene **(51)**, were reported for the first time together with 3*β*-(*p*-hydroxy-*trans*-cinnamoyloxy)-urs-12-en-28-oic acid **(50)**, 3*β*,6*β*,19*α*,23-tetrahydroxy-urs-l2-ene **(52)**, 1-*β*-hydroxy-3*β*-hexanoyllup-20 (29)-ene-23, 28-dioic acid **(53)** and ursolic acid (Jahan *et al.*, 2000; Jahan *et al.*, 2001). The fatty oils of *M. elengi* were found to consist of myristic acid **(8)**, oleic acid **(4)**, palmitic acid **(2)**, stearic acid **(3)** and a new compound 9-keto-octadec-15(*Z*)-enoic acid **(54)** (Daulatabad and Bhat, 2003). Hazar *et al*. identified the known flavones quercetin **(22)** and dihydroquercetin **(23)** from the seeds, both indicating strong antibacterial activity (Hazra *et al.*, 2007).



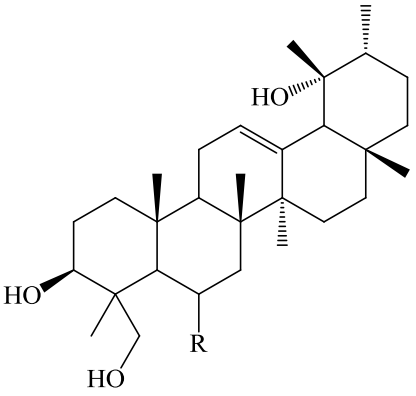
(42) $R_1 = R_2 = R_3 = R_4 = H$
(43) $R_1 = R_2 = H$, $R_3 = OH$, $R_4 = H$
(44) $R_1 = R_2 = R_3 = H$, $R_4 = \alpha$-L-rhamnosyl
(45) $R_1 = H$, $R_2 = \beta$-D-glucopyranosyl, $R_3 = R_4 = H$
(46) $R_1 = \beta$-D-glucopyranosyl, $R_2 = R_3 = R_4 = H$
(47) $R_1 = \beta$-D-glucopyranosyl, $R_2 = H$, $R_3 = OH$, $R_4 = H$



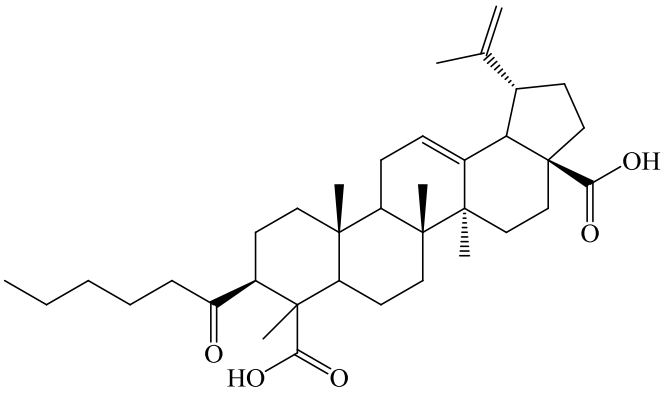
(48)



(49) R = *p*-hydroxy-*cis*-cinnamoyloxy
(50) R = *p*-hydroxy-*trans*-cinnamoyloxy



(51) R = H
(52) R = *β*-OH



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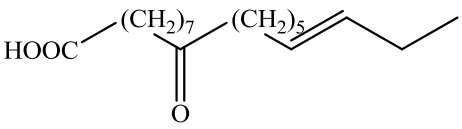
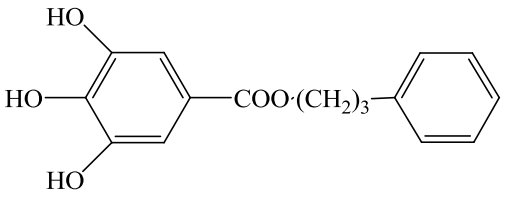


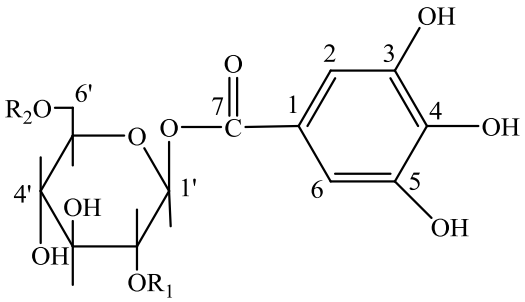
(54)

Akhtar *et al*. identified from the bark elengigallate **(55)**, mimusopstannin **(56)**, elengitannin **(57)**, gallic acid diglucoside **(58)**, gallic acid phenoxy diglucoside **(59)**,

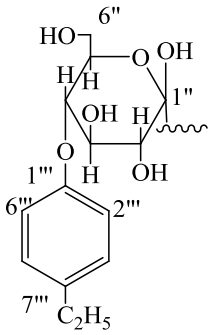
elengibenzyl diglycoside **(60)**, gallic acid triphenoxy diglycoside **(61)**, mimusopfarnanol **(62)**, farnan-3-one **(63)**, lupeol, olean-18-en-2-one-3-ol **(64)**, *β*-sitosterol, *β*-sitosterol glucopyranoside, stigmasta-5,22-dien-3*β*-ol-3*β*-D-glucuropyranosyl-(6'*β*→1")-D-glucopyranoside **(65)** and *β*-sitosterol-3*β*-(3"',6"',7"'-trihydroxynaphthyl-2"'-carboxyl)-4"-glucopyranosyl-(1"→4')-glucopyranoside **(66)** (Akhtar *et al.*, 2009; Akhtar *et al.*, 2010a; Akhtar *et al.*, 2010b).



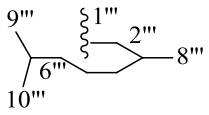
(55)



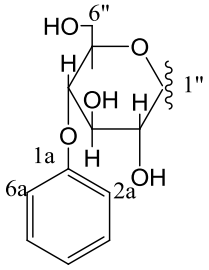
(56) R_1 = H, R_2 =



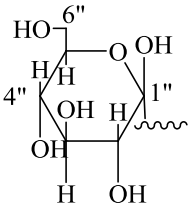
(57) R_1 =



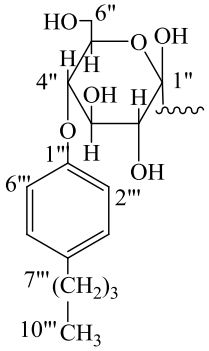
R_2 =



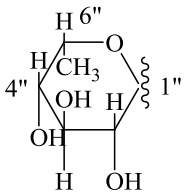
(58) R_1 = H, R_2 =



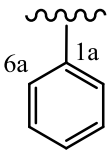
(59) R_1 = H, R_2 =



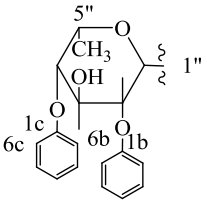
(60) R_1 = H, R_2 =

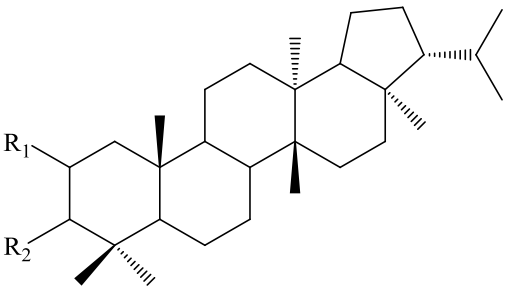


(61) R_1 =

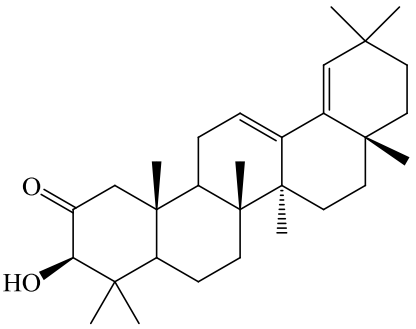


R_2 =

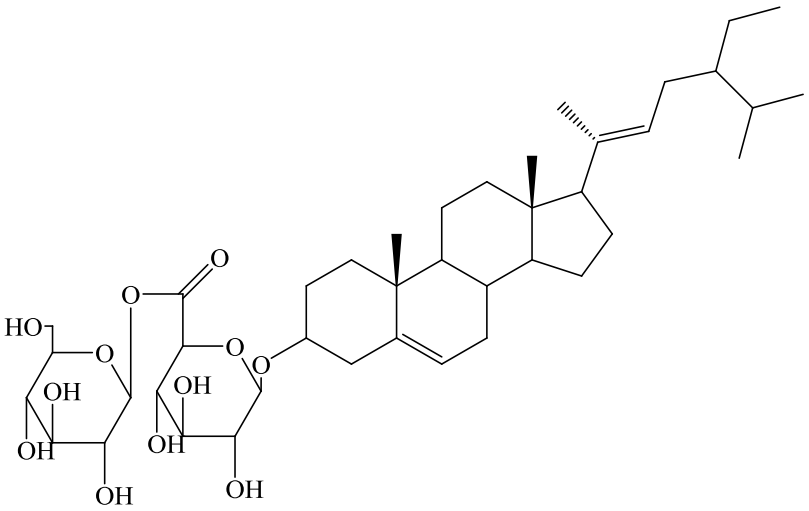




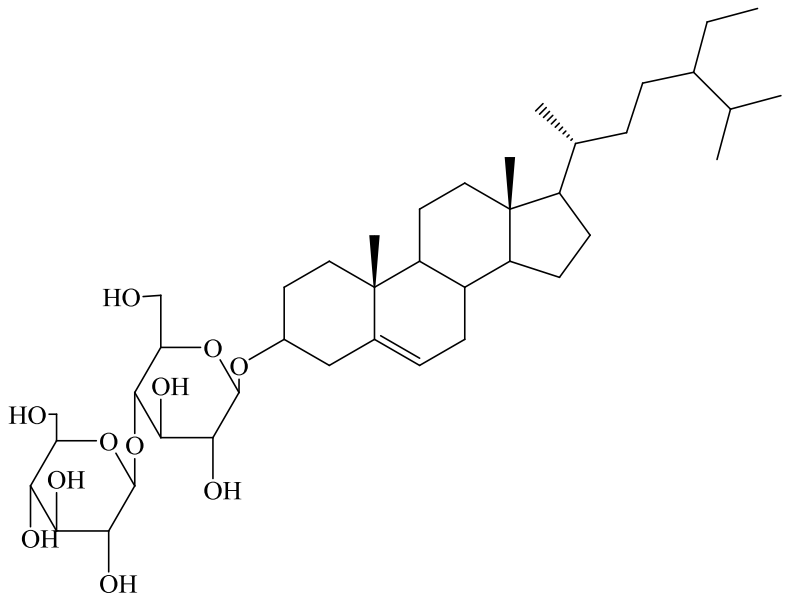
(62) R_1 = O, R_2 = β-OH
(63) R_1 = H, R_2 = O



(64)



(65)



(66)

1.5.2 Studies on the volatile constituents of *M. elengi*

Wong and Teng (1994) identified a total of 74 compounds among the volatile constituents of the flowers. The major constituents of headspace extraction were 2-phenylethanol (37.8%), methyl benzoate (13.4%), *p*-methylanisole (9.9%) and 2-phenylethyl acetate (7.1%), respectively, and those of solvent extraction were 2-phenylethanol (38.7%), (*E*)-cinnamyl alcohol (13.7%) and 3-hydroxy-4-phenyl-2-butanone (4.7%), respectively.

Aromdee and Rattanadon (2009) investigated the volatile constituents of the dried and fresh flowers of *M. elengi*, the dried flowers gave a long chain carboxylic acid ester (5.3%) and (*Z*)-9-octadecenoic acid (4.7%), while the fresh flowers yielded 2-phenylethanol (10.4%), 4-hydroxybenzyl alcohol (8.6%) and cinnamyl alcohol (6.1%).

Rout *et al*. (2010) identified 27 compounds in a comparative study on the flower fragrance obtained from the water-soluble volatile, hexane and liquid carbon dioxide

extracts using GC and GC/MS. Major constituents such as phenol, benzyl alcohol, 2-phenylethyl alcohol, anisyl alcohol, *E*-cinnamyl alcohol and ethyl *p*-anisate were found in varying percentages in the different extracts. The study focused on the proportions of benzenoids and waxy materials obtained from the different extracts. The hexane extract (concrete) was partitioned with methanol to give hexane extract absolute. The absolute and concrete hexane extracts contained waxy materials (30%) and benzenoids (45-51%). The water-soluble volatile extract was partitioned with diethyl ether to give a diethyl ether extract which contained a higher quantity of benzenoids, but their yield was low. The liquid carbon dioxide extract of the dried flowers contained waxy materials (60%) and benzenoids (14%). The liquid carbon dioxide extract of the fresh flowers showed the most promising results with 15% of waxy materials and 61% of benzenoids. Certain components such as heptanal, phenol, *p*-cresol, dimethyl ether, *p*-methoxycresol, *p*-anisaldehyde, anisyl alcohol, carvacrol, ethyl *p*-anisate, methyl palmitoleate, methyl palmitate, ethyl palmitoleate, palmitic acid, heneicosane, 6,13-octadecadienyl acetate, ethyl stearate and 5-methyltricosane had not been reported previously from the flowers. The examination of the volatile constituents of the bark indicated the presence of α-cadinol, T-muurolol and hexadecanoic acid as the major constituents (Ruikar *et al.*, 2009).

1.6 Objectives of the present work

The objectives of the present work are:

1. To isolate and identify chemical constituents from the chloroform extracts of the bark and leaves of *M. elengi*.
2. To determine the composition of the volatile constituents of the flowers of *M. elengi* isolated by low temperature vacuum distillation.
3. To evaluate the antibacterial and anti-inflammatory activities of the crude chloroform extracts, isolated compounds and flower volatile oil.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Plant materials

The bark, flowers and leaves of *M. elengi* were collected from the USM campus. The plant was identified by Mr. Baharuddin Sulaiman of the School of Biological Sciences, USM, Penang, Malaysia, where a voucher specimen (USM 9255) has been deposited.

2.2 Extraction of the leaves

Fresh leaves (3 kg) were macerated in 30 L of MeOH-H_2O (4:1 v/v). The extract after filtration was concentrated under reduced pressure at a bath temperature <40ºC to 1/10 of its original volume using a rotary evaporator, acidified with aqueous 2M H_2SO_4 to a pH of 2-3, and 500 mL portions of it extracted with chloroform (200 × 2 mL, then 100 mL) to give a combined chloroform extract of 31 g (1.03% w/w of fresh leaves) (Harborne, 1998).

2.3 Extraction of the bark

Fresh bark (4 kg) was dried in the shade for two weeks at room temperature (28ºC) to yield 1 kg of dry bark, which was powdered using a commercial crusher, and 150 g each was extracted using a Soxhlet extractor with hexane for 4 hrs, then with chloroform for 8 hrs, yielding a combined chloroform extract of 14 g (0.35% w/w of fresh bark).

2.4 Isolation of the flower volatiles

Intact flowers (350 g) collected 30 min earlier were immersed in distilled water and vacuum-distilled for 6 hrs, yielding approximately 250 mL of distillate which was collected in a liquid-nitrogen-cooled trap. Two small liquid-nitrogen-cooled traps connected in series between the first trap and the vacuum pump were used for preventing the back diffusion of impurities (Figure 2.1) (Joulain, 1987). The distillate was extracted with freshly-distilled dichloromethane (5 × 20 mL). The dichloromethane extract was then concentrated using a Kuderna-Danish concentrator at a bath temperature of 50ºC, and was carefully reduced to a volume of 0.1 mL under a gentle stream of high purity N_2 gas at room temperature prior to gas chromatographic analysis.

Figure 2.1 Vacuum-distillation of *M. elengi* flowers

2.5 Chromatography

2.5.1 Thin layer chromatography

Thin layer chromatography was used for the preliminary screening of the crude extracts, for the determination of a suitable solvent system for the subsequent column chromatographic separations, and for the purity determination of the isolated compounds. Pre-coated TLC plates (20 × 20 cm, coated with 0.2 mm silica gel F_{254} on aluminium sheets, Merck) were cut into smaller pieces of 5.0 cm × 2.0 cm, which were used throughout this work. For visualization, a UV lamp (Vilber Lourmet, multiband UV-254/356 nm) was used. Additionally, 95% methanolic sulphuric acid was used as a detection reagent, in which plates after dipping were charred using a heat gun (Jork *et al.*, 1990; Kirchner *et al.*, 1951).

2.5.2 Column chromatography

Column chromatography (CC) was used to separate the chemical constituents of the crude extracts and for the isolation of pure compounds. Silica gel 60 (0.040-0.060 mm, 230-400 mesh ASTM, Merck) was used as the adsorbent. Samples were either dissolved in a minimum quantity of a suitable solvent and adsorbed onto the silica gel by drying on a rotary evaporator to yield a powdered material which was loaded on the stationary phase, or were dissolved in a suitable solvent and applied to the top of the stationary phase (Sharp *et al.*, 1989).

2.6 Instrumentation

2.6.1 Specific optical rotation measurement

The specific optical rotations $[\alpha]_D^{25}$ of the isolated compounds were determined using an ATAGO AP-300 automatic polarimeter. A solution of 1.0% sucrose was

used as a standard and the sodium lamp was set at 589 nm. Samples were dissolved in chloroform and/or methanol according to their solubility and a cell of length 200 mm was used for the measurements which were all taken at room temperature (25ºC).

2.6.2 Melting point determination

A Stuart scientific SMP-1 melting point apparatus was used for the melting point measurements.

2.6.3 Infrared spectroscopy

Infrared (IR) spectra were recorded on a Perkin-Elmer System 2000 FT-IR spectrometer. The sample was pressed with potassium bromide (KBr) to form a disk which was scanned in the range 4000-650 cm^{-1}.

2.6.4 Direct-probe mass spectrometry

The electron impact (EI) and fast atom bombardment (FAB) mass spectra were recorded using an Agilent 5975C MSD and a Thermo Finnigan MAT95XL mass spectrometer, respectively.

2.6.5 Nuclear magnetic resonance spectroscopy

NMR spectra were recorded using a Bruker Avance 400 spectrometer operated at 400 MHz for 1H and 2D NMR experiments (^{1}H-^{1}H-COSY, HMQC, HMBC, NOESY), and at 100 MHz for ^{13}C NMR and DEPT experiments (DEPT 90 and DEPT 135). The samples were dissolved in an appropriate solvent [chloroform-d (Merck), methanol-d_4 (Acros Organics) and pyridine-d_5 (Sigma-Aldrich)] with

tetramethylsilane (TMS) as internal standard, and placed in 5 × 180 mm NMR tubes (Sharp *et al.*, 1989).

2.7 Column chromatography of chloroform leaf extract

The chloroform extract (31 g) was fractionated using silica gel column chromatography. Fifteen grams of each of the extracts absorbed on silica gel were loaded on a 100 × 15 cm glass column packed with 360 g of silica gel. The column was eluted using a hexane : ethyl acetate : methanol gradient (1:0:0 → 0:0:1 v/v/v). The eluates were collected in test tubes in 150 mL portions which were combined into ten fractions (F1 to F10) on the basis of TLC profile, and the solvent mixture was evaporated under reduced pressure: F1 (test tubes 1-7, 0.9 g, yellow oil, eluted with 100% hexane), F2 (test tubes 8-18, 1.3 g, yellow oil, eluted with hexane : ethyl acetate 9:1 v/v), F3 (test tubes 19-27, 1.1 g, green solid, eluted with hexane : ethyl acetate 8:2 v/v), F4 (test tubes 28-35, 1.0 g, green solid, eluted with hexane : ethyl acetate 6:4 v/v), F5 (test tubes 36-47, 1.0 g, green solid, eluted with hexane : ethyl acetate 4:6 v/v), F6 (test tubes 48-54, 1.0 g, green solid, eluted with hexane : ethyl acetate 2:8 v/v), F7 (test tubes 55-61, 1.6 g, green solid, eluted with 100% ethyl acetate), F8 (test tubes 61-64, 1.0 g, white solid, eluted with ethyl acetate : methanol 8:2 v/v), F9 (test tubes 65-76, 1.0 g, brown liquid, eluted with ethyl acetate : methanol 5:5 v/v) and F10 (test tubes 77-79, 2.0 g, brown liquid, eluted with 100% methanol).

Fraction F2 (1.3 g) was rechromatographed using a 60 × 8 cm glass column packed with 30 g silica gel and eluted with hexane : ethyl acetate (1:0 → 7:3 v/v). A total of 16 fractions (fr 1-16) were obtained, each 25 mL, which were combined into three