BIOACTIVITIES AND CHEMICAL CONSTITUENTS OF LEAVES OF SELECTED APOCYNACEAE SPECIES IN PENINSULAR MALAYSIA

Allamanda cathartica

Alstonia angustiloba

Calotropis gigantea

Catharanthus roseus

Cerbera odollam

Dyera costulata

Kopsia fruticosa

Nerium oleander

Plumeria obtusa

Vallaris glabra

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MONASH UNIVERSITY

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This thesis includes two original papers published in peer reviewed journals of which the candidate is the lead author. The idea, development and writing up of the papers in the thesis were the principal responsibility of the candidate, working under the supervision of Assoc. Prof. LIM Yau Yan of Monash University Sunway Campus (MUSC).

The inclusion of co-authors in the publications reflects that the work was based on team research through active collaboration between researchers of MUSC Malaysia and the Institute of Medical Research (IMR), Malaysia.

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ABSTRACT

Leaves of ten Apocynaceae species (*Allamanda cathartica*, *Alstonia angustiloba*, *Calotropis gigantea*, *Catharanthus roseus*, *Cerbera odollam*, *Dyera costulata*, *Kopsia fruticosa*, *Nerium oleander*, *Plumeria obtusa* and *Vallaris glabra*) were assessed for antiproliferative (APF) activity against selected cancer cells using the sulphorhodamine B (SRB) assay. Leaves were sequentially extracted with hexane (Hex), dichloromethane (DCM) and methanol (MeOH). Extracts were also analysed for total alkaloid content (TAC), total phenolic content (TPC), free radical scavenging (FRS) and antityrosinase (AT) activity using the Dragendorff precipitation, Folin-Ciocalteu, 1,1-diphenyl-2-picrylhydrazyl and dopachrome assays, respectively. Five species (*Alstonia angustiloba*, *Calotropis gigantea*, *Dyera costulata*, *Kopsia fruticosa* and *Vallaris glabra*) were also assessed for antiplasmodial (APM) activity using the lactate dehydrogenase assay.

Leaf extracts of *Alstonia angustiloba*, *Calotropis gigantea*, *Catharanthus roseus*, *Nerium oleander*, *Plumeria obtusa* and *Vallaris glabra* displayed positive APF activity. DCM extract of *Calotropis gigantea*, and DCM and DCM:MeOH extracts of *Vallaris glabra* showed strong APF activity against all six human cancer cell lines (MCF-7, MDA-MB-231, HeLa, HT-29, SKOV-3 and HepG2). Against MCF-7 and MDA-MB-231 breast cancer cells, DCM extracts of *Calotropis gigantea* and *Nerium oleander* were stronger than or comparable to standard drugs of xanthorrhizol, curcumin and tamoxifen.

In term of antiplasmodial (APM) activity, all five species were effective against chloroquine-resistant K1 strain of *Plasmodium falciparum.* All four solvent extracts of leaves of *Vallaris glabra* displayed effective APM activity which is stronger than mefloquine. Against chloroquine-sensitive 3D7 strain, only extracts of *Dyera costulata*, *Calotropis gigantea* and *Kopsia fruticosa* showed positive APM activity.

Among the ten species studied, leaf extracts of *Kopsia fruticosa* had the highest TAC while those of *Dyera costulata* had the highest TPC, FRS and AT activity. There was a strong correlation between TPC and FRS of extracts (R^2 = 0.991) but not between TPC and TAC (R^2 = 0.193), and not between TAC and FRS (R^2 = 0.168)

Vallaris glabra was chosen as the main species for further study. Stem and flower extracts displayed effective inhibition against MCF-7 and SKOV-3 cancer cells. Both leaves and flowers possessed broad-spectrum APF activity.

From the MeOH leaf extract of *Vallaris glabra*, three types of caffeoylquinic acids and a flavonol were isolated by column chromatography. They were identified by ^{1}H-NMR, ^{13}C-NMR and ESI-MS analyses to be 3-*O*-caffeoylquinic acid (3-CQA) or neochlorogenic acid, 4-*O*-caffeoylquinic acid (4-CQA) or cryptochlorogenic acid and 5-*O*-caffeoylquinic acid (5-CQA) or chlorogenic acid (CGA). The flavonol was identified as quercetin 3-*O*-glucoside (QG) or isoquercitrin. From the DCM leaf extract of *Vallaris glabra*, a fatty acid and a triterpenoid were isolated. They were identified as stearic acid (SA) and ursolic acid (UA), respectively.

Caffeoylquinic acid content of fresh leaf extracts of *Vallaris glabra, Dyera costulata, Alstonia angustiloba, Nerium oleander, Kopsia fruticosa* and *Plumeria obtusa* were quantitatively analysed using reversed-phase HPLC. *Nerium oleander* had the highest content of 5-CQA followed by *Vallaris glabra* which had the highest content of 3-CQA and 4-CQA. The 5-CQA content of *Vallaris glabra* was twice that of *Lonicera japonica* flowers, the commercial source of CGA, while its 3-CQA and 4-CQA content was about 16 times higher.

APF activity of the five compounds (3-CQA, 4-CQA, 5-CQA, QG and SA) isolated from leaf extracts of *Vallaris glabra* were tested against four cancer cell lines. Only SA displayed weak inhibitory activity. Although the yield of UA was too low for analysis of its inhibitory activity, a review of existing literature showed that there is a wealth of references on the potent anticancer properties of UA including those of the cancer cells used in this study. Most publications concluded that UA is a potent chemo-preventive agent for cancer.

MDA-MB-231 breast cancer cells treated with DCM leaf extract of *Vallaris glabra* and stained with fluorescent dye Hoechst 33342, provided evidence that the extract had an apoptotic effect on the MDA-MB-231 cells, based on nuclear morphological changes. Caspase colorimetry showed that the apoptotic effect involved activation of caspase-8, -9 and -3, but not caspase-6.

TABLE OF CONTENTS

LIST OF BOXES

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AA	Ascorbic acid
AEAC	Ascorbic acid equivalent antioxidant capacity
AGE	Advanced glycation end-products
AIDS	Acquired immunodeficiency syndrome
AIF	Apoptosis-inducing factor
ANOVA	Analysis of variance
APAD+	3-Acetylpyridine adenine dinucleotide
APAF1	Apoptotic protease activating factor-1
APCI	Atomic pressure chemical ionisation
APF	Antiproliferative
APM	Antiplasmodial
AT	Antityrosinase
ATCC	American Type Culture Collection
ATPase	Adenosine triphosphatase
AT-rich	Adenine thymine-rich
BE	Boldine equivalent
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
CD_3OD	Deuterated methanol
CGA	Chlorogenic acid
CGAE	Chlorogenic acid equivalent
$CHCl_3$	Chloroform
CO_2	Carbon dioxide
COSY	Correlation spectroscopy
COX	Cycloxygenase
CQA	Caffeoylquinic acid
CQAC	Caffeoylquinic acid content
Cyt c	Cytochrome c

1D	One-dimensional
2D	Two-dimensional
Da	Dalton
DCM	Dichloromethane
DIABLO	Direct IAP-binding protein with low pI
DISC	Death-inducing signalling complex
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
DPPH	1,1-Diphenyl-2-picrylhydrazyl
DW	Dry weight
EC	Effective concentration
EDTA	Ethylenediamine tetraacetic acid
EGFR	Epidermal growth factor receptor
EI	Electron ionisation
EndoG	Endonuclease G
ER	Estrogen receptor
ESI	Electrospray ionisation
EtAc	Ethyl acetate
EtOH	Ethanol
FADD	Fas-associated death domain
FC	Folin-Ciocalteu
FRIM	Forest Research Institute Malaysia
FRS	Free radical scavenging
FW	Fresh weight
GA	Gallic acid
GAE	Gallic acid equivalent
GC	Gas chromatography
GI	Growth inhibition
GR	Glucocorticoid receptor
GSK	Glycogen synthase kinase

HBA	Hydroxybenzoic acid
HCA	Hydroxycinnamic acid
HDL	High-density lipid
Hex	Hexane
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HSD	Honestly significant difference
H_2SO_4	Sulphuric acid
HTRA2	High-temperature-requirement protein A2
IAP	Inhibitors of apoptosis protein
IC	Inhibitory concentration
IL	Interleukin
IMR	Institute of Medical Research, Malaysia
IUPAC	International Union of Pure and Applied Chemistry
K^+	Potassium ion
LC	Liquid chromatography
LDH	Lactate dehydrogenase
LDL	Low-density lipid
L-Dopa	3,4-Dihydroxy-L-phenylalanine
MAPK	Mitogen-activated protein kinase
mAU	Milli absorption unit
MBTH	3-Methyl-2-benzothiazoninone hydrazone
MeOH	Methanol
MHz	Mega hertz
MMP-2	Matrix metalloproteinase-2
MS	Mass spectrometry
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MUSC	Monash University Sunway Campus
m/z	Mass-to-charge ratio

Na^+	Sodium ion
NAD	Nicotinamide adenine dinucleotide
NMR	Nuclear magnetic resonance
NOESY	Nuclear over-hauser effect spectroscopy
ODS	Octadecyl-derivatised silica
-OH	Hydroxyl
ORAC	Oxygen radical scavenging absorbance capacity
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate-buffered saline
pLDH	*Plasmodium* lactate dehydrogenase
pNA	p-Nitroanilide
ppm	Parts per million
PPO	Polyphenol oxidase
QG	Quercetin 3-*O*-glucoside
R^2	Correlation coefficient
RF	Radio frequency
RP	Reversed-phase
RSA	Radical scavenging activity
RT	Retention time
SA	Stearic acid
SD	Standard deviation
SI	Selectivity index
SMAC	Second mitochondria-derived activator of caspases
SOD	Superoxide dismutase
SRB	Sulphorhodamine B
TAC	Total alkaloid content
TC	Total cholesterol
TCA	Trichloroacetic acid

TFA	Trifluoroacetic acid
TLC	Thin-layer chromatography
TMS	Tetramethylsilane
TOCSY	Total correlation spectroscopy
TOF	Time of flight
TPC	Total phenolic content
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
UA	Ursolic acid
UPM	Universiti Putra Malaysia
USD	US dollars
u-PA	Urokinase plasminogen activator
UV	Ultraviolet

BOTANICAL NAMES

Apocynaceae

Allamanda blanchetti
Allamanda cathartica
Allamanda hendersonii
Allamanda schottii
Alstonia angustiloba
Alstonia augustifolia
Alstonia boonei
Alstonia calophylla
Alstonia coriacea
Alstonia macrophylla
Alstonia scholaris
Apocynum venetum
Asclepias gigantea
Calotropis gigantea
Calotropis procera
Catharanthus roseus
Cerbera fruticosa
Cerbera lactaria
Cerbera manghas
Cerbera odollam
Cryptolepis sanguinolenta
Dyera costulata
Dyera lowii
Dyera polyphylla
Kopsia arborea
Kopsia fruticosa
Kopsia singapurensis
Kopsia vinciflora
Nerium indicum
Nerium odorum
Nerium oleander
Paladelpha angustiloba
Plumeria acuminata
Plumeria obtusa
Plumeria rubra
Rauvolfia caffra
Rauvolfia serpentine
Rauvolfia tetraphylla
Streptocaulon tomentosum
Trachelospermum jasminoides
Vallaris glabra
Vallaris solanacea
Vinca major
Vinca minor
Vinca rosea

Other families

Annona squamosa
Camellia sinensis
Corchorus olitorius
Cynara scolymus
Diodia teres
Desmostachya bipinnata
Elaeis guineensis
Erica andevalensis
Erica cinerea
Etlingera elatior
Etlingera fulgens
Etlingera littoralis
Eucommia ulmoides
Euonymus alatus
Eupatorium perfoliatum
Eurycoma longifolia
Gentiana lutea
Hedyotis herbacea
Hibiscus sabdariffa
Hibiscus tiliaceus
Houttuynla cordata
Hyptis fasciculata
Ipomoea batatas
Ixora coccinea
Lonicera japonica
Malus pumila
Narcissus tazetta
Oldenlandia diffusa
Pandanus amaryllifolius
Papaver somniferum
Polylepis racemosa
Prunus domestica
Psidium guajava
Rosmarinus officinalis
Salix matsudana
Vaccinium macrocarpon

Chapter I

INTRODUCTION

1.1 APOCYNACEAE

1.1.1 Botany

Tropical plants of the family Apocynaceae consist of trees, shrubs and vines. Almost all species of the family produce milky sap (Wiart 2006; Ng 2006). Other characteristic features are that leaves are simple, opposite or whorled; flowers are large, colourful and slightly fragrant with five contorted lobes; and fruits are in pairs.

1.1.2 Uses

In traditional medicine, Apocynaceae species have been used to treat gastro-intestinal ailments, fever, malaria, pain and diabetes (Wiart 2006). In Kenya, Apocynaceae species are used to treat skin and ecto-parasitic diseases (Omino & Kokwaro 1993). Of the species studied:

Allamanda cathartica: Species of *Allamanda* have traditionally been used in East and Southeast Asia for their purgative and emetic properties (Rahayu 2001).

Alstonia angustiloba: Stems, leaves and latex have been used for gynaecological problems and skin sores in Indonesia (Mulyoutami *et al*. 2009). Leaves are applied to treat headache in Malaysia (Lin 2005).

Calotropis gigantea: Roots and leaves are used to treat skin and liver diseases, leprosy, dysentery, worms, ulcers, tumours and earaches (Rajakaruna *et al.* 2002). Its latex has been reported to have wound healing properties (Nalwaya *et al*. 2009).

Catharanthus roseus: Plant parts are used to treat malaria, diarrhoea, diabetes, cancer and skin diseases (Sutarno & Rudjiman 1999). Leaves are used as antiseptic agent for wounds and haemorrhage, and as mouthwash for toothache.

Cerbera odollam: Plants are applied for itch and sores (Khanh 2001). In Thailand, the bark is used as a laxative, antipyretic and in the treatment of dysuria.

Dyera costulata: Bark and leaves have been used for treating fever, inflammation and pain (Subhadhirasakul *et al*. 2003).

Kopsia fruticosa: Leaves are used to treat sore and syphilis, and has cholinergic effects (Johnson 1999).

Nerium oleander: Sold as Anvirzel, the extract is promoted to treat cancer, AIDS and congestive heart failure (Pathak *et al.* 2000). It induces cell death in human but not murine cancer cells.

Plumeria obtusa: In Asia, leaves of *Plumeria* are used for treating wounds and skin diseases (Burkill 1935; Kardono *et al.* 1990). Their latex and bark are known to have purgative and diuretic properties. In Indonesia, the bark is used to treat gonorrhea, and in Philippines, the bark is used for its purgative, emmenagogue, and febrifuge effects.

Vallaris glabra: Well known because the scent of its flowers is similar to that of pandan leaves and aromatic rice (Wongpornchai *et al*. 2003). Its use in traditional medicine has not been reported.

Apocynaceae species have a wide array of non-medicinal uses. In Southeast Asia, genera such as *Dyera* are important timber species. Many species are planted as ornamentals in gardens and along roadsides because of their colourful and showy flowers. In Kenya, they are also used for non-medicinal purposes such as food, poisons, fodder, wood, ornamentals, dye and perfume (Omino & Kokwaro 1993).

1.2 RATIONALE FOR STUDY

Although plants of the family Apocynaceae are commonly grown for ornamental purposes, many of them are also used in traditional medicine. Leaves were the main focus of this study because the collection of leaves is non-destructive and they represent a significant portion of plant biomass. Leaves of cultivated plants can be continually harvested as a source of natural products. Furthermore, most of the plant samples were collected from private and municipal gardens in Selangor, Malaysia.

Apocynaceae species have been reported to possess anticancer properties. The best known is *Catharanthus roseus* (Madagascar periwinkle). It contains indole alkaloids (vinblastine and vincristine) which are used to treat Hodgkin's disease and leukaemia, respectively (Cragg & Newman 2004; Wiart 2006; Patel *et al*. 2010). Other species having cytotoxic activity include those of *Allamanda* (Schmidt *et al*. 2006), *Alstonia* (Keawpradub *et al*. 1999a; Jagetia & Baliga 2006), *Calotropis* (Wang *et al.* 2008a & 2008b), *Cerbera* (Chang *et al*. 2000; Nurhanan *et al*. 2008), *Nerium* (Siddiqui *et al*. 1995; Pathak *et al*. 2000), *Plumeria* (Kardono *et al*. 1990) and *Tabernaemontana* (Lee & Houghton 2005). Species of Apocynaceae, notably those of *Alstonia*, are also known to have antimalarial properties (Wright *et al*. 1993; Keawpradub *et al*. 1999b; Schwikkard & van Heerden 2002). Of the indole alkaloids isolated from *Alstonia*, villastoninine has been reported to be the most active.

This study aims to determine if antiproliferative activity is widespread among the different genera of the family. Additionally, we wish to determine if there are other bioactive properties such as antimalarial, antioxidant and antityrosinase properties in the species studied.

In this study, leaf extracts of ten selected species belonging to ten genera were assessed for antiproliferative activity against six human cancer cell lines and for antiplasmodial activity against two strains of *Plasmodium falciparum*. The extracts were also analysed for total alkaloid content, total phenolic content, caffeoylquinic acid content, radical-scavenging activity and antityrosinase activity.

Of the ten species, *Vallaris glabra* was selected for further study as the species displayed outstanding antiproliferative and antiplasmodial activity, and the literature on the plant is scarce. Furthermore, leaves of the species are readily available. This study would contribute new and additional knowledge on the bioactivities and chemical constituents of Apocynaceae species.

1.3 OBJECTIVES OF STUDY

Objectives of the thesis were:

1. To assess the antiproliferative and antiplasmodial activities of leaf extracts of ten selected Apocynaceae species

2. To analyse the total alkaloid content, total phenolic content, caffeoylquinic acid content, radical-scavenging activity and antityrosinase activity of their leaf extracts

3. To select a species with outstanding antiproliferative and antiplasmodial activity for further studies of its bioactivities and chemical constituents

4. To isolate phytochemicals from the selected species, to assess their antiproliferative activity and to elucidate possible mechanisms

Chapter II

LITERATURE REVIEW

2.1 APOCYNACEAE

2.1.1 Introduction

The family Apocynaceae has been enlarged from two to five sub-families with the inclusion of species of the family Asclepiadaceae, under the unified classification (Endress & Bruyns 2000). The new sub-families are Apocynoideae, Asclepiadoideae, Periplocoideae, Rauvolfioideae and Secamonoideae. The expanded family now comprises more than 250 genera and 2000 species of tropical trees, shrubs and vines (Wiart 2006).

Ten species of Apocynaceae belonging to ten genera were studied in this thesis. Nine genera are of the sub-family Apocynoideae (*Allamanda*, *Alstonia*, *Catharanthus*, *Cerbera*, *Dyera*, *Kopsia*, *Nerium*, *Plumeria* and *Vallaris*) with the exception of *Calotropis* which belongs to the sub-family Asclepiadoideae.

2.1.2 Uses

a. Medicinal uses

In traditional medicine, Apocynaceae species in the Asia-Pacific region are used to treat gastrointestinal ailments, fever, malaria, pain, diabetes and infectious diseases (Wiart 2006). In Kenya, Apocynaceae species are used to treat skin and ecto-parasitic diseases (Omino & Kokwaro 1993). Medicinal uses of the studied genera of Apocynaceae are compiled in **Table 2.1**.

Table 2.1 Medicinal uses of the studied genera of Apocynaceae

Genus	Medicinal use	Source
Allamanda	*Allamanda* species have traditionally been used in East and Southeast Asia for their purgative and emetic properties.	Rahayu (2001)
Alstonia	The bark and latex are used in traditional medicine in Southeast Asia because of their astringent and antihelmintic properties. They are used to treat liver and intestinal disorders, heart diseases, asthma, various skin diseases and fever.	Teo (2001)
Calotropis	Plants are used for ulcers, tumours, piles, earache, toothache, headache, sprain and stiff joints. Leaves are applied on burns, headache and rheumatic pains, and as tincture for fever. Flowers are given for cough, cold and asthma. Latex is used as antiseptic, vermifuge, emetic and purgative.	Kiew (2001); Pathak & Argal (2007); Agrawal *et al.* (2010)
Catharanthus	Plant parts are used to treat malaria, diarrhoea, diabetes, cancer and skin diseases. Leaves are used as an antiseptic agent for wounds and haemorrhage, and as mouthwash for toothache.	Sutarno & Rudjiman (1999)
Cerbera	Plants are applied for itch and sores. In Thailand, the bark is used as a laxative, antipyretic and in the treatment of dysuria.	Khanh (2001)
Dyera	Leaves and barks have been used for treating fever, inflammation and pain.	Subhadhirasakul *et al.* (2003)
Kopsia	Leaves of *Kopsia* are used to treat sores and syphilis, and have cholinergic effects.	Johnson (1999)
Nerium	Sold as Anvirzel, the extract is promoted to treat cancer, AIDS and congestive heart failure. It induces cell death in human but not murine cancer cells.	Pathak *et al.* (2000)
Plumeria	In Asia, leaves of *Plumeria* are used for treating wounds and skin diseases. Their latex and bark are known to have purgative and diuretic properties. In Indonesia, the bark is used to treat gonorrhea, and in Philippines, the bark is used for its purgative, emmenagogue, and febrifuge effects.	Burkill (1935); Kardono *et al.* (1990)
Vallaris	*Vallaris* species do not have any documented medicinal uses.	

Table 2.2 Non-medicinal uses of studied genera of Apocynaceae

Genus	Non-medicinal use	Source
Allamanda	Grown as ornamentals for their bright and large flowers in pots and in gardens.	Rahayu (2001)
Alstonia	The wood is used for making pencils, matches and carvings, and is suitable for furniture and flooring. The latex or bark extract is used as eye lotion in the Pacific Islands. Some species are planted as ornamental trees because of their pagoda-like crown.	Teo (2001)
Calotropis	In Vietnam, *Calotropis* is planted as a hedge plant. In Africa, the wood is also used as toothbrush. In Southeast Asia, flowers are used as rosaries and in wedding ceremonies. Trees are also planted in home gardens as ornamentals.	Kiew (2001)
Catharanthus	Plants are a popular garden ornamental and are valued for their bushy habit, colourful flowers and dark green foliage.	Sutarno & Rudjiman (1999)
Cerbera	Trees are planted along roadsides as ornamentals. Their poisonous seeds are used for stupefying fish in Philippines and Thailand.	Khanh (2001)
Dyera	In Southeast Asia, the latex was formerly an important source of chewing gum. The light and soft timber of this species is excellent for manufacturing a variety of wood products e.g. carvings, toys, pencils, etc.	Middleton (2004)
Kopsia	*Kopsia* species are sometimes cultivated as ornamental or medicinal plants.	Li *et al.* (1995)
Nerium	Plants are planted as ornamentals in gardens and along roadsides.	
Plumeria	Trees are planted as ornamentals in gardens and along roadsides.	
Vallaris	With its attractive clusters of white flowers that emit a strong pandan fragrance, *Vallaris* is becoming a popular ornamental plant in gardens of Southeast Asia. Potted plants can be purchased from nurseries.	

b. Non-medicinal uses

In Southeast Asia, some genera of Apocynaceae are important timber species. Many are planted as ornamentals. In Kenya, Apocynaceae species are also used for non-medicinal purposes such as food, poisons, fodder, wood, ornamentals, dye and perfume (Omino & Kokwaro 1993). Non-medicinal uses of the studied genera of Apocynaceae are compiled in **Table 2.2**.

2.1.3 Phytochemistry

a. Alkaloids

Alkaloids are broadly classified as compounds with a basic nitrogen atom at any position in the molecule, excluding amino acids, peptides, purines and antibiotics (Guggisburg & Hesse 2004; O'Connor 2010). More than 20,000 types of alkaloids are known and are mostly isolated from plants (Cordell *et al.* 2001; Verpoorte 2005). The functions of alkaloids remain poorly understood though these compounds are now believed to play an important ecological role, enabling the producing organism to defend itself and interact with its environment (O'Connor 2010). Important plants families rich in alkaloids are shown in **Table 2.3**. Of the five families, Apocynaceae is the richest with 2664 alkaloids isolated from 400 species belonging to 76 genera.

Table 2.3 Plant families rich in alkaloids (Cordell *et al.* 2001)

Family	No. of genera (species) in the family	No. of genera (species) with alkaloid	No. of alkaloids isolated
Apocynaceae	215 (2100)	76 (400)	2664
Rutaceae	150 (1500)	91 (459)	1730
Ranunculaceae	50 (2000)	22 (352)	1559
Fabaceae	657 (16,400)	174 (884)	1452
Papaveraceae	41 (660)	30 (298)	1309

Alkaloids are often classified according to their molecular skeleton (Verpoorte 2005). Indole alkaloids and isoquinoline alkaloids are the two largest groups (each with more than 4000 compounds). Other important groups are tropane alkaloids (~300 compounds), steroidal alkaloids (~450 compounds), pyridine alkaloids (~250 compounds) and pyrrolizidine alkaloids (~570 compounds).

Alkaloids can also be classified into true alkaloids, proto-alkaloids and pseudo-alkaloids based on their common molecular precursors and biological pathway (Aniszewski 2007). True alkaloids and proto-alkaloids are derived from amino acids, whereas pseudo-alkaloids are not. True alkaloids are derived from amino acids such as L-ornithine, L-lysine, L-phenylalanine, L-tyrosine, L-tryptophan and L-histidine. They all share a heterocyclic ring with nitrogen. These alkaloids are highly reactive with bioactivity even in low doses. All true alkaloids have a bitter taste and appear as a white solid, with the exception of nicotine which is brown. They form water-soluble salts. Examples include cocaine, codeine, quinine, dopamine and morphine [**Figure 2.1**].

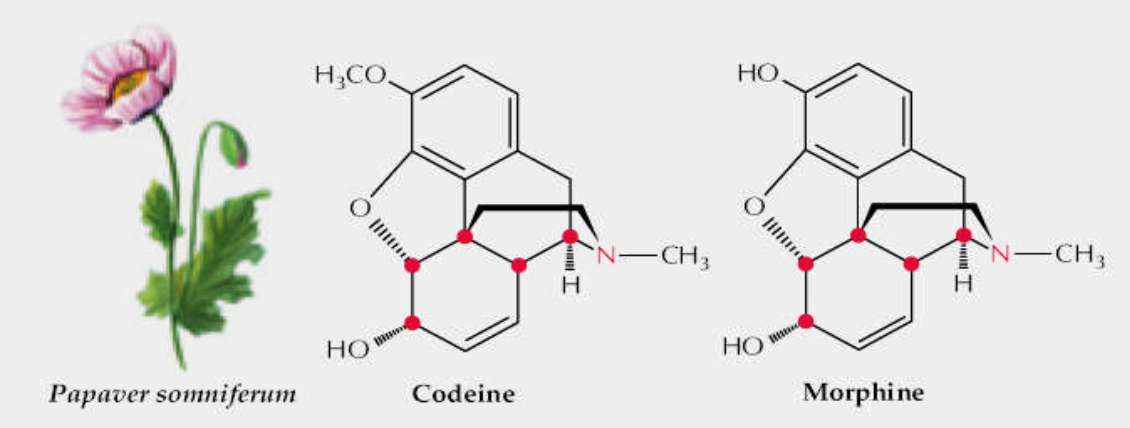


Figure 2.1 Codeine and morphine are alkaloids of opium (*Papaver somniferum*) (Croteau *et al.* 2000)

Proto-alkaloids are compounds, in which the nitrogen atom derived from an amino acid is not a part of the heterocyclic ring (Aniszewski 2007). Such kinds of alkaloid are derived from L-tyrosine and L-tryptophan. Proto-alkaloids are those with a closed ring and are structurally simple alkaloids. They form a minority of the alkaloids. Pseudo-alkaloids are compounds with the basic carbon skeletons that are not derived from amino acids Examples are coniine, capsaicin, ephedrine, solanidine and caffeine.

A number of alkaloids are of commercial pharmaceutical interest (Verpoorte 2005). They include quinine, vinblastine, vincristine, cocaine, codeine, morphine, nicotine and caffeine. Some alkaloids are widely found as drugs of abuse (e.g. cocaine and morphine) as doping compounds (e.g. strychnine and ephedrine), and as poisons (e.g. strychnine, coniine, nicotine and tetrodotoxin). The pharmaceutical drug industry has succeeded in using natural plant alkaloids to develop antimalarial agents (e.g. quinine and chloroquinine), anticancer agents (e.g. taxol, vinblastine and vincristine) and agents promoting blood circulation in the brain (e.g. vincamine) (Aniszewski 2007).

Apocynaceae species are particularly rich in L-tryptophan derived alkaloids (Aniszewski 2007). Notable examples are vinblastine and vincristine (*Catharanthus roseus*), and quinine (*Rauvolfia caffra*). Alkaloids reported in Apocynaceae species of the studied genera are shown in **Table 2.4**.

Table 2.4 Alkaloids reported in the studied genera of Apocynaceae

Genera	Alkaloid	Source
Alstonia	Alstonine, angustilobine, angustilodine, alstonerine, alstilobanine, villalstonine, macralstonine, macrocarpamine, undulifoline, scholaricine, macralstonidine, vincamajine, alstophylline, pleiocarpamine, echitamidine, echitamine	Hart *et al.* (1972); Banerji & Siddhanta (1981); Yamauchi *et al.* (1990); Massiot *et al.* (1992); Keawpradub & Houghton (1997); Macabeo *et al.* 2005; Elisabetsky & Costa-Campos (2006); Kam & Choo (2004a); Koyama *et al.* (2008)
Catharanthus	Vinblastine, vincristine	van der Heijden *et al.* (2004)
Dyera	Ochrolifuanines	Mirand *et al.* (1983)
Kopsia	Kopsine, kopsinine, kopsamine, kopsidine, afruticosine, fruticosamine, kinabalurins, prunifolines, mersinine, mersiloscine, mersifoline, mersidasine	Kam *et al.* (1993); Kam & Lim (2008); Subramanian *et al.* (2008b)

Alkaloids of Apocynaceae can be classified as simple indoles, carbolines, steroidal amines, isomeric quinindolines and quinindoles, and bisindoles (Raffauf & Flagler 1960). Indoles are the predominant alkaloids in Apocynaceae with 2431 types isolated (Cordell *et al.* 2001). Steroids are the next most important with 93 types isolated. The biochemical systematics of Apocynaceae has been studied using indole alkaloids of different skeletal types as markers (Bolzani *et al.* 1984).

The alkaloids of *Kopsia* have been reviewed (Kam & Lim 2008). The types of alkaloids found include monoterpene alkaloids (e.g. kinabalurins); simple indole alkaloids (e.g. *β*-carboline); aspidofractinine alkaloids (e.g. kopsine and kopsinine); heptacyclic alkaloids (e.g. kopsine, fruticosine and fruticosamine); kopsidasinine alkaloids (e.g. kopsidasinine); pauciflorine alkaloids (e.g. pauci-florines); kopsifoline alkaloids (e.g. kopsifolines); and bisindole alkaloids (e.g. nitaphylline).

In *Alstonia*, indole alkaloids (e.g. alstonine, angustilobine and angustilodine) have been isolated (Kam & Choo 2004a; Macabeo *et al.* 2005; Elisabetsky & Costa-Campos 2006) Bisindole alkaloids (e.g. villalstonine and macrocarpamine) have been isolated from *Alstonia augustifolia* (Kam & Choo 2006). From the root bark of *Alstonia macrophylla*, bisindole alkaloids of macralstonine, macrocarpamine, villalstonine and alstonerine have been isolated (Keawpradub & Houghton 1997).

Indole and bisindole alkaloids including those of Apocynaceae are known to have potent biological activities. This is partly due to their structural relationship with important signal compounds (neurotransmitters) such as dopamine, acetylcholine, nor-adrenaline and serotonin (Verpoorte 2005). Alkaloids are water soluble under acidic conditions and lipid soluble under neutral and basic conditions, giving them unique properties for medicinal use. The antipsychotic and anxiolytic properties of alstonine have been reviewed by Elisabetsky & Costa-Campos (2006). The antimalarial properties of indole alkaloids such as quinine, villalstonine, macro-carpamine and cryptolepine have been reviewed by Frederich *et al.* (2008). Cryptolepine has potent *in vitro* antiplasmodial activity against *Plasmodium falciparum* and possesses cytotoxic activity by inhibiting DNA synthesis in B16 melanoma cell (Lisgarten *et al.* 2002). The indole alkaloid was found to bind tightly to DNA and behaves as a typical intercalating agent. Cryptolepine accumulates in the

parasite structure and may have chloroquine-like action by inhibiting detoxification of the heme produced by the malaria parasites in red blood cells resulting in the digestion of haemoglobin.

b. Cardiac glycosides

Cardiac glycosides are a large group of compounds that show considerable structural diversity. All members of the group share a common structure of having a steroidal nucleus (Prassas & Diamandis 2008). The steroid core is double-substituted with an unsaturated lactone ring at position 17 and a sugar portion at position 3. The nature of the lactone moiety characterises the functional class of the glycosides. Depending on the lactone moiety, cardiac glycosides can be classified into cardenolides and bufadienolide [**Figure 2.2**]. Cardenolides are C_{23} steroids with a five-membered unsaturated butenolide ring (Singh & Rastogi 1970; Prassas & Diamandis 2008). Bufadienolides are C_{24} steroids with a six-membered unsaturated pyrone ring.

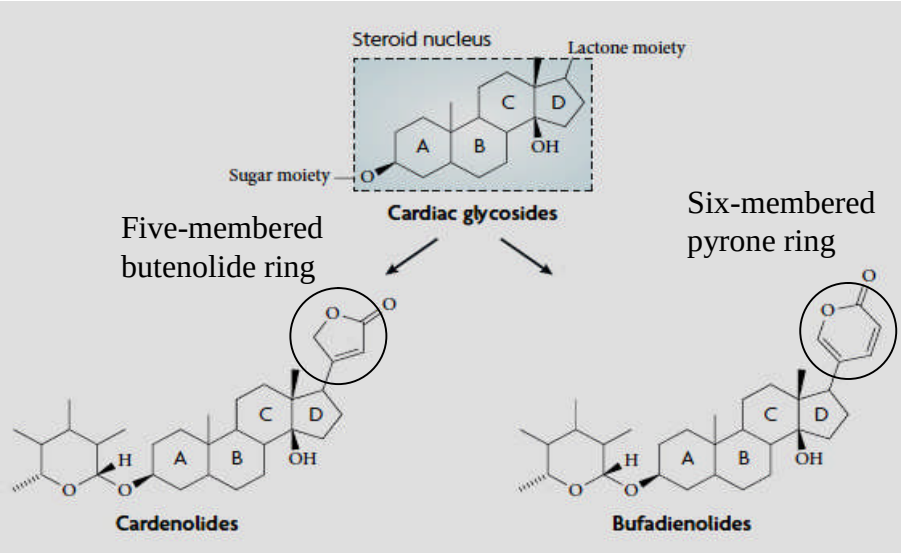


Figure 2.2 General structures of cardiac glycosides (Prassas & Diamandis 2008)

Sugars attached to cardiac glycosides include glucose, galactose, mannose, rhamnose and digitalose (Melero *et al.* 2000; Prassas & Diamandis 2008). Although sugars themselves have no activity, they do contribute to the bioactivity of the steroid. Free aglycones are absorbed and metabolised more readily than their glycosylated

counterparts, and the type of the attached sugar influences the potency of the compound. Rhamnose was shown to increase potency several times whereas mannose had no effect.

Cardenolides have important therapeutic applications (Prassas & Diamandis 2008; Newman *et al.* 2008) and there is now better understanding of their structure-activity relationship (Melero *et al.* 2000). Bufadienolides, on the other hand, are mainly of theoretical interest because of their very low therapeutic index and serious side-effects (Singh & Rastogi 1970).

Cardiac glycosides have a long history of therapeutic applications. For many years, they have been used as drugs for treating congestive heart failures and their mechanisms of action are now known (Manunta & Ferrandi 2006; Schoner & Scheiner-Bobis 2007). The mechanism of action of cardiac steroids in the heart involves inhibition of plasma membrane Na^+, K^+-ATPase, leading to increased intracellular Na^+ and Ca^{2+} ions and decreased intracellular K^+ ions (Winnicka *et al.* 2006).

Recently, considerable *in vitro*, *in vivo* and epidemiological data support their roles as drugs for the treatment of several diseases (Prassas & Diamandis 2008). Most notably, it is now established that cardiac glycosides can induce apoptosis and inhibit the growth of cancer cell lines. Studies of animal models have validated the anticancer effects of these compounds and the first cardenolide anticancer drugs are now under-going clinical trials. In contrast to the apoptotic effects of these drugs on cancer cells, low concentrations have cytoprotective effects by stimulating proliferation and inhibiting cell death in normal cells. Findings in recent years have revealed that cardiac glycosides are involved in complex cell-signal transduction mechanisms, resulting in selective control of human tumor but not normal cellular proliferation (Newman *et al.* 2008). Cardiac glycosides, therefore, represent a promising form of targeted cancer chemotherapy.

Cardenolides found in species of the studied genera of Apocynaceae are listed in **Table 2.5**. Many of these compounds have been reported to have anti-proliferative and/or apoptotic effects on cancer cells. They include calotropin, calactin, neriifolin, oleandrin and ouabain (Prassas & Diamandis 2008; Newman *et al.* 2008).

Table 2.5 Cardenolides reported in the studied genera of Apocynaceae

Genera	Cardenolide	Source
Calotropis	Calotropin, frugoside, calactin, calotoxin, calactinic acid	Singh & Rastogi (1970); Kiuchi *et al.* (1998); Lhinhatrakool & Sutthivaiyakit (2006); Seeka & Sutthivaiyakit (2010)
Cerbera	Cerberin, neriifolin, solanoside, cerberoside, cerbetin, thevetin B, tanghinin, tanghinigenin	Yamauchi (1985); Chang *et al.* (2000); Cheenpracha *et al.* (2004); Shen *et al.* (2007); Wang *et al.* (2010); Zhang *et al.* (2010)
Nerium	Oleandrin, ouabain, neriifolin, cardenolides N1-4, odoroside, nerigoside, neritaloside, neriantin, strospeside, adynerin, oleaside, neriumoside	Yamauchi *et al.* (1975); Yamauchi (1985); Zhao *et al.* (2007)

c. Iridoids

Iridoids are monoterpenes with a six-membered oxygen heterocycle annealed to a cyclopentane ring (Springob & Kutchan 2009). The hydroxy group of the oxygen containing heterocycle (dihydropyrane) is glucosylated [**Figure 2.3**]. Cleavage of the cyclopentane ring of the iridoid skeleton yields the secoiridoids, which are the building units of alkaloids.

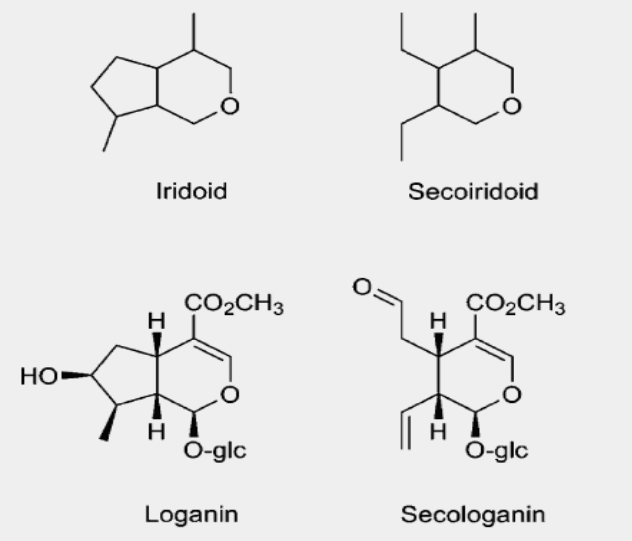


Figure 2.3 Structures of an iridoid (loganin) and secoiridoid (secologanin) (Springob & Kutchan 2009)

The four major groups of iridoids are iridoid glycosides, simple or non-glycosylated iridoids, secoiridoids, and bisiridoids (Villaseñor 2007). Simple iridoids have an iridane skeleton with methyl at C8 and another carbon bonded to C4. Glycosidic linkages are usually formed at the aglycone C1 or C11 hydroxyl. Cleavage of the 7,8-bond of the cyclopentane ring forms secoiridoids while bisiridoids result from the dimerization of both iridoids and secoiridoids.

There are several review articles which covered the new iridoids reported in the literature up to 1980 (El-Naggar & Beal 1980), from 1980–1989 (Boros & Stermitz 1991), from 1990–1993 (Al-Hazimi & Al-Khathlan 1996), and from 1994–2005 (Dinda *et al.* 2007a & 2007b).

Many iridoids have an intense bitter taste (Springob & Kutchan 2009). The plant *Gentiana lutea*, with its bitter iridoids of gentiopicroside and amarogentine, is used for the preparation of tonics against anorexia and dyspepsia.

Iridoids have a wide range of biological activities. Their neuroprotective, anticancer and anti-inflammatory properties have been reviewed with discussion on possible mechanisms of action and structure-activity relationships (Villaseñor 2007; Tundis *et al.* 2008).

In Apocynaceae, iridoids have been reported in the studied genera of *Allamanda*, *Cerbera* and *Plumeria* [**Table 2.6**]. Iridoids obtained from *Allamanda cathartica* are reported to have algicidal properties (Coppen 1983). The cytotoxic activity of plumericin isolated from the bark of *Plumeria rubra* was reported by Kardono *et al.* (1990). Bark extracts inhibited fibrosarcoma, melanoma, breast, lung and colon cancer cells. Administration of plumieride arrests spermatogenesis in male rats without noticeable side effects (Gupta *et al.* 2004). Sperm motility as well as sperm density was reduced significantly.

Table 2.6 Iridoids reported in the studied genera of Apocynaceae

Genera	Iridoid	Source
Allamanda	Allamandin, allamandicin, allamdin, isoallamandicin, allamcin, allamancin, allamcidin, allamcidin glucoside, allamanoid, isoplumericin, plumericin, plumieride, plumieride coumarate, plumieride coumarate glucoslde	Kupchan *et al.* (1974); Coppen (1983); Coppen & Cobb (1983); Abe *et al.* (1984); Yamauchi (1985); Abdel-Kader *et al.* (1997); Schmidt *et al.* (2006); Yu *et al.* (2010)
Cerbera	Theveside, theviridoside	Inouye & Nishimura (1972)
Plumeria	Isoplumericin, plumericin, plumieride, plumieride coumarate, plumieride coumarate glucoslde	Coppen & Cobb (1983); Kardono *et al.* (1990)

d. Triterpenoids

Triterpenoids constitute a large and diverse class of plant metabolites (Devarenne 2009; Gunatilaka 2012). There are over 1500 known triterpenoids and most are phytochemicals produced by plants to defend against fungal and insect pathogens. Triterpenoids contain 4- or 5-membered ring systems with 30 carbon atoms and 6 isoprene units. They have a common origin, and their structures are derived from squalene, the C30 precursor.

Plant triterpenoids belong to two main groups, i.e. tetracyclic and pentacyclic (Gunatilaka 2012). Tetracyclic triterpenoids include dammarane and tirucallane which are also considered as methylated steroids. Pentacyclic triterpenoids are by far the most diverse and are divided into five main groups, namely, friedelane, lupane, ursane, oleanane, and hopane. They are predominantly found in fruits such as apples, cranberries, figs and olives, and in medicinal herbs such as lavender, oregano, rosemary and thyme (Bishayee *et al.* 2011).

Currently, there is accumulating evidence on the broad spectrum pharmacological activities of triterpenoids coupled with a low toxicity profile and this has sparked their renewed interest with regard to human health and diseases (Bishayee *et al.* 2011). In many Asian countries, plants containing triterpenoids are used as medicines for their anti-inflammatory, analgesic, antipyretic, hepatoprotective, cardiotonic, sedative and tonic effects. An increasing number of triterpenoids has been reported to exhibit cytotoxicity against cancer cells without manifesting any toxicity in normal cells (e.g. Ovesná *et al.* 2006; Liby *et al.* 2007 & Petronelli *et al.* 2009). The potentials of using pentacyclic triterpenoids of oleanolic, ursolic and betulinic acids [**Figure 2.4**] in multi-functional drugs for potential clinical use have been discussed by Sporn *et al.* (2007).

In Apocynaceae, triterpenoids have been reported in the studied genera of *Nerium* and *Plumeria* [**Table 2.7**]. Two pentacyclic triterpenoids (cis-karenin and trans-karenin) isolated from leaves of *Nerium oleander* have shown cytotoxic properties with ED_{50} 15.0 and 7.5 µg/mL, on KB cell line, respectively (Siddiqui *et al.* 1995).

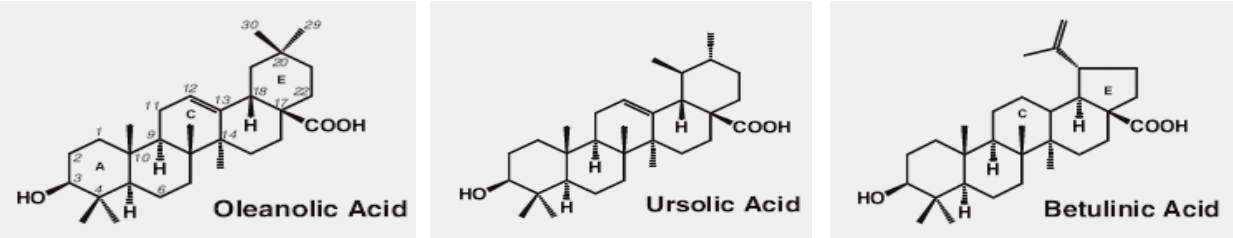


Figure 2.4 Structures of oleanolic, ursolic and betulinic acids (Sporn *et al.* 2007)

Table 2.7 Triterpenoids reported in the studied genera of Apocynaceae

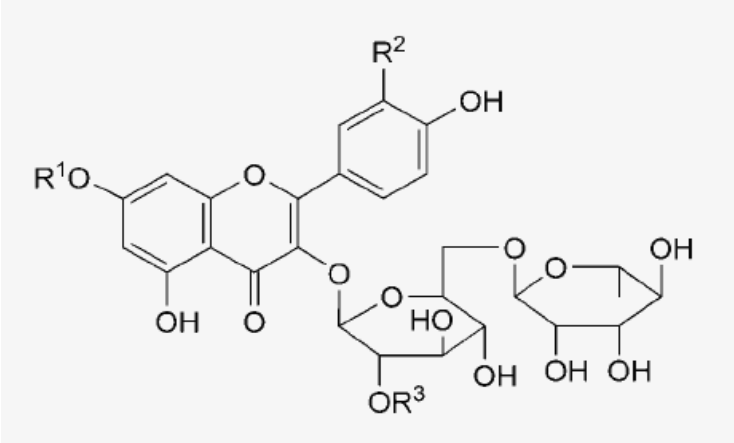
Genera	Triterpenoid	Source
Nerium	Oleanderol, betulin, betulinic acid, ursolic acid, oleanolic acid, kaneric acid, kanerin, kanerocin, 12,13-dihydroursolic acid,	Siddiqui *et al.* (1986a, 1988, 1989a & 1995); Begum *et al.* (1997)
Plumeria	Obtusol, obtusin, obtusilic acid, oleandrin, oleanonic acid, zamanic acid, ursolic acid, betulinic acid, β-amyrin, coumarobtusanoic acid, coumarobtusane	Siddiqui *et al.* (1989b, 1990, 1992, 1999 & 2004)

e. Other compounds

Other compounds reported in the studied genera of Apocynaceae are pregnanes, flavonols and caffeoylquinic acids.

Pregnanes are biological precursors to cardenolide genins. They are C_{21} steroids with methyl groups at C10 and C13, and an ethyl side-chain at C17 (Deepak *et al.* 1989). Pregnanes of calotroposide and calotropone have been isolated from *Calotropis* (Kitagawa *et al.* 1992; Shibuya *et al.* 1992; Wang *et al.* 2008b). Neridienones and other pregnanes were reported in *Nerium* (Abe & Yamauchi 1992a & 1992b; Kumar *et al.* 2005; Bai *et al.* 2007).

Flavonols are a class of flavonoids, with brief descriptions shown in **Box 2.1**. Flavonol glycosides of isorhamnetin have been isolated from aerial parts and latex of *Calotropis gigantea* (Sen *et al.* 1992; Agrawal *et al.* 2010), while flavonol glycosides of kaempferol, quercetin and isorhamnetin have been reported in seeds, stems, leaves and petals of *Catharanthus roseus* (Ferreres *et al.* 2008). From leaves of *Cerbera manghas*, flavonol glycosides of nicotiflorin, rutin, manghaslin and clitorin [**Figure 2.5**] have been isolated (Sakushima *et al.* 1976 & 1980).



	R^1	R^2	R^3
Nicotiflorin	H	H	H
Rutin	H	OH	H
Manghaslin	H	OH	Rhamnosyl
Clitorin	H	H	Rhamnosyl

Figure 2.5 Flavonol glycosides from *Cerbera manghas* (Shen *et al.* 2007)

Caffeoylquinnic acids (esters of caffeic acid with quinic acid) are phenolic acids, with brief descriptions shown in **Box 2.2**. They have been reported in stems and leaves of *Catharanthus roseus* (Ferreres *et al.* 2008).

Box 2.1 Flavonoids: chemistry and bioactivity

Flavonoids: chemistry

Flavonoids are widely distributed in plants and they constitute the largest group of phenolic compounds. They play important roles in the ecology of plants (Pietta 2000; Havsteen 2002). Their attractive colours may act as visual signals for pollinating insects, and their astringency can represent a defence system against insect pests. They are important in contributing to the flavour and colour of many fruits, vegetables and plants products such as wine and tea (Croft 1998; Bravo 1998). Occurring in plants as glycosides, they are widely recognised as antioxidants with health-promoting properties in human diets (Erlund 2004; Bravo & Mateos 2008).

Flavonoids, the largest class of polyphenols, are 15-carbon compounds with a C6-C3-C6 carbon skeleton (e.g. Croft 1998; Pietta 2000; Ross & Kasum 2002). The structure consists of two benzene rings (A and B), which are connected by an oxygen-containing pyrene ring (C) [**Figure 2.6**].

The chemical nature of flavonoids depends on their structures, substitutions, conjugations, degree of hydroxylation and degree of polymerisation (Aherne & O'Brien 2002; Ross & Kasum 2002). Flavonoids can be further divided into six major classes which include flavonols, flavones, flavanones, flavanols, anthocyanins and isoflavones.

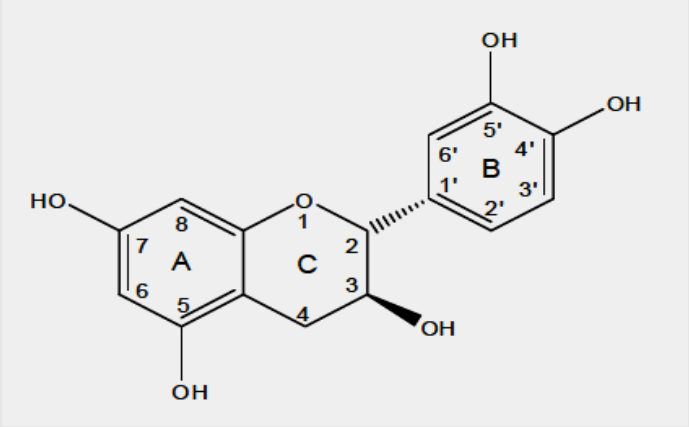


Figure 2.6 Generic structure of the flavonoid molecule (de Pascual-Teresa *et al.* 2010)

Flavonols are characterised by a non-saturated 3-C chain, a double bond between C2 and C3, and a hydroxyl group in the 3-position (Hollman & Arts 2000; Pietta *et al*. 2003). Occurring mainly as *O*-glycosides, the most common flavonols are quercetin, kaempferol, myricetin and isorhamnetin. Quercetin and its glycosides are ubiquitous in fruits and vegetables.

Flavones are characterised by a non-saturated 3-C chain, a double bond between C2 and C3, and an absence of the hydroxyl group in the 3-position (Hollman & Arts 2000; Pietta *et al*. 2003). The most common flavones are luteolin, apigenin and diosmetin.

Flavanones are derivatives of flavones after reduction of the double bond in the heterocycle (position C2-C3) (Pietta *et al*. 2003). The best known are naringenin and hesperidin. Citrus fruits are a major dietary source of flavanones.

Flavanols or catechins are characterised by a lack of the double bond at the 2-3 position, 4-oxo-group and the carbonyl group on C4 (Hollman & Arts 2000; Pietta *et al*. 2003). Major flavanols are (+)-catechin, (-)-epicatechin, epicatechin gallate and epigallocatechin-3-gallate. They are widely distributed in plants, and are dominant in wine, tea, fruits and vegetables (Yilmaz 2006; Yao *et al*. 2004a).

Anthocyanins are natural pigments responsible for the various colours of fruits and vegetables with more than 500 different types described in the literature (de Pascual-Teresa *et al.* 2010). Structurally, anthocyanins are characterised by hydroxylation in positions 3, 5 and 7 in the A ring, and 3' and 5' in the B ring (Pietta *et al*. 2003). Methoxyl groups can be attached to 3' and 5'. They lack the carbonyl group on C4. Common anthocyanins found in berries, flowers and wines are pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin (Yao *et al*. 2004a).

Isoflavones are structurally different from the other classes by having the B-ring bound to carbon 3 of the C-ring instead of carbon 2 (Erlund 2004; Yao *et al*. 2004a). They are almost exclusively found in soybean and its food products.

Flavonoids: bioactivity

Flavonoids have attracted much interest due to their ability to reduce free radical formation and to scavenge free radicals (Pietta 2000). Their capacity as antioxidants has been the subject of research and important structure-activity relationships of the antioxidant activity have been established.

The antioxidant activity of flavonoids involves two possible modes of action i.e. free radical scavenging and metal chelating (Croft 1998). Free radical scavengers are compounds that can neutralise free radicals to decrease their ability to cause oxidation. Metal chelators are compounds that can bind metals and decrease their reactivity in Fenton reaction. The scavenging activity of flavonoids increases with the number of hydroxyl groups in rings A and B (Husain *et al.* 1987). Flavonoids can act as effective chelators because of their carbonyl and hydroxyl groups (Pietta 2000). Flavonoids with multiple hydroxyl or carbonyl groups are effective chelators and thus their chelating ability is related to the number of substitutions.

Some flavonoids interact with and inhibit the functions of proteins (Pietta 2000). Flavonoids inhibit certain enzymes that produce superoxide radicals and other reactive oxygen species. Such protein-flavonoid interactions have therapeutic implications e.g. the inhibition of cyclooxygenase and lipoxygenase has anti-inflammatory effects (Ong & Khoo 1997).

Flavonols and flavones exhibit antibacterial activity (Xu & Lee 2001). Kim & Uyama (2005) reviewed the flavonoids with tyrosinase inhibition properties. They include flavanols, flavonols and flavones. Some flavonols such as kaempferol and quercetin, competitively inhibit tyrosinase activity by chelating with copper in the enzyme and then irreversibly inactivate the tyrosinase (Kubo *et al.* 2000). Flavonoids with the hydroxyl group in the B-ring are more potent inhibitors of tyrosinase (Xie *et al.* 2003). Flavonoids have been demonstrated to possess antiviral and anti-allergic properties (Kaul *et al*. 1985; Middleton 1996). Other bioactivities of flavonoids include antiproliferative, anti-inflammatory, anti-carcinogenic, hepato- and gastro-protective, anti-diabetic, and anti-neoplastic (Yao *et al.* 2004a; Ren *et al.* 2003).

Box 2.2 Phenolic acids: chemistry and bioactivity

Phenolic acids: chemistry

Phenolic acids are aromatic phenols with one carboxylic acid functionality and they consist of two groups, namely, hydroxybenzoic and hydroxycinnamic acids (e.g. Robbins 2003; Fleuriet & Macheix 2003; Mattila & Hellstrom 2007; Lafay & Gil-Izquierdo 2008; Veliŏglu 2009).

Hydroxybenzoic acids (HBA), with a C6-C1 structure, are derived from benzoic acid. Major HBA are *p*-hydroxybenzoic, protocatechuic, vanillic, syringic and gallic acids, and are mainly present in plant food as glucosides (Fleuriet & Macheix 2003; Shahidi & Naczk 2004; Mattila & Hellstrom 2007). As the content of HBA is generally low in most food except for some berries and vegetables (Lafay & Gil-Izquierdo 2008), they have not been extensively studied (Manach *et al.* 2004).

Hydroxycinnamic acids (HCA) have a C6-C3 structure. HCA commonly found in fruits, vegetables, grains and coffee are *p*-coumaric acid, caffeic acid, ferulic acid and sinapic acid (Rechner 2003; Fleuriet & Macheix 2003). Caffeic acid is most common in fruits while ferulic acid is abundant in cereal grains (Lafay & Gil-Izquierdo 2008). HCA frequently occur in foods as simple esters with quinic acid or glucose (Mattila & Hellstrom 2007).

The most common HCA derivatives are esters of caffeic and quinic acid or caffeoylquinic acids, predominantly chlorogenic acid (5-caffeoylquinic acid) (Rechner 2003; Lafay & Gil-Izquierdo 2008). It is the main constituent in coffee, apple juice, artichoke, egg plant and peach. Neochlorogenic acid (3-caffeoylquinic acid) is a major component of fruits e.g. cherry, plum, elderberry and apricot. Crytochlorogenic acid (4-caffeoylquinic acid) is a minor constituent of some fruits and vegetables.

Phenolic acids: bioactivity

In fruits and vegetables, HCA act as free radical scavengers and show strong antioxidant properties (Rice-Evans *et al.* 1996; Chen & Ho 1997; Natella *et al*. 1999; Kroon & Williamson 1999). Chen *et al.* (2004) have reported that the free radical scavenging activity of chlorogenic acid is higher than that of caffeic acid and α-tocopherol. Generally, the antioxidant activity of phenolic acids e.g. ferulic and caffeic acids is less than that of flavonoids e.g. quercetin and catechin (Rice-Evans *et al*. 1997). The antioxidant activity of phenolic acids and their esters depends on the number and position of hydroxyl groups in the molecule (Shahidi & Naczk 2004; Andjelkovic *et al*. 2006). HCA have been found to be more effective than their benzoic acid counterparts.

Chlorogenic and gallic acids have been reported to possess antimicrobial properties against human pathogens (Friedman & Jurgens 2000). Phenolic acids such as cinnamic acid, 3-coumaric acid, caffeic acid and ferulic acid showed weak antibacterial activity against Gram-negative bacteria (Puupponen-Pimia *et al.* 2001). Kampa *et al.* (2004) reported the antiproliferative and apoptotic effects of phenolic acids on T47D human breast cancer cells.

Other potential beneficial effects of phenolic acids demonstrated in laboratory studies include anti-inflammatory, anti-skin aging, anti-spasmodic and anti-glycemic activity (Harrison *et al.* 2008). The absorption, metabolism and bioactivities of chlorogenic acids have been reviewed by Morishita & Ohnishi (2001).

2.1.4 Bioactivities

a. Anticancer properties

Cancer is a generic term for a large group of diseases that can affect any part of the body (WHO 2011). One defining feature of cancer is the rapid creation of abnormal cells that grow beyond their usual boundaries, and which can then invade adjoining parts of the body and spread to other organs. Generally, there are four types of cancers (Ravindran *et al.* 2009). They are carcinoma, sarcoma, leukemia and lymphoma. The ability of tumor cells to expand in number is determined not only by the rate of cell proliferation but also by the rate of cell death. The two major pathways of cell death are apoptosis and necrosis (Braun & Albert 2005; Elmore 2007). Apoptosis or programmed cell death involves a series of biochemical events leading to characteristic cell morphology and death. It is characterised by chromatin condensation, cell shrinkage and the maintenance of plasma membrane integrity. Necrosis is caused by external factors such as infection, toxins or trauma. It is characterised by mitochondrial swelling, nuclear flocculation and disruption of the plasma membrane. Apoptosis is a major source of cell death, thus agents that trigger apoptotic cell death could be promising candidates as therapeutic for cancer. The search for natural phytochemicals as anticancer drugs continues intensively.

There are two main pathways to apoptotic cell death in mammalian cells [**Figure 2.7**] (Hengartner 2000; Zimmermann & Green 2001). The end-result of either pathway is cysteine-aspartic acid protease (caspase) activation and the cleavage of specific cellular substrates, resulting in the morphologic and biochemical changes associated with apoptosis. Caspases and their roles in regulating apoptosis in animal cells are shown in **Figure 2.8**.

The first is the extrinsic or death-receptor pathway. It involves the interaction of death receptors such as CD95. The binding of a ligand CD95L to CD95 induces receptor clustering and formation of a death-inducing signaling complex. This pathway is mediated by recruitment and activation of caspase-8, an initiator caspase, in the death-inducing signalling complex (DISC) followed by direct cleavage of downstream effector caspases. Thus, caspase 8 is an important proapoptotic protein

for the extrinsic apoptotic pathway. The second is the intrinsic or mitochondrial pathway. Members of the Bcl-2 family regulate the mitochondrial pathway. Caspase-9 is an important proapoptotic protein and activation of caspase-3, eventually leads to proteolysis and apoptotic cell death.

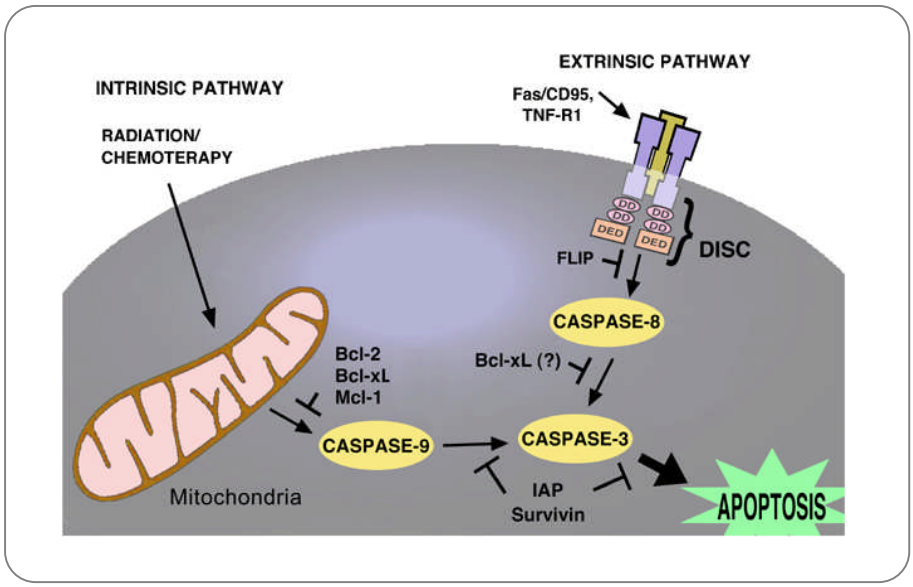
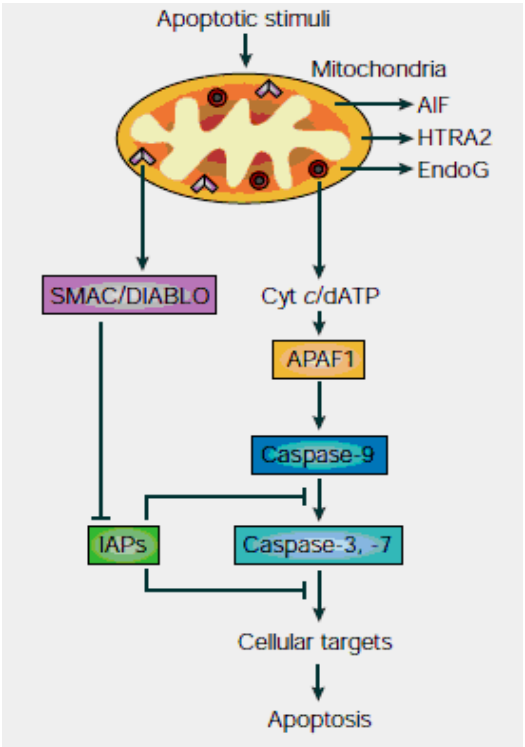


Figure 2.7 Two major apoptotic pathways in mammalian cells (Pai *et al*. 2006)



The mechanism of apoptosis in animal cells depends on caspases, a family of proteases that have a cysteine at their active site and cleave their target proteins at specific aspartic acids (Alberts *et al.* 2002). Caspases are synthesised in the cell as inactive precursors (pro-caspases), which are activated by cleavage at aspartic acids by other caspases. Once activated, caspases cleave, and thereby activate, other pro-caspases, resulting in an amplifying cascade. Activated caspases then cleave other key proteins in the cell e.g. nuclear lamina and DNAse. The dead cell is then digested by other cells. In mammals, caspase-9 is an initiator, whereas caspase-3 and caspase-7 are effectors (Riedl & Shi 2004). IAPs can suppress apoptosis by negatively regulating the caspases and SMAC/DIABLO can remove IAP-mediated negative regulation of caspases.

Abbreviations: DNAse = deoxyribonuclease; SMAC = second mitochondria-derived activator of caspases; DIABLO = direct IAP-binding protein with low pI; IAPs = inhibitors of apoptosis proteins; AIF = apoptosis-inducing factor; A2; EndoG = endonuclease G; HTRA2 = high-temperature requirement protein Cyt c = cytochrome c; APAF1 = apoptotic protease activating factor-1.

Figure 2.8 Schematic diagram of the roles of caspases in regulating apoptosis

Apoptosis or programmed cell death is an ongoing natural process in living systems and can progress very quickly, on a scale of minutes to hours (Ray & Corcoran 2009). Cells and apoptotic bodies are removed quite rapidly after the onset of apoptosis. They are engulfed and digested by phagocytes and by neighbouring cells. Thus, apoptotic cells die a clean death.

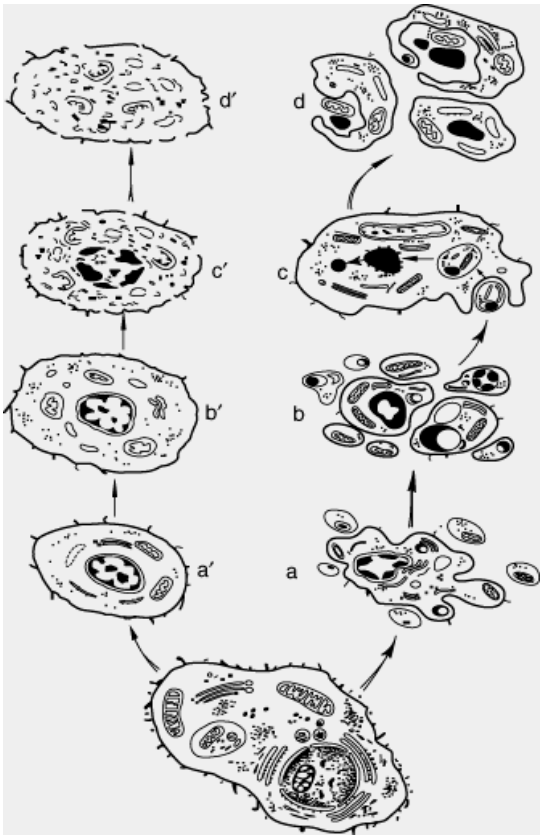
The entire apoptotic process can be divided into several steps (Häcker 2000; Martelli *et al.* 2001; Ray & Corcoran 2009). They are cell shrinkage; chromatin or nuclear condensation; formation of apoptotic bodies; and phagocytosis of apoptotic bodies [**Figure 2.9**]. In necrosis, cell death involves cell swelling followed by disintegration of organelles and cell lysis.

Necrosis
(left pathway)

a' Cells swell

b' Disintegration of organelles

c' & **d'** Cell lysis



Apoptosis
(right pathway)

a Cell shrinkage and chromatin or nuclear condensation

b Formation of apoptotic bodies

c & **d** Phagocytosis of apoptotic bodies

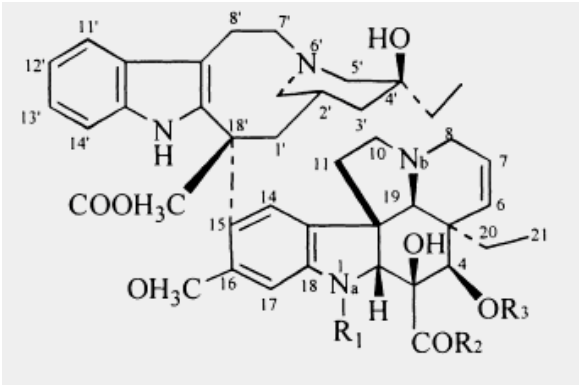
Figure 2.9 Morphological characteristics of apoptosis and necrosis (Ray & Corcoran 2009)

Species of Apocynaceae have been reported to possess anticancer properties (Wiart 2006; Patel *et al.* 2010). Genera of the studied Apocynaceae species with anti-proliferative activity have been compiled from the literature [**Table 2.8**]. Species having cytotoxic activity are those of the genera *Allamanda*, *Alstonia*, *Calotropis*, *Catharanthus*, *Cerbera*, *Kopsia*, *Nerium* and *Plumeria*.

Table 2.8 Apocynaceae species of the studied genera with antiproliferative activity

Species	Plant part	Source
Allamanda schottii	Root	Schmidt *et al.* (2006)
Alstonia macrophylla	Root bark	Keawpradub *et al.* (1999a)
Alstonia scholaris	Stem bark	Jagetia & Baliga (2006); Arulmozhi *et al.* (2007)
Calotropis gigantea	Leaf	Lhinhatrakool & Sutthivaiyakit (2006)
	Root	Wang *et al.* (2008a & 2008b)
Caloptropis procera	Root	Bhagat *et al.* (2010)
Catharanthus roseus	Leaf	El-Sayed & Cordell (1981);
Cerbera manghas	Root Leaf	Chang *et al.* (2000) Masuda *et al.* (2002)
Cerbera odollam	Leaf Seed	Nurhanan *et al.* (2008) Laphookhieo *et al.* (2004)
Kopsia singapurensis	Leaf & stem bark	Subramanium *et al.* (2008a)
Nerium oleander	Leaf	Siddiqui *et al.* (1995); Zhao *et al.* (2006); Fu *et al.* (2005)
	Stem & twig	Zhao *et al.* (2007)
Plumeria acuminata	Bark	Kardono *et al.* (1990); Atjanasuppat *et al.* (2009)

Catharanthus alkaloids are well-known for their anticancer properties. Indole alkaloids (vinblastine, vincristine and vindesine) from *Catharanthus roseus* [**Figure 2.10**] are used to treat the acute leukemias, lymphomas, and breast and lung cancer (Kuo & King 2001). Vinorelbine has recently been introduced for advanced breast cancer and for advanced non-small cell lung cancer.



Vincristine
R_1=CHO, R_2=OCH_3, R_3=CH_3CO
Vinblastine
R_1=CH_3, R_2=OCH_3, R_3=CH_3CO
Vindesine
R_1=CH_3, R_2=NH_2, R_3=H

Figure 2.10 Anticancer drugs of vincristine, vinblastine, and vindesine (Kuo & King 2001)

Catharanthus alkaloids block mitosis with metaphase arrest (Kuo & King 2001; Aronson 2008). Their antitumor activity is based on their high binding affinity to tubulin during the cell cycle, preventing polymerization of tubulin into microtubules, thus blocking spindle formation and arresting cells in metaphase. When cells are incubated with vinblastine, dissolution of microtubules occurs. Progressively, cells undergo changes characteristic of apoptosis (Smets 1994).

In addition to the anticancer properties of *Catharanthus* alkaloids, there is new knowledge on the anticancer mechanisms of other compounds isolated from species of Apocynaceae.

UNBS1450, a steroid cardiac glycoside derived from 2”-oxovoruscharin of *Calotropis procera* [**Figure 2.11**] was shown to be a potent sodium pump inhibitor, having anti-proliferative and cell death-inducing activities (Juncker *et al.* 2009). This anti-cancer potential of UNBS1450 is by binding to the sodium pump at the cellular membrane, by inducing cell death, by repressing NF-kB activation and by down-regulating c-Myc in cancer cells.

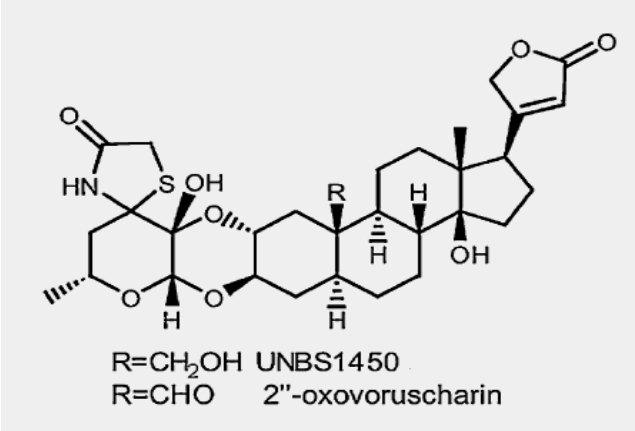
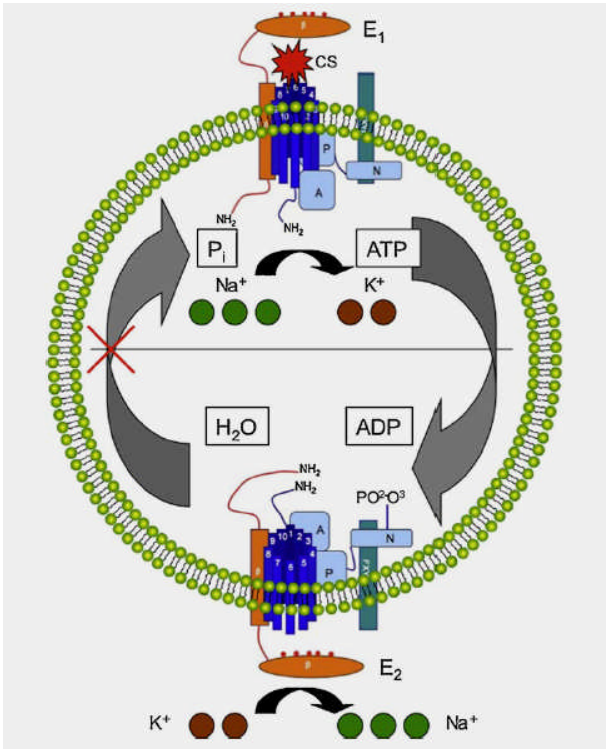


Figure 2.11 Cardiac glycoside UNBS1450 derived from 2”-oxovoruscharin of *Calotropis procera* (Juncker *et al.* 2009)

The cytotoxic effects of monoglycosidic cardenolides isolated from *Streptocaulon tomentosum* roots and *Nerium oleander* leaves involved inhibition of the plasma membrane bound Na^+/K^+-ATPase (Rashan *et al.* 2011). A schematic diagram of Na^+/K^+-ATPase inhibition by cardenolides is shown in **Figure 2.12**.



There are two different states (E_1 and E_2) for Na^+/K^+-ATPase.

In E_1, enzyme phosphorylation enables intracellular binding of three Na^+ in exchange to two K^+, thus leading to E_2. The three Na^+ are released in the extracellular fluid whereas two new K^+ are imported. Through hydrolysis, the enzymatic complex returns to E_1 with P_i release.

Cardiotonic steroids e.g. cardenolides become tightly bound to extracellular loops of the enzymes. This is blocked in E_2 and no further ion exchange is possible.

Figure 2.12 Schematic diagram of Na^+/K^+-ATPase inhibition by cardenolides (Juncker *et al.* 2009)

Recently, Juncker *et al.* (2011) reported that UNBS1450 induced apoptotic cell death in human leukemia cells by inhibiting NF-kB transactivation which triggers apoptosis by cleavage of pro-caspases 8, 9 and 3 or 7, by decreasing expression of anti-apoptotic Mcl-1 and by recruiting pro-apoptotic Bak and Bax protein.

b. Antimalarial properties

Malaria is caused by *Plasmodium* parasites transmitted by female *Anopheles* mosquitoes. It is still the most destructive and dangerous parasitic infection in many tropical and subtropical countries (Bastita *et al.* 2009; Bero *et al.* 2009). The disease is commonly associated with poverty, but is also a cause of poverty and a major hindrance to economic development. Malaria ranks third among the major infectious diseases in causing deaths and accounts for 2.6% of the total disease burden of the world (Vangapandu *et al.* 2007). The control of malaria requires a multi-faceted strategy of suppression of vector mosquitoes, protection of individuals from contact with mosquitoes, and treatment of those infected to prevent further transmission (Egan 2001).

Of the four species of parasites, *Plasmodium falciparum* is responsible for the most severe cases. In many parts of the world, the parasites have developed resistance to antimalarials such as chloroquine and derivatives, the most widely used treatment for malaria, and so there is an urgent need to discover new compounds with an original mode of action (Bastita *et al.* 2009; Bero *et al.* 2009). The mechanism of resistance to chloroquine and other antimalarial drugs is still unknown and is the subject of much debate (Egan 2001). There is a need to find out the parasite genes responsible for the resistance, physiological changes in the parasite associated with resistance, and structural changes in the drugs circumventing resistance.

Species of Apocynaceae, notably those of *Alstonia,* are also known to have antimalarial properties (Wright *et al.* 1993; Keawpradub *et al.* 1999b). In Africa, species of *Alstonia*, *Funtumia*, *Picralima* and *Rauvolfia* have been reported to show strong antiplasmodial activity (François *et al.* 1996; Okokon *et al.* 2007; Guédé *et al.* 2009, 2010). A review of indole alkaloids with antimalarial activity by Frederich *et al.* (2008) included villalstonine and macrocarpamine from *Alstonia*, cryptolepine from

Cryptolepis, and voacamine from *Tabernaemontana* [**Figure 2.13**]. Their IC_{50} values ranged from 0.114–0.411 µM with selectivity index of 9–47.

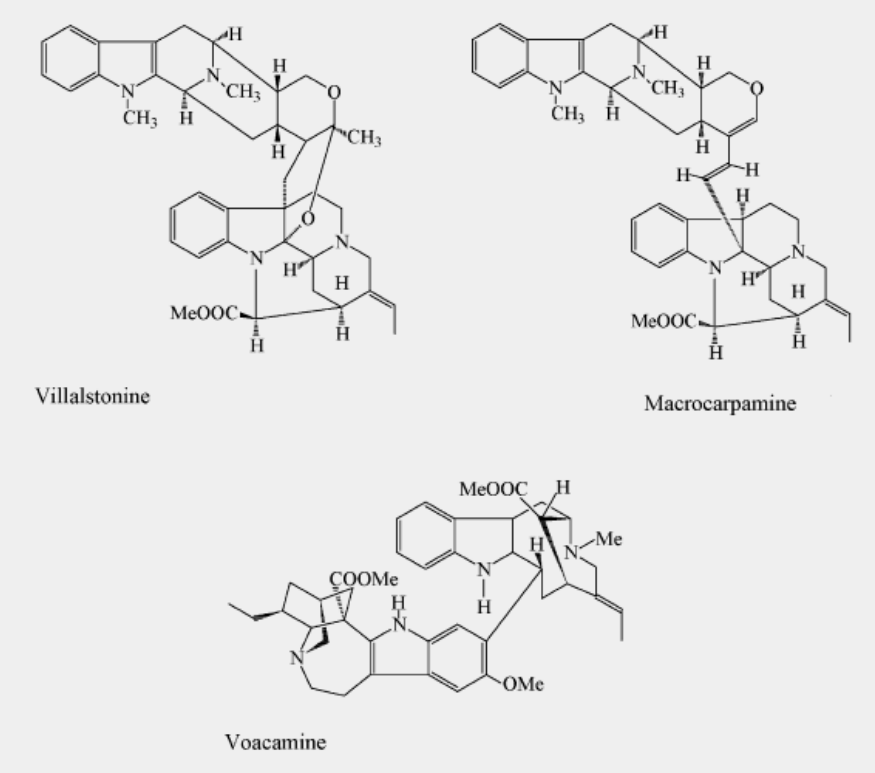


Figure 2.13 Indole alkaloids with antiplasmodial activity (Frederich *et al.* 2008)

Species of Apocynaceae, notably those of *Alstonia,* are also known to have anti-malarial properties. Apocynaceae species of the studied genera with anti-plasmodial activity have been compiled from the literature [**Table 2.9**]. Species having anti-plasmodial activity include those of the genera *Alstonia*, *Calotropis* and *Kopsia*.

The mechanisms of action of drugs such as chloroquine, quinine, mefloquine and artemisinin [**Figure 2.14**] have been reviewed by Christensen & Kharazmi (2001). The main mechanism is the inhibition of haem polymerisation of the haemoglobin. As the haem is toxic to the parasites because of its strong reducing power, the parasites convert the haem to the polymeric haemazoin, also known as β-haematin or malaria pigment. Inhibition of haem polymerisation which is lethal to the parasites is believed to be the mechanism of action of the quinoline antimalarials such as chloroquine, quinine and mefloquine. Artemisinin appears to be able to inhibit this polymerisation by alkylating the haem which inhibits haemazoin formation.

Table 2.9 Apocynaceae species of the studied genera with antiplasmodial activity

Species	Plant part	Source
Alstonia angustifolia	Root	Wright *et al.* (1992)
Alstonia boonei	Stem bark	Bello *et al.* (2009); Iyiola *et al.* (2011)
Alstonia coriacea	Bark	Wright *et al.* (1993)
Alstonia macrophylla	Stem & root bark	Keawpradub *et al.* (1999b)
Alstonia scholaris	Bark	Gandhi & Vinayak (1990)
Calotropis procera	All parts	Sharma & Sharma (2000)
Kopsia dasyrachis	Leaf	Khozirah *et al.* (2011)

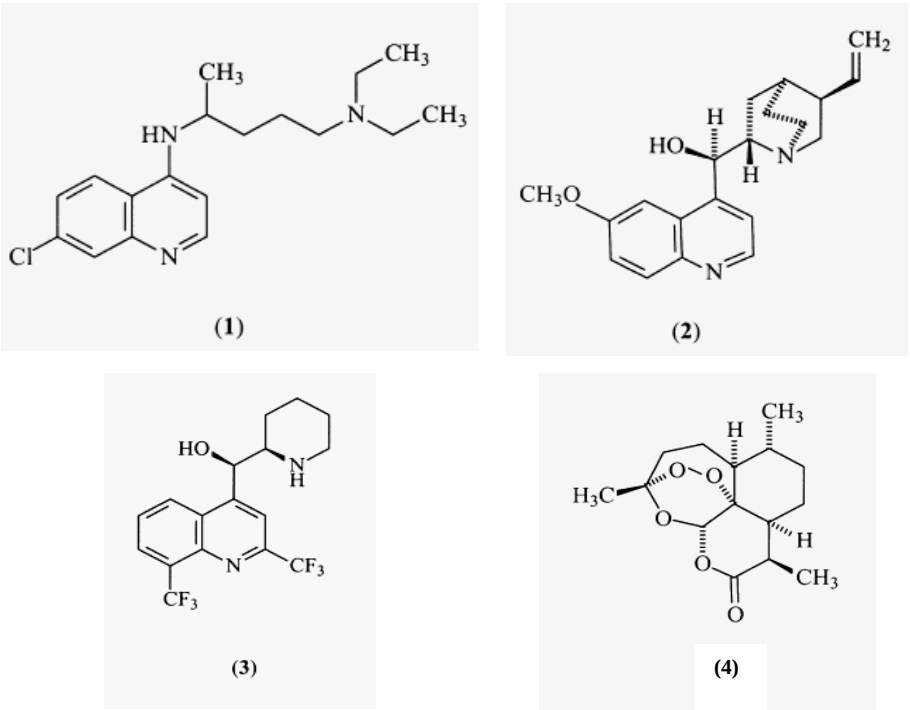


Figure 2.14 Antimalarial drugs of chloroquine (1), quinine (2), mefloquine (3) and artemisinin (4) (Christensen & Kharazmi 2001)

Cryptolepine [**Figure 2.15**] is an indole alkaloid isolated from the roots of *Cryptolepis sanguinolenta* (Lisgarten *et al.* 2002). It prevents formation of *β*-haematin suggesting that its antiplasmodial effect depends on a quinine-like mode of action, although it is possible that its effects on DNA synthesis and inhibition of topoisomerase II may also contribute (Wright *et al.* 2001). Fluorescence microscopy showed that cryptolepine accumulates inside the parasite structures that may correspond to the parasite nucleus (Arzel *et al.* 2001).

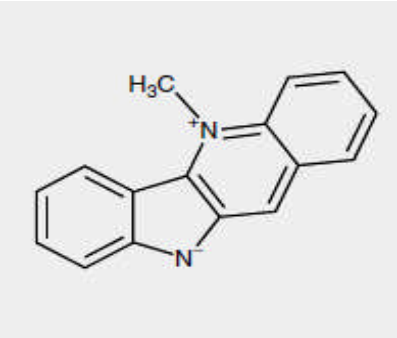


Figure 2.15 Cryptolepine from *Cryptolepis sanguinolenta* (Ghisalberti 2008)

Cryptolepine is also known to have antiproliferative activity. Its induced cell death is the result of necrosis and not apoptosis (Go 2003). The antimalarial activity of cryptolepine has not been actively pursued because of its cytotoxic effects,. Neocryptolepine, a minor alkaloid isolated from *Cryptolepis sanguinolenta*, also demonstrates antimalarial activity, but to a lesser degree when compared to cryptolepine. However, it is less cytotoxic.

2.2 SPECIES STUDIED

2.2.1 *Allamanda cathartica* L.

Synonym: *Allamanda hendersonii*
Common name: Common allamanda

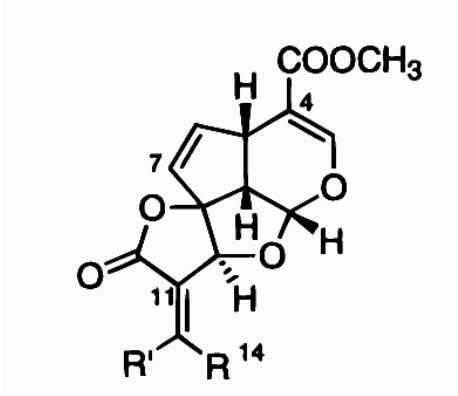
a. Botany and uses

Plants of *Allamanda cathartica* are robust climbing shrubs growing up to 6 m tall (Li *et al.* 1995; Rahayu 2001). Leaves are opposite or in whorls of 3–5 leaves, elliptical to obovate. Flowers are yellow and trumpet-shaped with corolla tube 5–7 cm. Flowers are similar in size as leaves. Fruits are capsules with spines and seeds are compressed and winged. Shrubs of *Allamanda cathartica* with their beautiful yellow flowers are popular ornamentals

Leaves of *Allamanda cathartica* are used as a purgative or emetic in Southeast Asia (Rahayu 2001). Leaves are also used as an antidote, and for relieving coughs and headaches. Plants of *Allamanda cathartica* are used in traditional medicine for treating malaria and jaundice (Nayak *et al.* 2006). The flowers act as a laxative. The species is also used for treatment of liver tumours (Schmidt *et al.* 2006).

b. Phytochemistry and bioactivities

Previous work on *Allamanda cathartica* included the isolation of iridoid lactones of allamandin, allamandicin and allamdin (Kupchan *et al.* 1974), iridoid glycosides of plumieride coumarate and plumieride coumarate glucoside (Coppen 1983), and iridoid lactones of isoplumericin and plumericin [**Figure 2.16**] (Abdel-Kader *et al.* 1997).



1 = isoplumericin (R = H, R' = CH_3),
2 = plumericin (R = CH_3, R' = H)

Figure 2.16 Iridoid lactones of *Allamanda cathartica* (Abdel-Kader *et al.* 1997)

Isolated from leaves of *Allamanda cathartica*, allamandin has antileukemic properties, and isoplumericin and plumericin are weakly cytotoxic (Abdel-Kader *et al.* 1997). Aqueous leaf extracts of *Allamanda cathartica* promote wound healing activity in rats (Nayak *et al.* 2006). Aqueous leaf extracts of *Allamanda cathartica* screened for gastrointestinal effects showed that the extracts induced dose-related purgation in mice by increasing the number of wet faeces and the propulsive movement of intestinal contents (Akah & Offiah 1992).

2.2.2 *Alstonia angustiloba* Miq.

Synonyms: *Alstonia calophylla*
Vernacular name: Pulai

a. Botany and uses

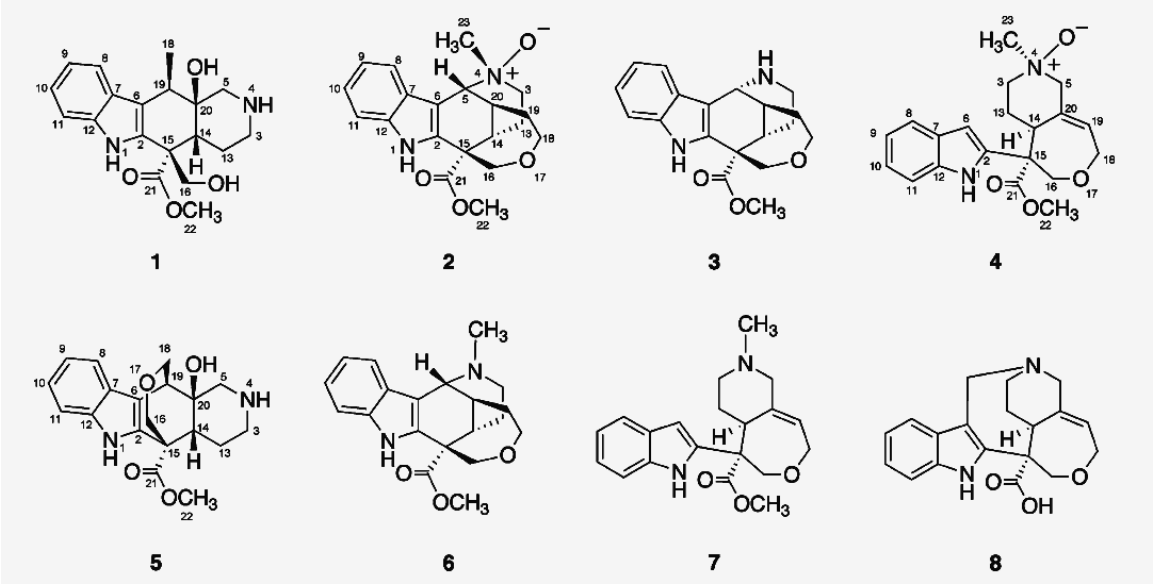
Alstonia angustiloba is a medium-sized to large tree that grows up to 45 m tall (Teo 2001). It has a tall, straight and fluted bole up to 100 cm in diameter. When slashed, the bole produces copious white latex. The bark is brown or grey to whitish, rough, fissured and peeling off in rectangular flakes. Leaves are in whorls of 4–7 leaves which are elliptical to obovate. The species occurs on a wide variety of soils in rain forests from the lowlands up to 200 m altitude.

In Malaysia, the leaves of *Alstonia angustiloba* are externally applied to treat fever and headache (Teo 2001; Lin 2005). The latex is used to heal boils and abscesses. An extract of the pounded bark is an ingredient of febrifuges and vermifuges. In Thailand, the latex is used to soothe toothache. Stems, leaves and latex of *Alstonia angustiloba* have been used for gynaecological problems and skin sores in Indonesia (Mulyoutami *et al.* 2009).

b. Phytochemistry and bioactivities

Five new alkaloids, alstilobanines A–E and three known alkaloids were isolated from methanol leaf extract of *Alstonia angustiloba* [**Figure 2.17**] (Koyama *et al.* 2008).

Using the vasodilation assay, the alstilobanines showed moderate vasorelaxant activity against phenylephrine-induced contraction of isolated rat aorta.



1-5 = Alstilobanines A-E, **6** = undulifoline, **7** = 6,7-seco-angustilobine B, **8** = alstonamic acid

Figure 2.17 Indole alkaloids of *Alstonia angustiloba* (Koyama *et al.* 2008)

2.2.3 ***Calotropis gigantea*** (L.) Aiton

Synonym: *Asclepias gigantea*

Common names: Crown flower; giant milkweed

a. Botany and uses

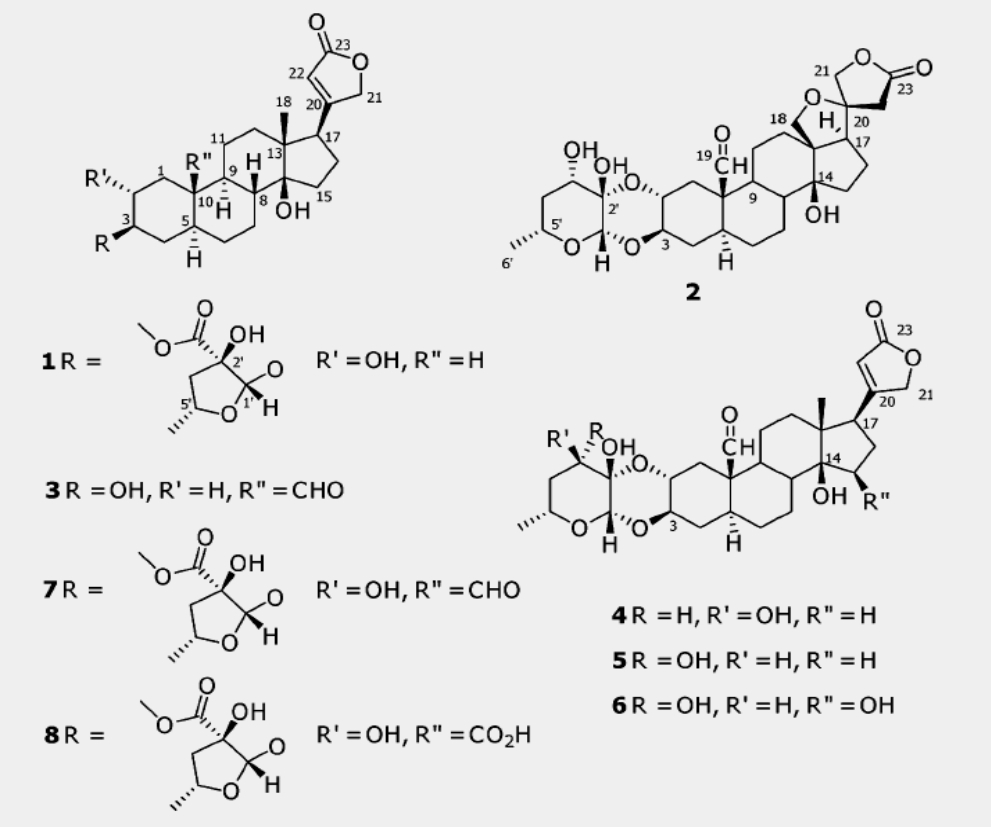
Calotropis gigantea is a large shrub or small tree of 3–4 m tall (Kiew 2001; Llamas 2003). Leaves are obovate, woolly with light coloured veins and sub-sessile. Flowers are pale lilac and cream coloured towards the tips. The elaborate crown structure of the flowers is due to the highly modified stigma and stamens. The species is fast growing and flowers throughout the year. It occurs naturally from India and Sri Lanka to Thailand and southern China. It is planted as a medicinal plant in Malesia. The species can be distinguished from *Calotropis procera* which has white petals with dark purple tips.

In Indonesia, the roots of *Calotropis gigantea* are used as an antidote for snake-bite and scabies (Kitagawa *et al.* 1992). In India, plants of *Calotropis gigantea* have been traditionally used for the treating diseases such as leprosy, ulcers, tumors and piles (Agrawal *et al.* 2010). The roots and leaves are used for the treatment of abdominal tumours, syphilis, leprosy, skin diseases, piles, wounds, rheumatism, insect-bites, ulceration and elephantiasis. Plants of *Calotropis gigantea* are used as analgesia for treating earache, toothache and headache, sprain, stiff joints and pain (Pathak & Argal 2007). Leaves of *Calotropis gigantea* are used to treat skin and liver diseases, leprosy, dysentery, worms, ulcers, tumours and earaches (Rajakaruna *et al.* 2002). Its latex has been reported to have wound healing properties (Nalwaya *et al.* 2009).

b. Phytochemistry and bioactivities

The chemical constituents of *Calotropis gigantea* have been extensively investigated, leading to the isolation of many cardenolides [**Figure 2.18**] (Singh & Rastogi 1972; Kiuchi *et al.* 1998; Mueen Ahmed *et al.* 2005; Lhinhatrakool & Sutthivaiyakit 2006; Wang *et al.* 2008a; Seeka & Sutthivaiyakit 2010), flavonoids (Sen *et al.* 1992), terpenes (Anjaneyulu & Row 1968; Thakur *et al.* 1984) and pregnanes (Kitagawa *et al.* 1992; Shibuya *et al.* 1992; Wang *et al.* 2008b).

Recently, isorhamnetin-3-O-α-rhamnopyranosyl(1‴→6‴)-β-glucopyranoside (flavonol glycoside) has been isolated from the latex of *Calotropis gigantea* (Agrawal *et al.* 2010). Pregnanes (calotroposides A–G) are new oxypregnane-oligoglycosides isolated from the methanol root extract of *Calotropis gigantea* (Kitagawa *et al.* 1992; Shibuya *et al.* 1992). Bioassay-guided fractionation of the ethanol extract from roots of *Calotropis gigantea* resulted in the isolation of coroglaucigenin (a cardenolide aglycone) and frugoside (its glycoside) (Wang *et al.* 2008a). A new pregnanone, named as calotropone, was isolated from the ethanol root extract of *Calotropis gigantea* together with glycoside gofruside, a known cardiac glycoside (Wang *et al.* 2008b).



1 = 19-nor-10-hydrocalactinic acid methyl ester, **2** = 18,20-epoxycalotropin, **3** = uzarigenin, **4** = calactin, **5** = calotropin, **6** = 15*β*-hydroxycalotropin, **7** = calactinic acid methyl ester, **8** = 19-carboxylcalactinic acid methyl ester, **9** = taraxasteryl acetate

Figure 2.18 Cardenolides of *Calotropis gigantea* (Lhinhatrakool & Sutthivaiyakit 2006)

Three cardenolide glycosides (calotropin, frugoside and 4'-*O*-*β*-D-glucopyranosyl frugoside) isolated from root extract of *Calotropis gigantea* were found to have cytotoxic activity against cancer lines of human origin but not against cancer lines of mouse origin (Kiuchi *et al.* 1998). Against KB cells, calotropin showed strong cytotoxicity while frugoside and 4'-*O*-*β*-D-glucoside showed moderate cytotoxicity. The ethanol extract from roots of *Calotropis gigantea* showed cytotoxic activity towards human chronic myelogenous leukemia (K562) and human gastric cancer (SGC-7901) cell lines *in vitro* using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay with IC_{50} values of 9.7 and 6.7 μg/mL, respectively (Wang *et al.* 2008a & 2008b). Coroglaucigenin and frugoside isolated from the extract exhibited significant cytotoxic activity against K-562 and SGC-7901 cell lines with IC_{50} values of 3.4–14.1 μg/mL. It was postulated that the presence of a deoxysugar at C3 was crucial for the cytotoxic activity. Seeka & Sutthivaiyakit (2010)

reported calotoxin and frugoside isolated from leaves of *Calotropis gigantea* had potent inhibitory activity against KB, MCF-7 and NCI-H187 cells.

The ethanol extract of *Calotropis gigantea* flowers has been reported to have an analgesic effect on chemically and thermally treated mice using the acetic acid induced writhing test and hot plate method, respectively (Pathak & Argal 2007). There was a significant decrease in the number of writhings and paw licking time. The analgesic effect, observed 30 min after dose administration, reached its maximum after 90 min.

Topical application of root bark extract of *Calotropis gigantea* formulated in ointment increased the percentage of wound contraction in rats and wound healing was completed by the third week (Deshmukh *et al.* 2009). The accelerated wound healing in rats supports its traditional use.

The ethanol root bark extract of *Calotropis gigantea* showed strong hepato-protective activity in Wistar albino rats with induced hepatic injury (Deshmukh *et al.* 2008). An oral dose of 200 and 400 mg/kg normalised the levels of aspartate and alanine amino transferase, alkaline phosphatase, total bilirubin and lactate dehydrogenase in the rats. The effect was comparable to silymarin, a known hepatoprotective drug.

The ethanol leaf extract of *Calotropis gigantea* has been reported to have antiviral activity against herpes simplex type-1 and vesicular stomatitis viruses, both with MIC of 0.01 mg/mL (Ali *et al.* 1996).

The anti-diarrhoeal activity of water-ethanol extract of aerial parts of *Calotropis gigantea* against rats with diarrhoea induced by castor oil was studied by Chitme *et al.* (2004). Results showed that the extract had remarkable anti-diarrhoeal effects on the treated rats, which included significant reduction in the number of stools, and in the weight and volume of intestinal content, as well as modest reduction in intestinal transit.

The latex from *Calotropis gigantea* was reported to have vasodilatory effect on the green frog (*Rana hexadactyla*) by Sheelaa *et al.* (2010). The crude extract diluted with distilled water to 1:10 and 1:100 concentrations produced 55% and 66% cardiac output, respectively. Results showed that the latex had vasodilatory effect at fixed dose concentration. On a higher dose treatment, the latex damaged the contractility of the cardiac muscle.

2.2.4 ***Catharanthus roseus*** (L.) G. Don

Synonym: *Vinca rosea*

Common name: Madagascar periwinkle

a. Botany and uses

Catharanthus roseus is a perennial herb that grows up to 1 m tall and produces white sap (Li *et al.* 1995; Sutarno & Rudjiman 1999; Aslam *et al.* 2010). Leaves are obovate or elliptic with rounded apex. The fragrant trumpet-shaped flowers are purple, red, pink or white with a purple, red, pink, pale yellow or white eye. Fruits consist of two cylindrical follicles producing many minute black seeds. It blooms throughout the year and can be propagated by seeds or cuttings. Horticulturists have developed more than 100 varieties of *Catharanthus roseus*. Native to Madagascar, the species is cultivated or naturalised in all tropical countries. The Madagascar periwinkle is a popular garden ornamental, grown as a perennial in tropical regions and as an annual in temperate regions. It is valued for its bushy habit, showy colourful flowers and dark green foliage. Plants of *Catharanthus roseus* are also cultivated for medicine.

The plant has historically been used to treat a wide variety of diseases (Aslam *et al.* 2010). Decoction of all parts is used in the treatment of malaria, skin diseases, Hodgkin's disease, diarrhea, hypertension and diabetes (Li *et al.* 1995). A decoction of all plant parts is used to treat malaria, diarrhoea, diabetes, cancer and skin diseases (Sutarno & Rudjiman 1999). The species is also well known as an oral hypoglycaemic agent. Extracts prepared from leaves have been used as an antiseptic agent for healing wounds and as a mouthwash to treat toothache. The

plant has long been used as a hypoglycemic agent for the treatment of diabetes (Kuo & King 2001).

Commercial drugs have been developed from alkaloids (vinblastine and vincristine) extracted from the plant (Aslam *et al.* 2010). Vinblastine sulphate (sold as Velban) is used to treat Hodgkin's disease. Vincristine sulphate (sold as Oncovin) is effective for treating acute leukemia in children and lymphocytic leukemia. The target of *Catharanthus* alkaloids is tubulin, a protein needed for cell division, and their mechanism of action is inhibition of mitosis which is the process of cell division (Itokawa *et al.* 2008).

b. Phytochemistry and bioactivities

Plants of *Catharanthus roseus* contain about 130 alkaloids of the indole group of which 25 are dimeric in nature (van der Heijden *et al.* 2004; Aslam *et al.* 2010). Some major products found are vinblastine, vindoline, catharanthine, ajmalicine and serpentine (van der Heijden *et al.* 2004). The species is particular rich in bisindole alkaloids (~40 compounds), most of them containing a vindoline or catharanthine moiety. Both the commercially important alkaloids of vinblastine and vincristine [**Figure 2.19**] have a large dimeric asymmetric structure composed of a dihydroindole nucleus (vindoline ring) and an indole nucleus, linked by a carbon-carbon bond (Aronson 2008).

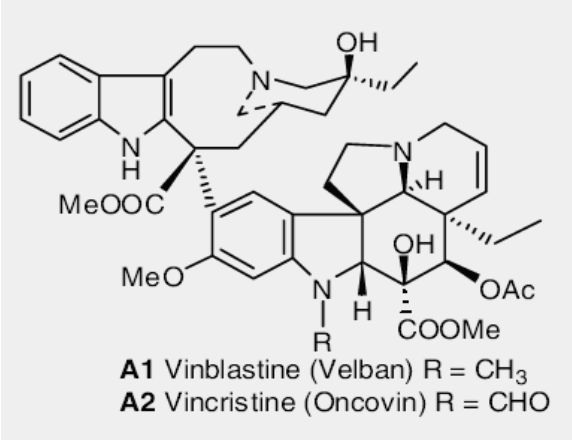


Figure 2.19 Vinblastine and vincristine of *Catharanthus roseus* (Itokawa *et al.* 2008)

Catharanthus alkaloids have been reported in a large number of publications (e.g. Cordell *et al.* 1974; El-Sayed *et al.* 1980; El-Sayed & Cordell 1981; Mukhopadhyay & Cordell 1981; Mukhopadhyay *et al.* 1983; Jossang *et al.* 1998; van der Heijden *et al.* 2004; Itokawa *et al.* 2008). Besides alkaloids, other compounds such as flavonoids and anthocyanins are also reported. Three caffeoylquinic acids and fifteen flavonol glycosides have been identified from seeds, stems, leaves and petals of *Catharanthus roseus* (Ferreres *et al.* 2008). Fifteen of these compounds were reported for the first time in this species. From the flowers of *Catharanthus roseus*, anthocyanins isolated and identified were 3-*O*-glucosides and 3-*O*-(6-*O*-*p*-coumaroyl) glucosides of hirsutidin, malvidin and petunidin (Piovan & Filippini 2007).

The ethanol flower extract of *Catharanthus roseus* has been reported to have wound healing properties in rats (Nayak & Pereira 2006). The extract promoted wound contraction, increased tensile strength, increased hydroxyproline content and has antibacterial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Leaf, stem, flower and root ethanol extracts of *Catharanthus roseus* displayed broad-spectrum antibacterial activity against *Escherichia coli*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Salmonella typhi* and *Aeromonas hydrophila* (Raza *et al.* 2009). No activity was found for *Staphylococcus aureus* while activity was weak for *Pseudomonas aeruginosa*, contrary to findings of Nayak & Pereira (2006). In a similar study, leaf, stem, root and flower methanol and ethanol extracts of *Catharanthus roseus* were reported to have antibacterial activity to both Gram-positive and Gram-negative bacteria (Ramya *et al.* 2008). Catharanthamine isolated from *Catharanthus roseus* was cytotoxic to the KB and P-388 cell lines (El-Sayed & Cordell 1981).

2.2.5 ***Cerbera odollam*** Gaertner

Synonyms: *Cerbera lactaria*; *Cerbera manghas*

Vernacular name: Pong-pong

a. Botany and uses

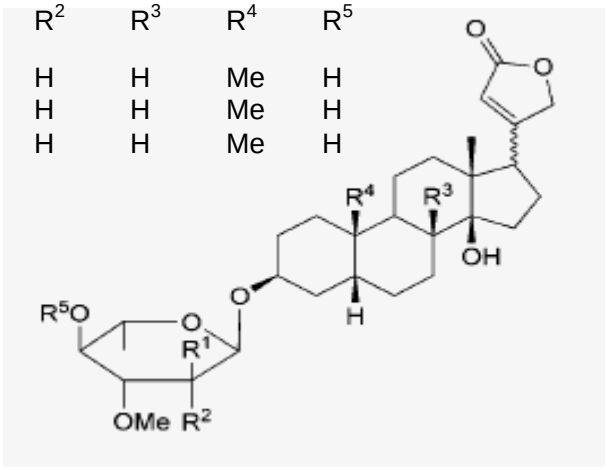
Cerbera odollam are evergreen shrubs or small to medium-sized trees up to 30 m tall (Khanh 2001). The bole when slashed exudes abundant white latex. Leaves are arranged spirally and clustered at the apices of twigs. Flowers are white with a pinkish centre. Fruits are a drupe with a single rounded seed. Trees are planted as ornamentals in gardens and along road sides. The species occur naturally in coastal areas of tropical Asia and Melanesia.

In Southeast Asia, leaves of *Cerbera odollam* are used in aromatic bath by women after childbirth (Khanh 2001). Leaves, bark and latex are emetic and purgative, and seeds are toxic and strongly purgative. The bark, latex and leaves are sometimes used as an emetic and a purgative (Li *et al.* 1995).

b. Phytochemistry and bioactivities

The chemical constituents of the genus *Cerbera* have been reviewed by Shen *et al.* (2007). Compounds isolated from *Cerbera odollam* are lignans (Abe *et al.* 1988), cardenolides (Yamauchi *et al.* 1987a & 1987b; Hanada *et al.* 1992) and terpenoids (Abe *et al.* 1989).

A new cardenolide glycoside was isolated from methylene chloride extract of the seeds of *Cerbera odollam*, together with four known compounds of cerleaside A, 17*α*-neriifolin, 17*β*-neriifolin and cerberin [**Figure 2.20**] (Laphookhieo *et al.* 2004). From the bark of *Cerbera odollam*, a 14β-(H) steroid characterised as triticusterol was isolated [**Figure 2.21**] (Hasan *et al.* 2011). Two benzoic acid derivatives identified as 2,6-dihydroxy-4-methoxy benzoic acid and 2-hydroxy-4-methoxy-6-methyl benzoic acid were also isolated.



1 = 17*α*-neriifolin, **2** = 17*β*-neriifolin, **3** = cerberin

Figure 2.20 Cardenolide glycosides of *Cerbera odollam* (Shen *et al.* 2007)

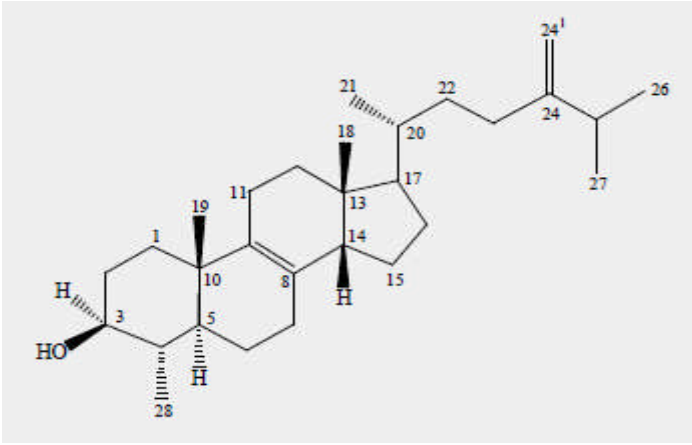


Figure 2.21 Triticusterol from *Cerbera odollam* (Hasan *et al.* 2011)

Methanol leaf extract of *Cerbera odollam* strongly inhibited MCF-7 and T47D cells (Nurhanan *et al.* 2008). Cardenolides from seeds had cytotoxic activity against KB, BC and NCI-H187 cells (Laphookhieo *et al.* 2004). Chloroform and carbon tetra-chloride soluble fractions of *Cerbera odollam* stem bark of demonstrated the strongest antioxidant activity with IC_{50} value 21.6 and 21.0 µg/mL, respectively, compared to BHT with a value 14.5 µg/mL (Hasan *et al.* 2011).

2.2.6 *Dyera costulata* Hook

Synonyms: *Dyera polyphylla*; *Dyera lowii*
Vernacular name: Jelutong

a. Botany and uses

Dyera costulata trees grow up to 80 m tall (Middleton 2004). Bark is dark grey, brown or black in colour. Leaves occur in whorls of 4–8 and flowers are white. Paired resembling horns, fruits dehisce when mature, releasing seeds with membranous wings. Occurring naturally in southern Thailand, Malaysia and Sumatra, the species are tall forest trees growing up to 1200 m altitude. Latex of *Dyera costulata* was formerly an important source of chewing gum. The light and soft timber is excellent for manufacturing a variety of wood products e.g. carvings, toys, pencils, etc. In traditional medicine, leaves and barks of *Dyera costulata* have been used for treating fever, inflammation and pain (Subhadhirasakul *et al.* 2003).

b. Phytochemistry and bioactivities

Six bisindole alkaloids have been isolated from leaf extracts of *Dyera costulata* (Mirand *et al.* 1983). They were novel ochrolifuanines E and F, and 18-dehydro-ochrolifuanines A, E and F, including known ochrolifuanine A. From the leaves, Subhadhirasakul *et al*. (2003) reported the isolation of β-amyrin (a pentacyclic triterpenoid) and rhamnazin (a methylated flavonol) from chloroform extract, and quercetin-3-*O*-α-L-rhamnopyranoside (a flavonol glycoside) from n-butanol extract. The molecular structures of ochrolifuanine A and quercetin-3-*O*-α-L-rhamno pyranoside are shown in **Figure 2.22**.

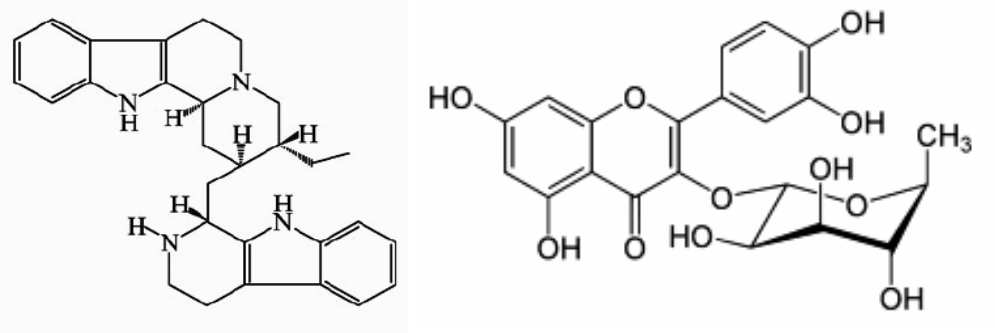


Figure 2.22 Ochrolifuanine A (left) and quercetin-3-*O*-α-L-rhamnopyranoside (right) reported in *Dyera costulata*

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of quercetin-3-*O*-α-L-rhamnopyranoside isolated from leaves of *Dyera costulata* was 8.6 times stronger than butylated hydroxyltoluene (Subhadhirasakul *et al*. 2003). The chloroform extract from leaves of *Dyera costulata* showed marked analgesic effect but no antipyretic activity in mice (Reanmongkol *et al.* 2002). Out of 185 plant samples belonging to more than 30 families screened for central nervous system receptor activity, the leaf extract of *Dyera costulata* had positive activity against 5HT1a receptor with 90% inhibition (Chung *et al.* 2005).

2.2.7 ***Kopsia fruticosa*** (Ker.) A. DC.

Synonyms: *Cerbera fruticosa*; *Kopsia vinciflora*

Common name: Pink kopsia

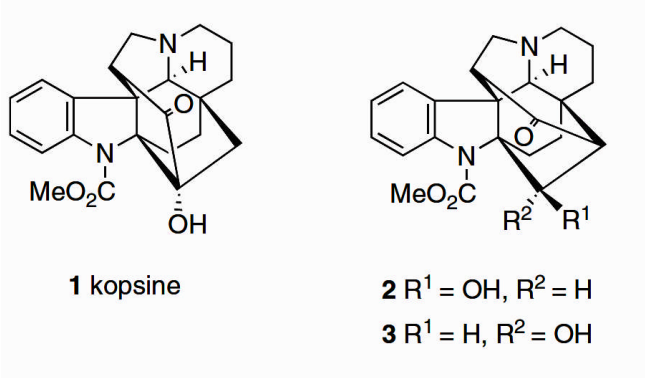
a. Botany and uses

Kopsia fruticosa is an evergreen shrub that grows up to 4 m tall (Sévenet *et al.* 1994; Li *et al.* 1995). Leaves are elliptic or oblong, thin, shiny above and pale beneath. Inflorescences are a compact cyme bearing few pink flowers that resemble those of *Ixora*. The corolla tube is slender. Fruits are a flattened drupe bearing a single seed. The species is native to Myanmar and has naturalised in India, Indonesia, Malaysia, Philippines and Thailand. It is sometimes cultivated as an ornamental or medicinal

plant. Leaves of *Kopsia fruticosa* are used to treat sores and syphilis, and have cholinergic effects (Johnson 1999).

b. Phytochemistry and bioactivities

From leaves of *Kopsia fruticosa*, alkaloids of kopsine, fruticosine and fruticosamine have been isolated [**Figure 2.23**] (Kam & Lim 2008). Kopsine was the first *Kopsia* alkaloid isolated. The NMR spectral assignments of these three compounds have been reported by Glover *et al.* (2005). Novel indole alkaloids of kopsifolines A-F (Kam & Choo 2003), venacarpine A-B and kopsorinine (Kam & Choo 2004b) have been reported.



1 = kopsine, **2** = fruticosine, **3** = fruticosamine

Figure 2.23 Alkaloids of *Kopsia fruticosa* (Kam & Lim 2008)

2.2.8 ***Nerium oleander*** L.

Synonyms: *Nerium indicum*; *Nerium odorum*

Common name: Oleander

a. Botany and uses

Nerium oleander is an evergreen shrub or small tree ranging from 2–6 m tall with spreading to erect branches (Gruppe 2010). Leaves are in pairs or whorls of three, thick and leathery, dark green, narrow lanceolate with entire margin. Flowers are

borne in clusters at the end of each branch. They vary from white, pink, red or yellow in colour and 2.5–5.0 cm in diameter. The corolla is deeply 5-lobed fringing round the central corolla tube. They are often sweetly scented. The fruit is a long narrow capsule which splits open at maturity to release numerous downy seeds. Plants of *Nerium oleander* are extensively used as an ornamental plant in landscapes, parks, and along roadsides. There are over 400 varieties with additional flower colours of red, purple and orange (Sharma *et al.* 2010). The pink and white varieties are the most common.

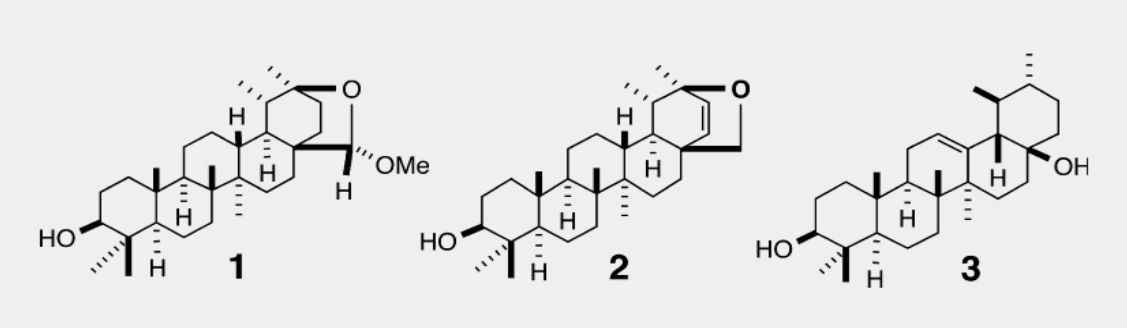
b. Phytochemistry and bioactivities

Plants of *Nerium oleander* are highly poisonous and contain toxic cardiac glycosides such as oleandrin, oleandrigenin and neriine (Sharma *et al.* 2010). Anvirzel, a hot water extract of oleander, has been used on a limited, compassionate basis to treat small numbers of patients with malignant disease in Europe and the U.S. (Newman *et al.* 2001). A Phase I clinical trial of Anvirzel has also recently been initiated in the U.S. Oleandrin (2.5 µg/mg) and its aglycone, oleandrigenin (4.4 µg/mg) were detected in the oleander extract. The extract was reported to induce cell death in human prostrate cancer cells but not murine melanoma cells (Pathak *et al.* 2000). Cytotoxicity studies showed that the extract inhibited human melanoma BRO cells (IC_{50} of 1.6 µg/mL), but not as potent as compounds of oleandrin (IC_{50} of 4.0 ng/mL) and oleandrigenin (IC_{50} of 17 ng/mL) (Newman *et al.* 2001).

Triterpenoids are major constituents of *Nerium oleander* [**Figure 2.24**] (Siddiqui *et al.* 1995, 1986a, 1988 & 1989a; Begum *et al.* 1997; Fu *et al.* 2005; Zhao *et al.* 2006). Other compounds such as pregnanes (Bai *et al.* 2007) and cardenolides (Siddiqui *et al.* 1986b; Paper & Franz 1989; Abe *et al.* 1992a; Zhao *et al.* 2007) were frequently isolated and characterised. The phytochemical and pharmacological properties of *Nerium oleander* have recently been reviewed (Gupta & Mittal 2010).

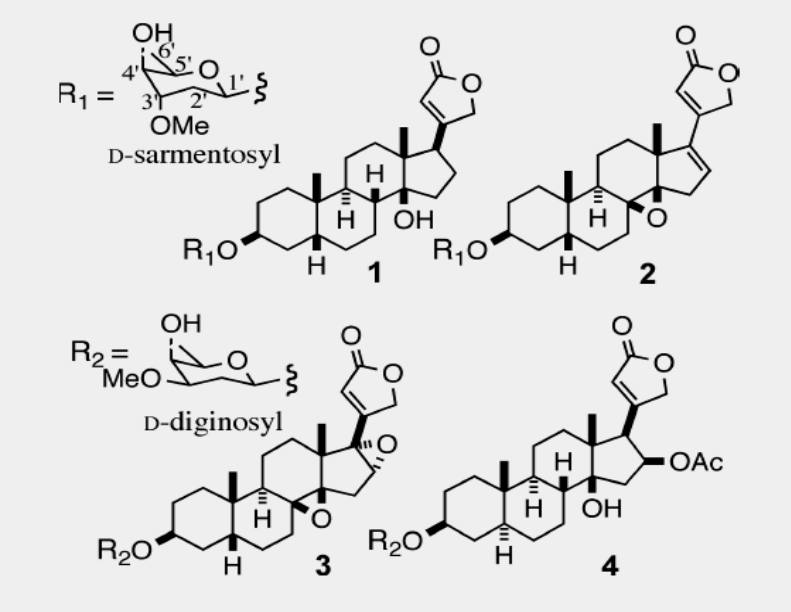
Extracts from roots and leaves of *Nerium oleander* displayed antibacterial activity against *Bacillus pumilus*, *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli* (Hussain & Gorsi 2004). Triterpenes isolated from leaves of *Nerium oleander* (Zhao *et al.* 2006) and cardenolides isolated from stems and twigs (Zhao *et al.* 2007)

were cytotoxic to WI-38, VA-13 and HepG2 human cell lines. Leaf, stem and root extracts have cytotoxic effects on leukaemia cell lines (Turan *et al.* 2006).



Triterpenoids

1 = 20*β*,28-epoxy-28α-methoxytaraxasteran-3*β*-ol, **2** = 20*β*,28-epoxytaraxaster21-en-3*β*-ol, **3** = 28-nor-urs-12-ene-3*β*,17*β*-diol



Cardenolides

1 = 3*β*-*O*-(*β*-D-sarmentosyl)-14-hydroxy-5*β*,14*β*-card-20(22)-enolide, **2** = 3*β*-*O*-(*β*-D-sarmentosyl)-5*β*,14*β*-card-20(22)-enolide, **3** = 3*β*-*O*-(*β*-D-diginosyl)-8,14;16α,17-diepoxy -5*β*,14*β*-card-20-(22)-enolide, **4** = 3*β*-*O*-(*β*-D-diginosyl)-5α-card-20(22)-enolide

Figure 2.24 Triterpenoids and cardenolides of *Nerium oleander* (Zhao *et al.* 2006 & 2007)

2.2.9 *Plumeria obtusa* L.

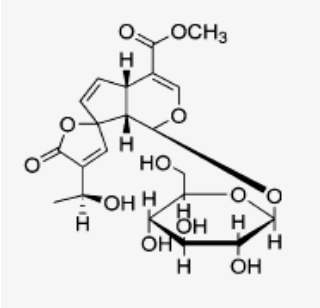
Common name: Frangipanni

a. Botany and uses

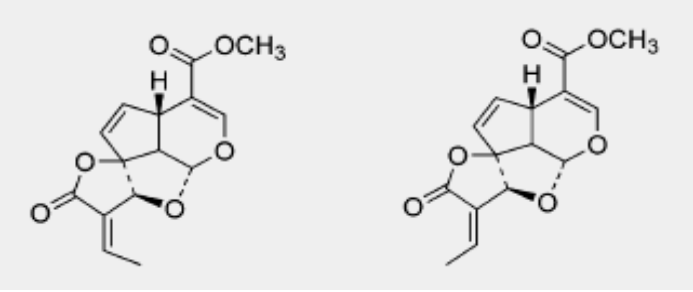
Plumeria obtusa trees grow up to 5 m tall (Li *et al.* 1995). Stems are succulent and produce a milky sap. Leaves are simple, spirally arranged and clustered at the stem tips. They are obovate, leathery, glossy dark green on the upper surface and have a rounded apex. Flowers are funnel-shaped, white, ~4 cm in diameter with a yellow throat yellow and slightly recurved. Flowering is continuous throughout the year. The species is native to the Caribbean Islands. It is commonly planted as an ornamental. In Asia, a decoction of leaves of *Plumeria obtusa* is used for treating wounds and skin diseases (Burkill 1935). Its latex and bark are known to have purgative and diuretic properties.

b. Phytochemistry and bioactivities

Triterpenoids are the major constituents of leaves of *Plumeria obtusa* (Siddiqui *et al.* 1989b, 1990, 1992 & 1999) with iridoids also reported [**Figure 2.25**] (Siddiqui *et al.* 1994). From the flowers, an iridoid β-glucoside (plumieride coumarate glucoside) was isolated (Boonclarm *et al.* 2006).



Plumieride



Two isomers of plumericin

Figure 2.25 Iridoids of *Plumeria obtusa* (Dobhal *et al.* 2004)

2.2.10 *Vallaris glabra* (L.) Kuntze

Common name: Bread flower

Vernacular name: Kesidang

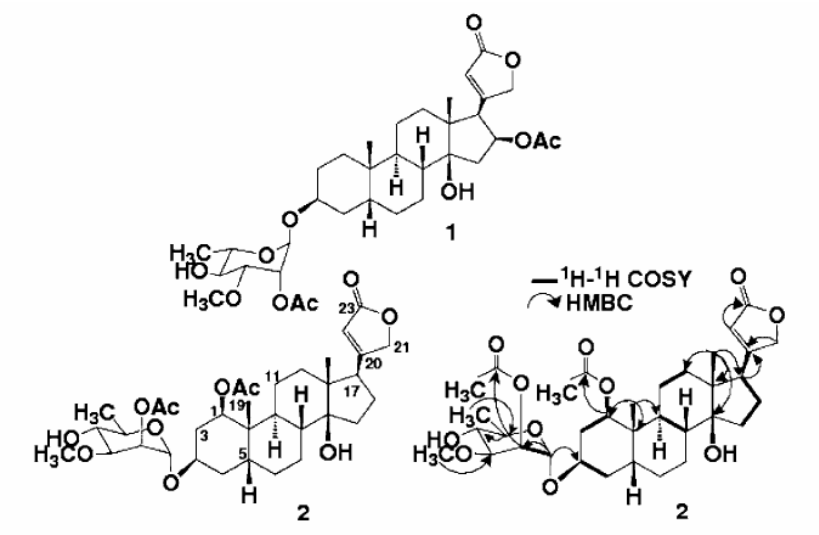
a. Botany and uses

Vallaris glabra or bread flower is a woody climber with broadly elliptic leaves of 7–9 x 4–6 cm in size (Wongpornchai *et al*. 2003). Inflorescences are long-stalked with clusters of fragrant cup-like white flowers of 1.0–1.5 cm in diameter. The plant is well known in Thailand and Malaysia because its flowers have a scent of leaves of pandan (*Pandanus amaryllifolius*) or newly cooked fragrant rice. The aromatic compound is 2-acetyl-1-pyrroline, first reported in cooked rice and in pandan leaves. Originated from Java in Indonesia, the species grows in full sun and can be propagated by marcotting (Ng 2006). With its attractive clusters of white flowers that emit a strong pandan fragrance, *Vallaris glabra* is becoming a popular ornamental plant in botanic and home gardens of Southeast Asia. Potted plants can be purchased from nurseries. The use of *Vallaris glabra* in traditional medicine has not been reported.

b. Phytochemistry and bioactivities

Its bioactivity and phytochemistry have yet to be studied. Some work has been done on the phytochemistry of *Vallaris glabra* and *Vallaris solanacea*.

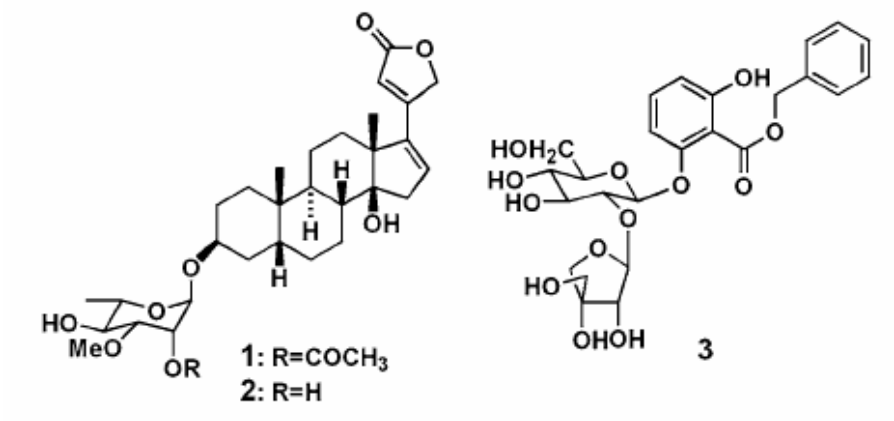
Recently, bioassay-guided separation of leaves of *Vallaris glabra* led to the isolation of two cardiac glycosides (Rifai *et al.* 2011). Compound **1** was identified as acoschimperoside P, 2'-acetate and compound **2** was a new cardiac glycoside [**Figure 2.26**]. Compound **1** was active in hedgehog signalling inhibition, and showed strong cytotoxicity against human pancreatic (PANC1) and human prostate (DU145) cancer cells.



1 = acoschimperoside P, 2'-acetate, **2** = new cardiac glycoside

Figure 2.26 Cardiac glycosides of *Vallaris glabra* (Rifai *et al.* 2011)

A glycoside isolated from leaves of *Vallaris solanacea* identified as *O*-acetyl-solanoside had been reported to possess potent cardiotonic activity (Vohra *et al.* 1966). Vallarisoside (a new cardenolide glycoside) [**Figure 2.27**] had been isolated from the leaves of *Vallaris solanacea* (Ahmed *et al.* 2010). A new glycoside and a known cardenolide glycoside were also isolated. Vallarisoside from *Vallaris solanacea* leaves showed potent tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) resistance-overcoming activity in human gastric adenocarcinoma cells, and cell growth inhibitory activity against HeLa and SW480 cells.



1 = vallarisoside, **2** = 3β-*O*-(α-acofriosyl)-16-anhydrogitoxigenin, **3** = benzyl-2-*O*-β-apiofuranosyl-(1→2)-β-D-glucopyranosyl-2,6-dihydroxy-benzoate

Figure 2.27 Cardenolide glycosides of *Vallaris solanacea* (Ahmed *et al.* 2010)

2.3 BIOACTIVITY ASSAYS

2.3.1 Sulphorhodamine B Assay

The sulphorhodamine B (SRB) assay was developed by the National Cancer Institute and has been adopted for routine use *in vitro* anticancer screening (Skehan *et al.* 1990; Rubinstein *et al.* 1990). The assay has become one of the most widely used methods for *in vitro* cytotoxicity screening, substituting other tetrazolium-based assays (Rubinstein *et al.* 1990; Papazisis *et al.* 1997; Vichai & Kirtikara 2006). The assay relies on the ability of SRB to bind to protein components of cells that have been fixed to tissue-culture plates by trichloroacetic acid (TCA) (Vichai & Kirtikara 2006). SRB is a bright-pink aminoxanthene dye with two sulphonic groups that bind to basic amino acid residues under mild acidic conditions, and dissociate under basic conditions. As the binding of SRB is stoichiometric, the amount of dye extracted from stained cells is directly proportional to the cell mass.

The strong intensity of SRB staining allows the assay to be carried out in a 96-well microtitre plates (Skehan *et al.* 1990; Vichai & Kirtikara 2006). The assay can detect densities as low as 1000–2000 cells per well, and with a signal-to-noise ratio of 4.83 at a density of 5000 cells per well. The level of sensitivity is comparable to other dye-staining methods using conventional visible dyes. Furthermore, the SRB method has proven to be practical, because fixing with TCA and staining with SRB, the cell mono-layers can be dried and stored indefinitely (Vichai & Kirtikara 2006). Colour extracted from SRB-stained cells is also stable. With its high level of sensitivity and endpoint stability, the SRB assay is well suited to large-scale screening applications, as well as research.

The effectiveness of the SRB assay is frequently compared to other *in vitro* cytotoxicity testing methods especially the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Rubinstein *et al.* 1990; Keepers *et al.* 1991; Martin & Clynes 1993; Pauwels *et al.* 2003). Developed by Mosmann (1983), the MTT assay requires cellular metabolic activity to convert the colourless tetrazolium to the purple-coloured formazan dye (Vichai & Kirtikara 2006). The MTT assay detects only viable cells,

whereas the SRB method does not distinguish between viable and dead cells. Although studies have shown that results from both assays correlated well, the SRB assay has several advantages over the MTT assay. SRB staining is independent of cell metabolic activity and involves fewer steps (Keepers *et al.* 1991), and has better linearity, higher sensitivity and lower cost (Rubinstein *et al.* 1990; Monks *et al.* 1991; Martin & Clynes 1993). The SRB assay is an efficient and highly cost-effective method for screening of cytotoxicity as it requires only inexpensive reagents and simple equipment, and allows large number of samples to be tested within a few days (Voigt, 2005; Vichai & Kirtikara 2006).

2.3.2 *Plasmodium* Lactate Dehydrogenase Assay

Various diagnostic technologies have been developed for the detection of malaria. Of these technologies, the MALSTAT reagent is widely used in testing drug efficacy against the malaria parasite *Plasmodium* spp. (Makler *et al.* 1998; Piper *et al.* 1999; Noedl *et al.* 2003). At present, the MALSTAT-based assay is the only commercially available colorimetric assay system for detecting malaria in blood samples (Malik *et al.* 2004; Berger 2005). The assay detects the level of an enzyme of the parasite, *Plasmodium* lactate dehydrogenase (pLDH), which takes part in glycolysis. The activity of pLDH was found to increase progressively following the level of infection or parasitemia based on *in vitro* cultures (Makler & Hinrichs 1993; Makler *et al.* 1993). MALSTAT reagent contains an analog of nicotinamide adenine dinucleotide (NAD), i.e. 3-acetylpyridine adenine dinucleotide (APAD+). The assay is based on the greater ability of *Plasmodium* lactate dehydrogenase (pLDH), to make use of the 3-acetylpyridine analogue of NAD as a cofactor in converting lactate to pyruvate (Gomez *et al.* 1997). Human LDH isozymes do not use this analog readily (Piper *et al.* 1999). An aliquot of infected blood is lysed by detergent and incubated with APAD, lactate, diaphorase, and nitroblue tetrazolium for the reaction shown in **Figure 2.28**. The resulting blue colour can be quantified at 630 nm using a spectrophotometer.

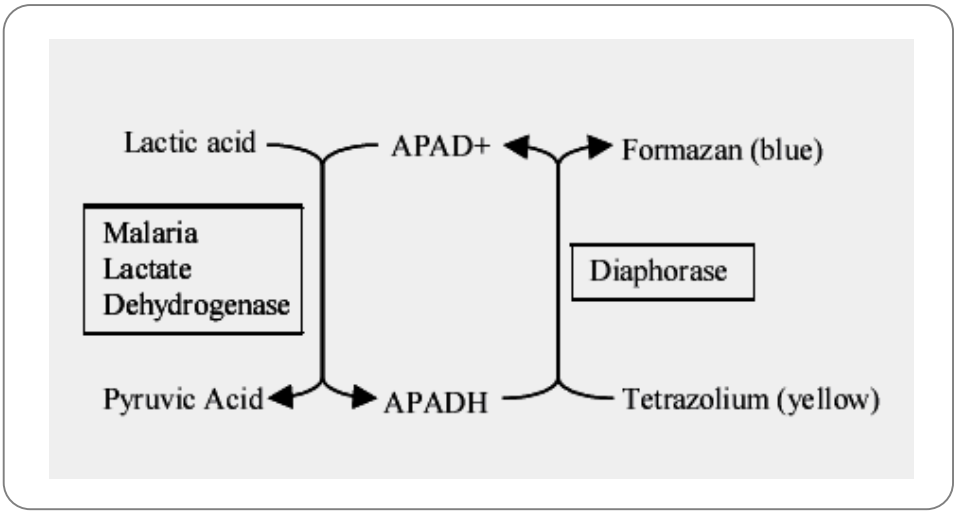


Figure 2.28 MALSTAT reaction for detecting *Plasmodium* lactate dehydrogenase (Berger 2005)

The colorimetric assay system using MALSTAT is unique in that it allows different concentrations of drugs to be incubated with the infected blood samples prior to lysis and the addition of colorimetric reagent. This allows more accurate assessment on drug susceptibility of different strains. The assay is quantitative, reproducible and inexpensive.

However, a more sensitive but non-quantitative OptiMAL kit is preferred for clinical diagnosis (WHO 1996; Palmer *et al.* 1998). The method is based on immuno-chromatographic separation of lysed blood samples and labelled antibodies down a strip which holds an immobilised antibody (Berger 2005). The kit has an additional advantage of being able to differentiate between the various strains and non-strains of *Plasmodium falciparum*.

2.3.3 DPPH Radical Scavenging Assay

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay is one of the most widely employed method to evaluate free radical scavenging activity of plant and food extracts, and single compounds (Scalzo 2008). This assay is based on the measurement of the scavenging ability of antioxidants towards the 1,1-diphenyl-2-picrylhydrazyl radical ($DPPH^{\bullet}$).

The DPPH assay is widely used because $DPPH^{\bullet}$ is stable, easily accessible, does not have to be generated before the assay (Brand-Williams *et al.* 1995; Magalhaes *et al*. 2008; Locatelli *et al*. 2009). The method is rapid, simple, sensitive and does not require expensive reagents or sophisticated instruments, and the results are highly reproducible and comparable to other free radical scavenging methods. The method was developed by Brand-Williams *et al.* (1995). Since then, the DPPH assay has become a reference point to evaluate *in vitro* antioxidant activity.

$DPPH^{\bullet}$ is a stable radical of organic nitrogen, characterised by a typical deep purple colour and a maximum absorbance in the range of 515–520 nm (Magalhaes *et al*. 2008; Locatelli *et al*. 2009). The assay involves hydrogen atom transfer (Huang *et al.* 2005) and the reactions are as follows:

$DPPH^{\bullet}$ (purple) + AH → DPPH-H (yellow) + $A^{\bullet}$ (stable free radical)
$DPPH^{\bullet}$ (purple) + $R^{\bullet}$ → DPPH-R (yellow)

2.3.4 Tyrosinase Inhibition Assay

Tyrosinase is the key enzyme in melanin synthesis, catalysing the first two steps of the pathway i.e. the hydroxylation of L-tyrosine to 3,4-dihydroxy-L-phenylalanine (L-dopa) and the oxidation of L-dopa to dopaquinone (Winder & Harris 1991; Winder 1994). Many assays have been devised to measure the tyrosine hydroxylase and dopa oxidase activities of tyrosinase.

The most widely used *in vitro* assay for the dopa oxidase activity of tyrosinase is the dopachrome method (Winder & Harris 1991; Winder 1994). Oxidation of L-dopa by tyrosinase produces dopaquinone which undergoes autoxidation to the orange coloured dopachrome measured at 475 nm. A major disadvantage of this method is that dopachrome is unstable in aqueous solution and is further oxidised even while its accumulation is being monitored. This instability results in the assay being linear for only a short period of 2–3 min. The absorption coefficient of dopachrome is relatively low and the assay is therefore of low sensitivity.

Other spectrophotometric dopa oxidase methods such as the melanochrome assay (Vachtenheim *et al.* 1985) and the 3-methyl-2-benzothiazoninone hydrazone (MBTH) assay (Winder & Harris 1991) are more sensitive than the dopachrome assay. The MBTH assay was further improved by stopping the enzymatic oxidation of L-dopa by addition of perchloric acid to enable convenient spectrophotometric measurements (Winder 1994).

2.4 PHYTOCHEMICAL ASSAYS

2.4.1 Dragendorff Precipitation Assay

The Dragendorff's reagent has often been used for phytochemical screening of plants for alkaloids. There are at least six different types of Dragendorff's reagent, each containing potassium iodide (Aniszewski 2007).

A rapid, easy and simple spectrophotometric method for quantification of total alkaloidal content precipitated by Dragendorff's reagent in plant materials has been reported by Sreevidya and Mehrotra (2003). The Dragendorff precipitation assay is based on the formation of yellow bismuth complex in nitric acid medium with thiourea. Absorbance of the yellow solution was measured at 435 nm against colourless reagent blanks. This method can be applied to single alkaloid or to alkaloid-bearing plants. It can be used for routine analysis of commercial samples by industries dealing with herbal drugs for standardization of plant materials or pharmaceutical products containing alkaloids. The method was adopted by Ribeiro *et al.* (2008) in their work on wild edible mushrooms.

2.4.2 Folin-Ciocalteu Assay

The Folin-Ciocalteu (FC) assay is used to determine the total phenolic content (TPC) in food and plant samples (Waterhouse 2002). The assay has been adopted as the official procedure for total phenolic levels in wine. This is probably the most popular method for quantifying total phenols in foods since it is a rapid and simple procedure (Huang *et al.* 2005; Bravo & Mateos 2008). FC reagent is commercially available and the procedure has been standardised. It is a commonly accepted assay and routinely

practised in research laboratories throughout the world, and so it is possible to compare results with values reported in the literature. The FC assay is simple, sensitive and repeatable (Phipps *et al*. 2007; Magalhaes *et al.* 2008). Several studies have established linear correlations between TPC and antioxidant capacity, thus confirming that phenolic compounds are likely to contribute to the antioxidant activity of these samples (Kähkönen *et al.* 1999).

The FC assay is based on electron transfer and involved redox reaction and the main components of FC reagent are phosphomolybdic and phosphotungstic acids (Magalhaes *et al*. 2008; Bravo & Mateos 2008). Under alkaline condition, there is transfer of electrons from phenolic compounds to phosphomolybdic-phosphotungstic reagent and resulting in the formation of a stable blue complex which can be detected spectrophotometrically at 750–765 nm. The reaction can be summarised as follows (Prior *et al.* 2005):

$$\text{Mo(VI) (yellow)} + e^- \rightarrow \text{Mo(V) (blue)}$$

TPC is usually expressed in terms of molar or gram equivalent of a commonly occurring phenol such as gallic acid or quercetin (Robards 2003). Gallic acid is more often used as standard because it is a common phenolic compound, and is stable, cheap, water soluble and easy to prepare (Singleton *et al.* 1999).

A disadvantage of this assay is the interference of reducing substances such as ascorbic acid which reacts with FC reagent and thus overestimated results are obtained (Robards 2003; Phipps *et al*. 2007; Bravo & Mateos 2008). Correction for interfering substances should be made.

2.4.3 Hoechst Nuclear Staining Assay

There are many *in vitro* and *in vivo* methodologies of morphological, biochemical and functional assays for monitoring cell death (Braun & Albert 2005). As apoptotic cell death is characterised by marked condensation of nucleus and cytoplasm, and nuclear fragmentation (Kerr *et al.* 1972), a common method used to monitor nuclear morphological changes is Hoechst nuclear staining assay.

Hoechst stain is a bisbenzimidazole dye which fluoresces strongly under ultraviolet (UV) light (Smith *et al.* 1988; Kiechle & Zhang 2002). Being cell permeable, it rapidly diffuses into cells and binds specifically and quantitatively to DNA. Because of its low toxicity to viable cells, it is extensively used to stain DNA for evaluating the cell cycle, apoptosis and quantifying viable cells by flow cytometry. Hoechst stain is DNA-specific and intercalates between adenine and thymine (Exbrayat 2001). Hoechst 33342 dye emits a blue fluorescence at 450 nm while Hoechst 33258 dye emits a green fluorescence at 470 nm.

Control and treated cells are stained with a fluorescent Hoechst dye and visualised under a fluorescence microscope (Zakaria *et al.* 2009). Hoechst stain binds to adenine and thymine regions of double-stranded DNA of the nuclei which exhibit enhanced fluorescence. Non-apoptotic cells have intact structures. Treated cells will show shrinkage followed by nuclear condensation and fragmentation. Stained cells with such morphological features are indicative of apoptosis. Nuclei of apoptotic cells stain more brightly from their condensed chromatins compared to the nuclei of non-apoptotic cells.

2.5 NATURAL PRODUCT RESEARCH

2.5.1 Introduction

Natural product research for drug discovery generally involves three fundamental steps (Lahlou 2007). They are: i) the appropriate selection of plants for biological screening; ii) the development of quantitative techniques for measuring biological activity; and iii) bioassay-guided fractionation of plant extracts and the structural elucidation of bioactive chemicals. The whole process of drug discovery takes more than 10 years and cost more than USD 800 million on the average (Balunas & Kinghorn 2005). Only one in 5000 lead compounds will successfully advance to clinical trials and approval for use. Research leading to the discovery of new drugs needs a multi-disciplinary research team and collaborative efforts of botanists, chemists, pharmacologists and biologists (Balunas & Kinghorn 2005; Lahlou 2007; Pan *et al.* 2009).

Over the past decade, pharmaceutical companies have reduced their investment in natural product research (Koehn & Carter 2005; Beutler 2009). Among the reasons are the perceptions that discovery and development of natural products is too slow compared to high-throughput screening; the synthesis of natural products is too difficult as the structures are too complex; and combinatorial chemistry is viewed as superior to natural products research.

In recent decades, chemists are able to synthesise medicinal compounds which led to the production of effective medicines (Lesney 2004; McRae *et al.* 2007). However, as new diseases and drug-resistant strains of pathogens continue to emerge, the potential of synthetic compounds with simple structures and known modes of action has started to diminish. As such, attention is again being focused on lead compounds from natural sources where there is a wealth of complex compound structures and novel modes of action.

Natural product research has therefore gained renewed attention with a remarkable resurgence of interest over the last decade (Sarker *et al*. 2006). Strategies for research on natural products have evolved quite significantly. There are two broad categories. They are:

1) Older strategies focus on chemistry of compounds from natural sources, but not on activity. They involve isolation and identification of compounds from natural sources followed by testing of bioactivity.

2) Modern strategies involve bioassay-guided isolation and identification of active compounds from natural sources. Focus is on isolating target compounds rather than all compounds present in any extract.

The main objectives of medicinal plant research are qualitative and quantitative analysis of plant constituents; isolation and identification of biologically active, purified fractions and molecules with new structures; and optimization of the amount and/or ratio of compounds responsible for therapeutic effects (Nyiredy 2004). As one single plant can contain up to several thousand secondary metabolites, high-

performance and rapid separation methods are absolutely necessary for all three categories.

2.5.2 Plant Selection

The two main approaches used in plant selection for natural product research i.e. random screening and ethnobotany. Since there are many thousands of plant species each with leaves, bark and roots having different phytochemical composition, the likelihood of finding an active plant sample from a random screening survey is indeed very small (Hostettman & Wolfender 1997; Lesney 2004). It is even less likely that the active compound isolated from the plant sample is novel or has a novel mode of action that is more efficient than any product currently in the market. These problems can often be overcome by using ethnobotanical or traditional knowledge (Hostettman & Wolfender 1997). Studies have shown high correlation between the traditional use of plants and the presence of active compounds within the plant extract (Leven *et al.* 1979; Zavala *et al.* 1997).

2.5.3 Assays

In natural product research, screening of extracts of plant samples is carried out to provide preliminary information on their pharmacological potential (Ghisalberti 2008). Assays used are intended to guide fractionation of the crude material towards isolation of the pure bioactive substances (Vlietinck & Apers 2001). Assays need not be quantitative, but they have to be fast and cheap, have high capacity, and be readily available to the scientist. The methods for the detection of activity of natural products can be divided into general and specialised assays.

General bioassays can be conducted by scientists who lack the resources or expertise to carry out more elaborate bioassays. Examples are tests for brine shrimp lethality, nematocidal, antimicrobial, antihelmintic and insecticidal activities (Sarker *et al.* 2006; Setzer & Vogler 2006; Ghisalberti 2008). Specialised bioassays are becoming more sophisticated and would require scientific skills and expertise. Examples are tests for antimalarial, antiviral, anticancer, immunosuppressive, antimycobacterial and amoebicidal activities.

Chemical assays involve various chemical tests for identifying the chemical nature of compounds, e.g., Folin-Ciocalteu reagent for phenolics, Dragendorff's reagent for alkaloids and 2,2-diphenyl-1-picrylhydrazyl reagent for antioxidant compounds (Sarker *et al.* 2006).

2.5.4 Extraction

When choosing a solvent for extraction, its ability to extract components of a solute has to be considered (Ghisalberti 2008). The more efficient the extraction step, the greater is the range of compounds present in an extract. The choice of solvents is important to maximise the yields of the compounds of interest, while minimizing the extraction of unwanted compounds (Jones & Kinghorn 2005). Water, ethanol and methanol are used for extraction of polar compounds; ethyl acetate and dichloromethane for compounds of medium polarity; and n-hexane, chloroform for non-polar compounds (Sarker *et al*. 2006). The use of organic solvents also prevents microbial growth, which is one of the most serious problems associated with aqueous extraction (Shimizu & Li 2005). Organic solvents are much easier to evaporate than water.

The typical extraction process involves drying and grinding of plant materials or macerating fresh plant parts e.g. leaves and flowers with a solvent (Sarker *et al.* 2006; Ghisalberti 2008). Samples after freeze drying are normally extracted with a variety of solvents, and sometimes sequentially from low to high polarity, if a crude fractionation of metabolites is sought. Fresh plant materials can be ground to a powder using liquid nitrogen in a mortar and pestle (Zheng *et al*. 2004; Ghisalberti 2008). With this method, endocellular material is also extracted. The fresh tissue can also be finely chopped using a food processor before grinding in a mortar and pestle to rupture the cells (Cseke *et al.* 2006). This would facilitate the exit of cellular components and consequently the extraction process (Escribano-Bailón & Santos-Buelga 2003). The bottom line is that the finer the material, the better is the final extraction of compounds.

The extraction conditions applied for sample preparation can have an important influence on the type of compounds isolated as well as on the yield of extraction (Bravo & Mateos 2008). Methanol and ethanol are the most commonly used solvents. Alcohols cause instability of cell membranes, facilitating extraction of phenolic compounds, and they inactivate enzymes such as polyphenol oxidase, thus contributing to an increased stability of the extracted compounds (Arts & Hollman 1998). Sequential extraction with different solvents has also been applied to ensure more complete extraction of compounds (Bravo & Mateos 2008).

2.5.5 Chromatography

Chromatography is one of the most useful techniques for separating mixtures of compounds in extracts or fractions (Cseke *et al.* 2006; Sarker *et al.* 2006). Chromatographic techniques are available for both preparative and analytical separations of compounds. Preparative techniques are used to isolate large sample loads to get enough of a compound for further study. Common preparative techniques include the classical gravity fed open-column chromatography and the more modern vacuum liquid and flash chromatography. Analytical chromatography techniques are used to identify and quantify analytes. Resolution of individual components and reproducibility are of paramount importance. Common analytical chromatographic techniques include thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC).

The great variety of compounds makes separation and isolation of unknown active compounds from plant material a difficult task (McRae *et al.* 2007). Activity-guided fractionation is the most frequently used strategy for isolating natural products with column chromatography [**Figure 2.29**]. Analytical chromatography techniques are used to determine the components of each fraction and determine if they are pure enough for structural analysis.

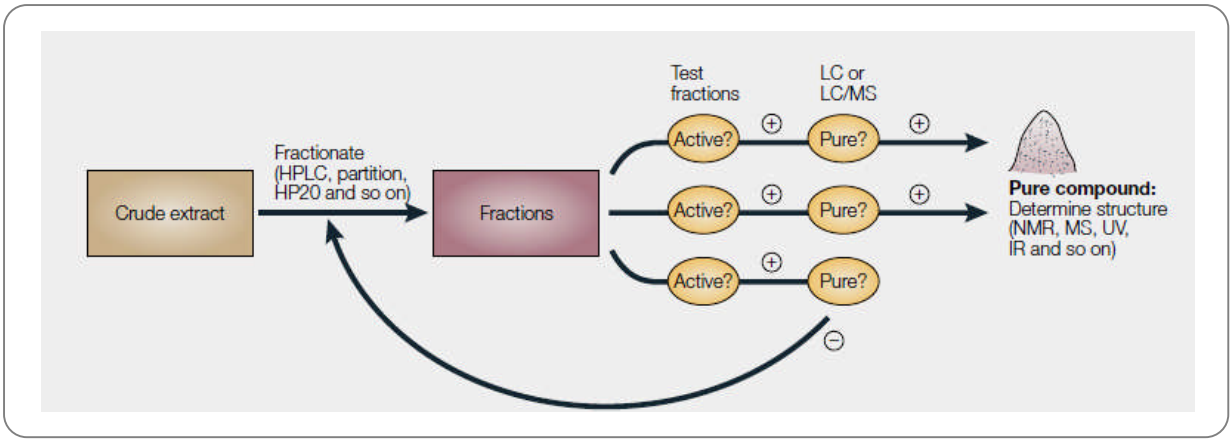


Figure 2.29 Generic scheme of activity-guided fractionation (Koehn & Carter 2005)

a. Activity-guided fractionation

Activity-guided fractionation involves various techniques of column chromatography for separating phytochemical components [**Table 2.10**], followed by testing of the bioactivity of each separated fraction. The use of different types of stationary phases or media provides more efficient separation of compounds than reliance on one type.

Table 2.10 Summary of the characteristics of stationary phases for separating plant compounds (McRae *et al.* 2007)

Stationary phase/media	Separation stage	Separation mechanism	Solvent used
Silica	Initial	Polarity	Wide range [a]
Styrene divinylbenzene	Initial [b]	Hydrophobicity	Methanol/water
C-18	Secondary	Non-polar	Methanol/water
Sephadex LH-20	Tertiary	Molecular size	Methanol/water

[a] Excluding acetone; [b] Best for aqueous extracts

After extraction of plant material using a range of different solvents with different polarities, column separation of compounds usually begins with large diameter columns packed with inexpensive media such as silica gel and styrene divinyl benzene (McRae *et al.* 2007). Larger columns are necessary at the earlier stages of purification procedures as the amount of sample loaded is higher. The properties of silica gel and other commonly used stationary phases (McRae *et al.* 2007) are summarised as follows:

Silica gel is a polar stationary phase that is selective toward polar compounds. It is one of the cheapest stationary phase available. Silica is usually discarded after each use because it is harder to be regenerated back to its original condition. When water or protic organic solvents are introduced to silica, the hydration state of the media becomes significantly altered which makes regeneration of media difficult. In addition, highly polar compounds with hydroxyl or amine groups can permanently adhere to the silica. Thus, silica is not ideal for the separation of such highly polar compounds.

Macroporous resins comprising styrene divinylbenzene are increasingly used to isolate natural products. The resin is a hydrophobic solid adsorbent media covered with aromatic groups. It has excellent selectivity towards phenolic compounds. Unlike silica, styrene divinylbenzene can be easily regenerated. Thus, it is often used as an alternative to silica to isolate highly polar compounds. Styrene divinylbenzene being a solid adsorbent media can also effectively separate structural isomers by steric interaction.

Sephadex LH-20 is a hydrophobic size exclusion media which separates compounds based on molecular size and has a slight selectivity toward hydrophobic compounds. The porosity of this gel allows large compounds to be eluted first, while smaller compounds move through the pores and are eluted later. Separation of this type can be useful for removing large molecules such as tannins and chlorophylls from the plant extract.

C-18 is a hydrophobic partition stationary phase comprising a long 18 carbon liquid stationary phase bonded onto silica particles. The material is selective towards

non-polar compounds. Some columns may also use C-8 and phenyl derivatised silica with slightly different selectivity. Phenyl columns exhibit slightly better retention of aromatic compounds.

As the fractionation process continues the number of compounds in each fraction decreases (McRae *et al.* 2007). Of the thousands of compounds in the initial extract, less than ten may be present in the active fraction after repeated chromatography. These stages of separation can be immensely time-consuming and difficult.

At later stages of fractionation, these compounds tend to elute more closely together. As a result, separation of these compounds becomes more challenging. Finer separation of such compounds is required and using columns of a smaller diameter and media of different separation mechanisms and selectivity can assist separation of these closely eluting compounds (McRae *et al.* 2007). Sometimes, media of more refined particle size is used. C-18 and Sephadex LH-20, which are more expensive media, can be used in smaller columns. These media are used to separate compound that have medium to low polarity.

b. Thin-layer chromatography

One of the fastest and most widely used chromatographic techniques is thin-layer chromatography (TLC). This method employs glass or aluminum plates pre-coated with adsorbent (e.g. silica gel and alumina) to varying thickness depending on the amount to be loaded (Gurib-Fakim 2006). Commercially available TLC plates (60 Å silica gel, 250 µm thick and with polyester or aluminum backing) are suitable for the rapid analysis of crude plant extracts (Cseke *et al.* 2006).

TLC is often used to monitor fractions eluted from column chromatography (Houghton & Raman 1998). Compounds are detected by illumination under 254 and 365 nm UV light and spraying the TLC plate with different reagents such as sulphuric acid, a general purpose spray reagent. More specialised sprays such as Dragendorff's, Folin-Ciocalteu and ninhydrin reagents may also be employed to detect alkaloids, phenols and amines, respectively

The sample is loaded at the bottom of the TLC plate which is lowered in a tank containing the solvent. The latter migrates up the plates and separates the compound mixture according to the polarity of the components. Different solvent systems are used depending on the types of compounds to be analysed [**Table 2.11**]. A schematic diagram of a thin-layer chromatographic set-up is shown in **Figure 2.30**.

Table 2.11 Solvent systems for thin-layer chromatography (Cseke *et al.* 2006)

Steroids
Benzene or benzene-ethyl acetate (9:1 or 8:2)
Chloroform-ethanol (96:4), alumina
Ethyl acetate-cyclohexane
Benzene-isopropanol
Methanol-water (95:5)
Cyclohexane-heptane (1:1)
Cyclohexane-ethyl acetate (9:1)
Benzene-chloroform (9:1)
Benzene-methanol

Terpenoids
Hexane or hexane-ethyl acetate (85:15)
Benzene or benzene-petroleum ether or benzene-ethanol
Isopropylic ether or isopropylic ether-acetone (5:2 or 19:1)

Essential oils
Benzene-chloroform
Petroleum ether

Cardenolides
Chloroform-pyridine (6:1)

Fatty acids
Petroleum ether-isopropylic ether-acetic acid (70:30:1)
Chloroform-petroleum ether

Vitamins
Methanol-carbon tetrachloride-xylene, chloroform (alumina)
Chloroform-petroleum ether
Methanol, propanol, or chloroform
Acetone-hexane (9:1)

Alkaloids
Chloroform or ethanol or hexane-chloroform (3:7)
Benzene-ethanol (9:1)
Chloroform-acetone-diethylamine (5:4:1)

Flavonoids and coumarins
Petroleum ether-ethyl acetate (2:1)
Methanol-water (8:2 or 6:4)
Toluene-ethyl formate-formic acid (5:4:1)

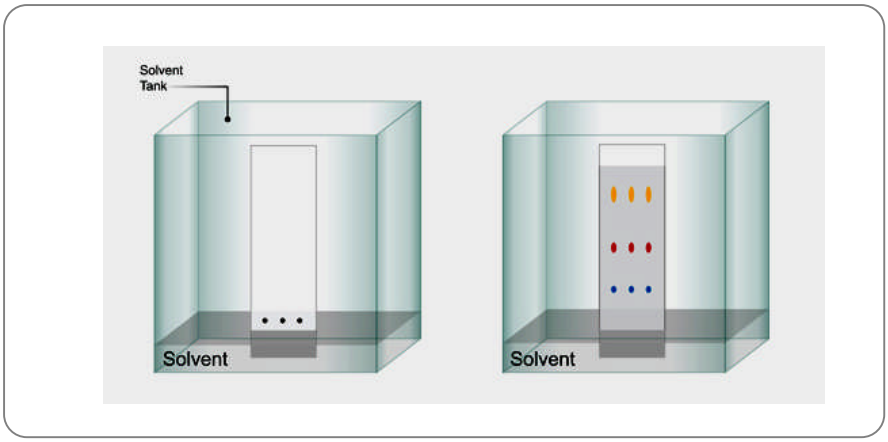


Figure 2.30 Schematic diagram of a thin-layer chromatographic set-up

c. High performance liquid chromatography

High performance liquid chromatography (HPLC) is commonly used in determining phenolic compounds present in a wide range of spices, herbs, beverages, fruits and vegetables (Jandera *et al.* 2005). Its main advantage over column chromatography is that higher pressure, applied with a pump, allows packing material with finer particle size, typically five microns, to be used. This can provide enhanced separation of compounds.

HPLC is the most powerful and frequently used technique for the separation of phenolic compounds (Bravo & Mateos 2008). It offers significant advantages in terms of simplicity, speed, cost, sensitivity, specificity, precision and sample preservation. It is versatile and adaptable for specific requirements through the use of adequate stationary phases, composition of mobile phases, and the possibility to couple with a wide range of selective detectors.

Of the various modes of HPLC, the reversed phase is the most commonly used, with the normal phase being an alternative mode (Shah & Maryanoff 2001). Normal phase HPLC is usually performed on a polar silica stationary phase with a non-polar mobile phase, while the reversed phase is performed on a non-polar stationary phase with a polar mobile phase. In reversed-phase HPLC, the analyte adsorbs to the stationary phase through the hydrophobic effect (Larson *et al.* 2001).

Reversed-phase columns are almost exclusively employed with HPLC because they provide more stable performance and better recovery of compounds compared to their normal-phase counterparts (Latif 2005). The performance of normal-phase packing materials such as silica gel are less consistent because they are hard to regenerate and are often disposed after each usage. This can be costly as analytical HPLC columns have to be factory packed for precision.

Typically, an HPLC system consists of the following components: solvent reservoir, injection system, column, HPLC pump, detector, sample collector (optional), and a computer serving as a data station for detector information as well as to control and automate the HPLC pump [**Figure 2.31**]. The performance characteristics of the chromatographic pump and solvent gradients fundamentally define and limit the kind of separation that can be performed with HPLC (Larson *et al.* 2001).

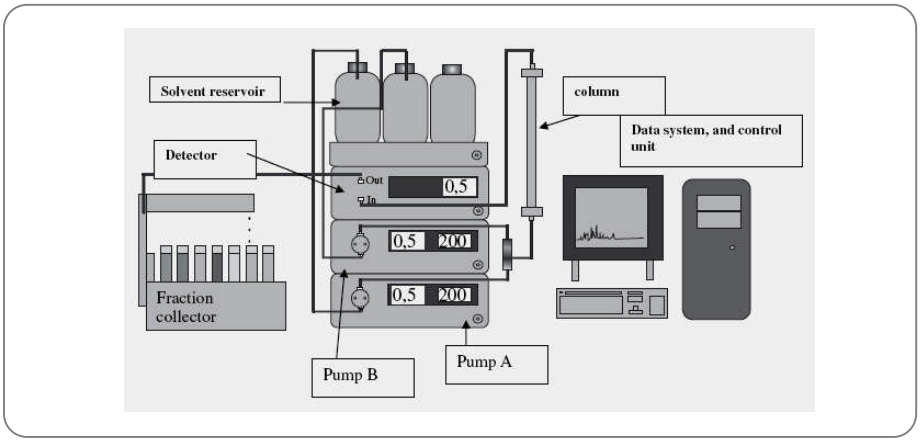


Figure 2.31 Typical HPLC system with two pumps to allow for gradients, column, detector, fraction collector and data station (Cseke *et al*. 2006)

The most commonly used columns are packed with C-18 octadecyl derivatised silica (ODS). It offers good selectivity for a wide range of compounds based on polarity (Proestos *et al.* 2006). Moreover, it allows samples to be loaded in an aqueous solvent, further improving its versatility over normal phased columns that often require pre-adsorption. Other less commonly used packing materials such as phenyl cater to more specific applications, namely, aromatic compounds. Most HPLC configurations often include a UV detector because it can be applied to a wide range of compounds. Monitoring absorbance at different wavelengths confer different

sensitivity and selectivity to the method. Identification is based upon both retention time and absorption spectrum.

2.5.6 Structural Elucidation of Compounds

a. Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy has proven to be a powerful and well-established method for determining the structures of organic compounds since the beginning of its applications in chemistry (Neri & Tringali 2001; Stobiecki & Kachlicki 2006). It is particularly useful in the field of natural products, where the problem occurs in elucidating the structure of a novel compound, sometimes with unusual structural features. The method is non-destructive and samples can be recovered after analysis for other purposes (McRae *et al.* 2007).

Basic principles of NMR are the nuclear spin and the splitting of energy levels in a magnetic field (Friebolin 2005). All nuclei that contain odd numbers of protons and neutrons possess an intrinsic magnetic moment and may adopt two different spins i.e. parallel and anti-parallel in a magnetic field. Transitions between the two spin states can be caused by the absorption of radio frequency electromagnetic waves. A schematic diagram of a NMR set-up is shown in **Figure 2.32**.

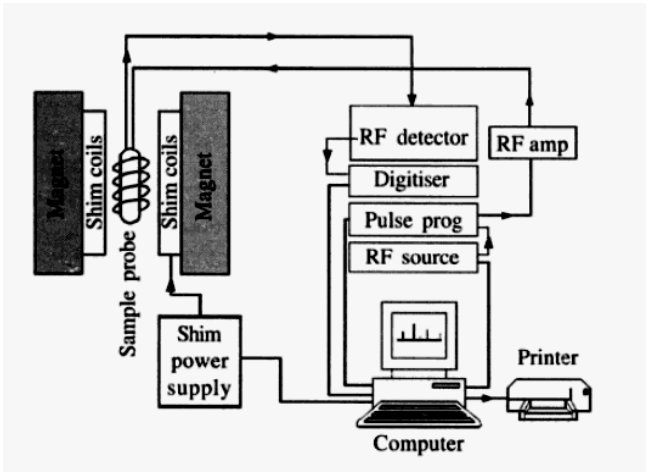


Figure 2.32 Schematic diagram of a nuclear magnetic resonance (NMR) set-up (Ghosh 2007)

One-dimensional (1D) NMR spectrum offers rich structural information of the compound (Huang *et al.* 2008). 1D NMR provides the basic information: chemical shifts, coupling constants and peak integration areas. An intense broad band radio frequency (RF) pulse is applied on to the sample and the absorbance of RF across the whole frequency range is measured. Different radio frequency is required to cause a spin transition of each nucleus depending on how they are bonded to adjacent nuclei i.e. their functional group. The unique absorption frequency of each nucleus is compared to tetramethylsilane and is used to calculate its chemical shift.

^{1}H and ^{13}C NMR spectra identify the functional groups of different hydrogen and carbon atoms respectively (Huang *et al.* 2008). A disadvantage of ^{1}H NMR is signal overlap due to the small range of ^{1}H chemical shift values. ^{13}C spectrum has much larger chemical shift values and much less signal overlap. However, because of the low abundance of the carbon-13 isotope, ^{13}C NMR has very low sensitivity and requires large amounts of sample.

Two-dimensional (2D) NMR techniques were developed to address the issues of signal overlap in ^{1}H NMR which may obscure interpretation of fine structures such as spin coupling (Vogler & Setzer 2006; Byrne 2008). 2D NMR applies more than one radio frequency pulse to induce spin transition. In most 2D NMR techniques, two RF pulses are applied with a small time lapse in between and measurements are taken after the second RF pulse. Common 2D NMR techniques include:

1) Correlation spectroscopy (COSY) where the different protons in spectrum that are coupled to each other are correlated.

2) Total correlation spectroscopy (TOCSY) where instead of correlating only one group of protons, a complete spin system of coupled protons is correlated.

3) The nuclear over-hauser effect spectroscopy (NOESY) allows the identification of those nuclei within a molecule that are close in space, irrespective of whether the nuclei are spin-spin coupled or not. It is most useful for studying large molecules.

b. Mass spectrometry

Mass spectrometry (MS) is an important tool in the structural determination of organic compounds (Tsao & Deng 2004; Vogler & Setzer 2006; Cheng *et al.* 2008). Mass spectrometers measure the mass-to-charge ratio of charged particles generated by an ion source. Mass analysers are used to separate ions based on their mass-to-charge (m/z) ratio.

Electron ionisation (EI), electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) are the most commonly used ion sources for analysing natural products. EI is the oldest and best characterised of all the ionisation methods (Vogler & Setzer 2006). A beam of electrons passes through the gas-phase sample and collides with the neutral analyte molecule. This collision can knock off an electron from the analyte molecule, resulting in a positively charged radical ion. EI is considered a hard ionisation technique that results in the fragmentation of molecules.

Most EI ion sources use electrons with energy of 40–70 eV. For molecules that are easily fragmented, analyses are often done at 40 eV (Vogler & Setzer 2006). The EI mass spectra provide reproducible structural information searchable from libraries of EI mass spectra. However, EI is less useful for analysis of molecules that are not available in mass libraries. Furthermore, because molecules have to be ionised in the gas phase, EI cannot be used for large or polar molecules which are not easily volatised.

ESI is often used to ionise polar and non-volatile molecules (Cuyckens & Claeys 2004). Because it is a soft ionisation technique that causes minimal fragmentation, it is often used for molecular weight analysis and can be easily interpreted without the aid of mass libraries (Vogler & Setzer 2006). It is often used to verify the identity of compounds analysed with NMR spectroscopy.

The ESI nebuliser can be easily linked to a HPLC instrument. Therefore, ESI is often used in combination with liquid chromatography [**Figure 2.33**]. Hybridisation of these two techniques, known as LC-MS, allows for rapid analysis and characterization of compounds in natural products which usually consist of a complex mixture of

compounds (Sherma 2003; Bravo & Mateos 2008; Cheng *et al.* 2008). LC-MS remains the most convenient and powerful method for structural identification of compounds and its use has been growing rapidly in recent years.

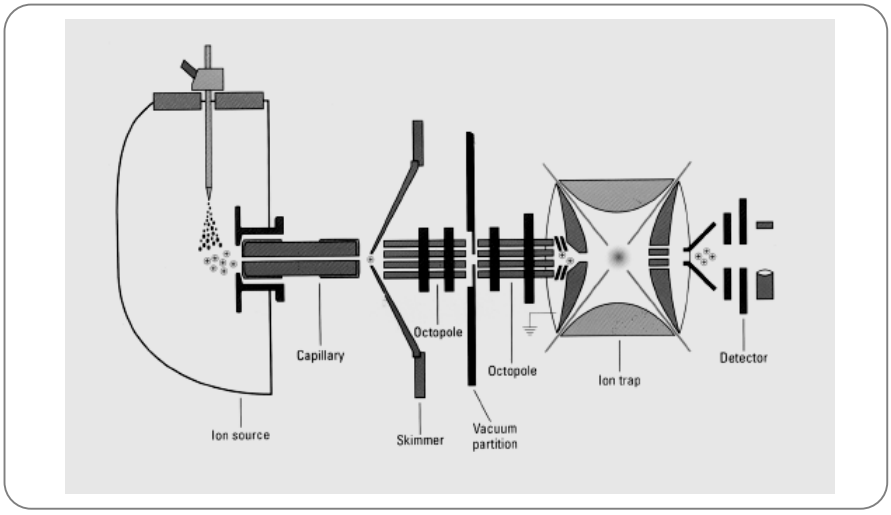


Figure 2.33 Schematic diagram of an electrospray ionization (ESI) source with mass analyser and detector (Sherma 2003)

APCI is used for less polar and non-ionic phytochemicals (Tsao & Deng 2004). Like ESI, it can be operated under both positive and negative ion modes. Because of the high temperature involved, APCI is not applicable to phytochemicals with high molecular weight (Sherma 2003). A schematic diagram of an APCI ion source is shown in **Figure 2.34**.

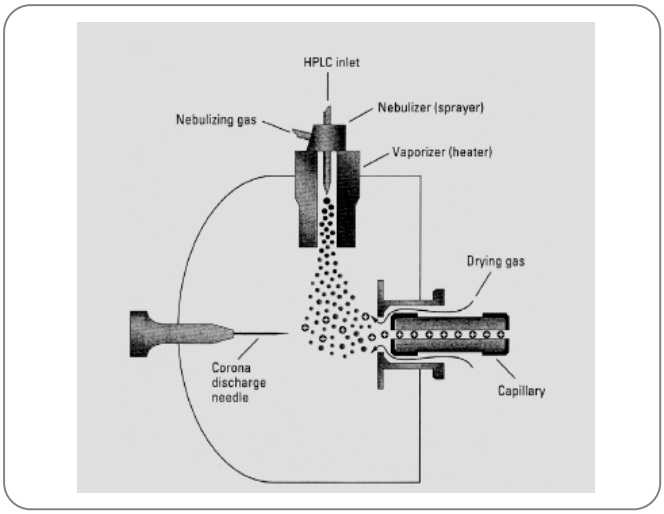


Figure 2.34 Schematic diagram of an atmospheric pressure chemical ionisation (APCI) ion source (Sherma 2003)

The two most common mass analysers used in modern MS instruments are the time of flight (TOF) and quadrupole mass filter. TOF mass analysers separate ions based on the time needed to reach the detector. Larger ions would travel more slowly than smaller ions. There is no size limit to the ions that can be analysed with TOF instruments. Therefore, TOF is often used to analyse large molecules such as proteins.

Quadrupole mass analysers consist of four parallel metal rods which have alternating positive and negative charges [**Figure 2.35**]. The alternating charges of the metal rods are used to control the flight path of the ions. The frequency of the alternating charges can be changed to allow only ions of a specific m/z ratio to reach the detector. The main disadvantage of a quadrupole mass analyser is that it cannot be used to analyse molecules with m/z greater than 3000.

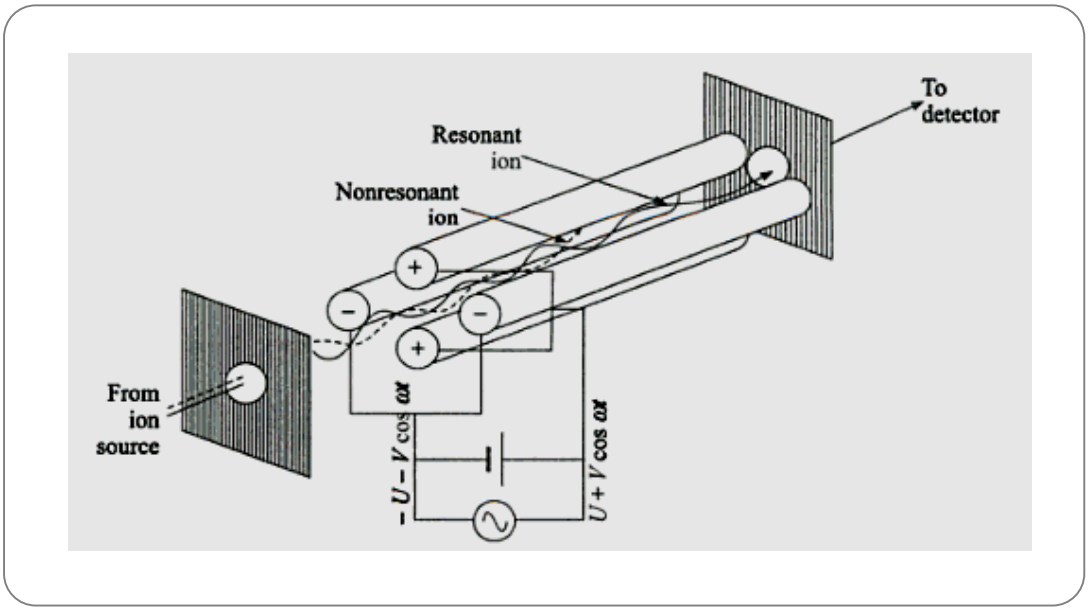


Figure 2.35 Schematic diagram of a quadrupole mass analyser (Ghosh 2007)

Chapter III

MATERIALS AND METHODS

3.1 CHEMICALS AND EQUIPMENT

Chemicals and equipment used are listed in **Appendix I** and **Appendix II**, respectively. They are classified under bioassays and natural product research.

3.2 PLANT MATERIALS

Ten Apocynaceae species were selected for study. They were *Allamanda cathartica*, *Alstonia angustiloba*, *Calotropis gigantea*, *Catharanthus roseus*, *Cerbera odollam*, *Dyera costulata*, *Kopsia fruticosa*, *Nerium oleander*, *Plumeria obtusa* and *Vallaris glabra* [**Figure 3.1**].

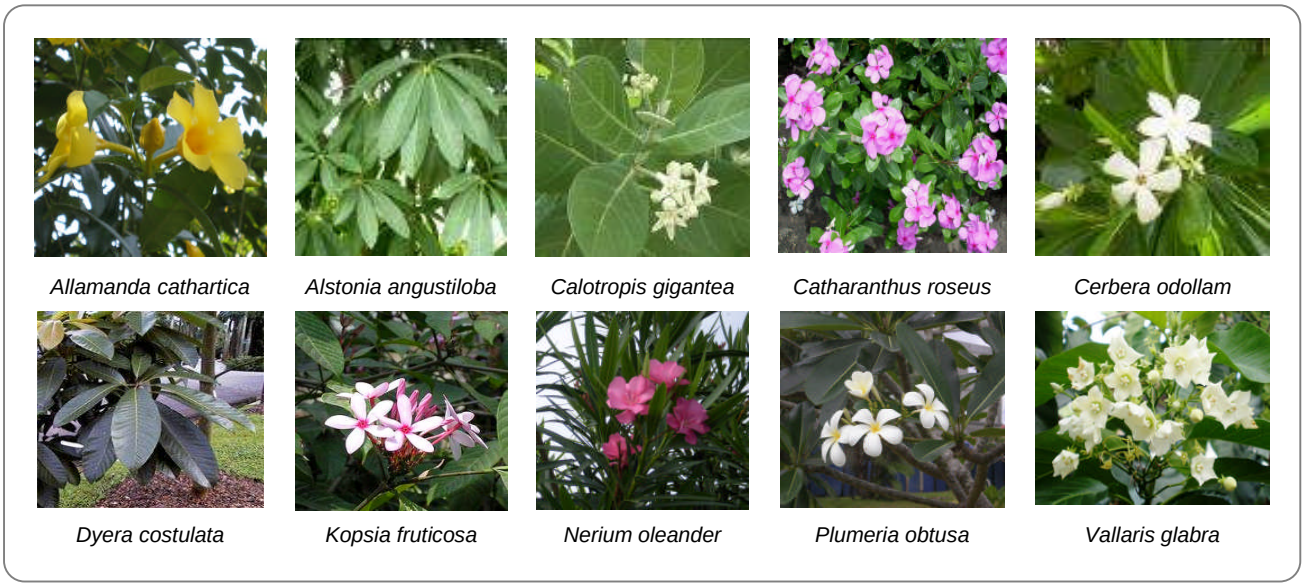


Figure 3.1 The ten Apocynaceae species studied

Their common or vernacular names and brief descriptions are given in **Table 3.1**. Leaves of the species were collected from Sunway (3°4’30”N; 101°36’8” E), Puchong (3°2’42”N; 101°37’12”E), Kepong (3°12’14”N; 101°37’50"E) and the Forest Research Institute Malaysia (3°14’6”N; 101°37’58”E), all in the state of Selangor, Malaysia.

Identification of species was based on descriptions and illustrations by Wiart (2006) and Ng (2006). Voucher specimens of these species were deposited in the herbarium of Monash University Sunway Campus.

Table 3.1 Common or vernacular names and brief descriptions of Apocynaceae species studied

Species (common or vernacular name)	Brief description
Allamanda cathartica L. (Common allamanda)	A shrub with trumpet-shaped yellow flowers similar in size as leaves which are in whorls
Alstonia angustiloba Miq. (Pulai)	A medium-sized tree with leaves in whorls and having fine secondary veins
Calotropis gigantea (L.) Aiton (Giant milkweed)	A shrub with pale green leaves and white or lilac flowers with a crown rising from the centre
Catharanthus roseus (L.) G. Don (Madagascar periwinkle)	A common ornamental shrub with oval to oblong leaves and white, pink or purple flowers with a dark coloured centre
Cerbera odollam Gaertner (Pong-pong)	A tree bearing white flowers in clusters and rounded fruits that are green when young and red when mature
Dyera costulata Hook (Jelutong)	A tall timber tree with straight columnar bole, leaves in whorls and latex which was an important source of chewing gum
Kopsia fruticosa (Ker.) A. DC. (Pink kopsia)	A shrub with large glossy leaves and clusters of light pink flowers resembling those of *Ixora*
Nerium oleander L. (Oleander)	An ornamental shrub with thick narrow leaves in pairs or whorls and bearing clusters of pink, red or purple flowers
Plumeria obtusa L. (Frangipanni)	A tree producing dark green, glossy and oval leaves and white fragrant flowers with a yellow centre
Vallaris glabra (L.) Kuntze (Kesidang)	A woody climber producing clusters of white flowers with a scent characteristic of *Pandanus amaryllifolius* (pandan) leaves or fragrant rice

3.3 EXTRACTION OF LEAVES

3.3.1 Extraction of Dried Leaves

a. Crude extraction

For crude extraction, fresh leaves of 10 Apocynaceae species (40 g each) were cut into small pieces and freeze-dried overnight. Dried samples were blended and extracted with 250 mL of methanol (MeOH) three times for 1 hour each time. Samples were filtered and the solvent was removed using a rotary evaporator (Eyela). The dried crude extracts were stored at -20^{o}C for further analysis.

b. Sequential extraction

For sequential extraction, fresh leaves of 10 Apocynaceae species (40 g each) were freeze-dried overnight, ground and extracted successively with hexane (Hex), dichloromethane (DCM), DCM:MeOH (1:1) and MeOH. For each solvent, the suspension of ground leaves in 200 mL of solvent was shaken for 1 hour on the orbital shaker. After filtering, the samples were extracted two more times for each solvent. Solvents were removed with a rotary evaporator to obtain the dried extracts, which were stored at -20^{o}C for further analysis.

3.3.2 Extraction of Fresh Leaves

a. Extraction efficiency

Extraction efficiency of methanol (70% and 100%) was tested on leaves of ten Apocynaceae species based on their total phenolic content (TPC) and ascorbic acid equivalent antioxidant capacity (AEAC) values.

b. Extraction procedures

Fresh leaves of six species (1 g each) were powdered with liquid nitrogen in a mortar and extracted with 50 mL of 70% methanol. Extracts were filtered under suction,

prepared in triplicate and stored at 4°C for analyses which were conducted within a week of extraction.

3.4 EXTRACTION OF OTHER PLANT PARTS

With the selection of *Vallaris glabra* as the main species of study, flowers and stems were also screened for antiproliferative activity, phenolic content and antioxidant activity for comparison with those of leaves.

For screening of antiproliferative activity of *Vallaris glabra*, freeze-dried flowers and stems (10 g each) were sequentially extracted with Hex, DCM, DCM:MeOH (1:1) and MeOH (50 mL each). Extraction followed the same protocol as in **item 3.3.1a**.

For analysing phenolic content and antioxidant activity, fresh flowers and stems of *Vallaris glabra* (1 g each) were extracted with 50 mL of 70% methanol, following the same protocol as in **item 3.3.2b**.

3.5 ANTIPROLIFERATIVE ACTIVITY

3.5.1 Cell Culture

Human cancer cells, namely, MCF-7 (breast), MDA-MB-231 (breast), HeLa (cervical), HT-29 (colon), SKOV-3 (cervical) and HepG2 (liver) were obtained from American Type Culture Collection (ATCC), Manassas, VA, USA. A brief description of these human cancer cells is given in **Appendix III**.

Three human cancer cell lines (MCF-7, MDA-MB-231 and HeLa) were used for the initial screening of the APF activity of leaf extracts of the ten Apocynaceae species [**item 4.1.1**]. Six species with positive inhibition were further tested to quantify their APF activity using six human cancer cell lines (MCF-7, MDA-MB-231, HeLa, HT-29, SKOV-3 and HepG2) [**item 4.1.2**]. These studies were conducted in the laboratory of my collaborator at the Institute of Medical Research (IMR), Malaysia.

Vallaris glabra was then chosen as the main species for further study. Four human cancer cell lines (MCF-7, MDA-MB-231, HT-29 and SKOV-3) were used for initial screening and further testing of the APF activity of stems and flowers [**section 4.6**], fractions [**section 4.7**], and compounds [**section 4.10**] of *Vallaris glabra*. The four human cancer cell lines were used for rapid screening and testing. These studies were conducted in the laboratory of Monash University Sunway Campus (MUSC), Malaysia.

The cells were either cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, USA) or Roswell Park Memorial Institute 1640 medium (RPMI-1640; Sigma-Aldrich, USA). Media were supplemented with 10% heat-inactivated foetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin. The cell cultures were kept at 37°C in an incubator with a humidified atmosphere of 5% CO_2 in air. To maintain cells in the logarithmic growth phase, the cells were sub-cultured using 0.05% trypsin-EDTA every 2–3 days. Methods described were adapted from Cheah *et al.* (2008).

3.5.2 Sulphorhodamine B Assay

Antiproliferative (APF) activity of sequential leaf extracts of 10 Apocynaceae species (25 µg/mL) was initially screened for inhibitory activity against three types of human cancer cells (MCF-7, MDA-MB-231 and HeLa) using the sulphorhodamine B (SRB) assay. Procedures of the assay were adopted from Vichai & Kirtikara (2006) and Cheah *et al.* (2008) with slight modifications. Sequential flower and stem extracts of *Vallaris glabra* were also screened for inhibitory activity against four types of human cancer cells (HT-29, MCF-7, MDA-MB-231 and SKOV-3) for comparison with that of leaves.

Control cultures received the same concentration of solvent without the leaf extracts. Standard drugs of xanthorrhizol, curcumin and tamoxifen were used as positive controls. They have been reported to induce apoptosis in a panel of five human breast cancer cell lines including MCF-7 and MDA-MB-231 used in this study (Cheah 2008).

Inhibitory activity of less than 50% cell density was considered positive while that of more than 50% cell density was considered negative (Vichai & Kirtikara 2006). The formula used to obtain percentage cell density was $[(T_c - T_i)/T_c] \times 100\%$ where T_i represents absorbance of treated cells and T_c represents absorbance of cells treated with vehicle (0.5–1.0% DMSO).

Sequential leaf extracts of the 10 Apocynaceae species with positive inhibitory activity were further tested against six types of human cancer cells (MCF-7, MDA-MB-231, HeLa, HT-29, SKOV-3 and HepG2) using six different concentrations of extracts.

The cells were seeded 24 hours prior to treatment in 96-well plates at appropriate densities (30,000 cells/mL for MCF-7 and HeLa; 60,000 cells/mL for MDA-MB-231, HT-29 and HepG2; and 120,000 cells/mL for SKOV-3) with each well containing 100 µL of cell culture. Extracts (5 mg/mL) were dissolved in 100% dimethyl sulphoxide (DMSO) and serially diluted two-fold to concentrations ranging from 3.13–50.0 µg/mL with medium. As 100 µL of the diluted samples were introduced to the existing 100 µL of cell cultures, the final concentration of extracts ranged from 1.56–25.0 µg/mL. The control cultures were treated with the same volume of DMSO (0.5–1.0%). The concentration of DMSO was kept within 1% to avoid any interference with cell viability. After the addition of extracts, the plates were incubated for 48 hours. After incubation, the cells were fixed with 50 µL of cold 50% TCA and incubated for 1 hour at 4°C. The plates were then washed with tap water and air dried. Cells were stained with 100 µL of 0.4% SRB solution diluted with 1% acetic acid followed by incubation for 10 min at room temperature. Unbound dye was removed by washing with 1% acetic acid. Bound stain was then solubilised with 200 µL of 10 mM trizma base.

Absorbance of each well at 505 nm was obtained using a microplate reader. Dose-response curves were constructed to obtain GI_{50} and IC_{50}. GI_{50}, the concentration of extract which causes 50% reduction in cell growth was calculated using the formula $[(T_z - T_i)/(T_c - T_i)] \times 100 = 50$. T_z is the absorbance of cells treated with extracts or drugs at the end of incubation, T_i is the absorbance of cells prior to treatment with extracts or drugs, and T_c is the absorbance of untreated cells treated with vehicle (0.5–1.0% DMSO) at the end of incubation. IC_{50}, the concentration of extract which

causes 50% inhibition of cell density, was obtained using the formula $T_z/T_c \times 100 = 50$. APF activity is considered to be effective when GI_{50} value ≤ 20 µg/mL (Gaidhani *et al.* 2009).

3.6 ANTIPLASMODIAL ACTIVITY

Antiplasmodial (APM) activity of sequential leaf extracts of five Apocynaceae species was determined against the chloroquine-resistant (K1) and chloroquine-sensitive (3D7) strains of *Plasmodium falciparum* that were continuously cultured according to the methods described by Trager & Jensen (1976). Plant extracts were assessed for antiplasmodial activity *in vitro* in human blood using parasite lactate dehydrogenase method with slight modifications (Makler & Hinrichs 1993; Makler *et al*. 1993; Chan *et al.* 2004). Microtitration techniques were used to measure the activity of samples over a wide range of concentrations. All tests were performed in duplicate.

Chloroquine-resistant K1 and chloroquine-sensitive 3D7 strains of *Plasmodium falciparum* were tested. Standard drugs of artemisinin (Sigma) and mefloquine (Sigma) were used as positive controls.

Leaf extracts dissolved in DMSO (10 mg/mL) were diluted with deionised water to 320 µg/mL. The solution was serially diluted two-fold six times to give seven different concentrations. Aliquots of each concentration (10 µL) were transferred into 96-well microtiter plates. Parasitised red blood cell suspensions (1% parasitaemia, 190 µL were added to each well. Parasitised and non-parasitised red blood cells were used as negative controls. The plates were incubated for 24 hours at 37^{o}C in a candle jar and were subsequently chilled at -20^{o}C to lyse the red blood cells. The plates were then allowed to cool to room temperature, and 20 µL of blood suspension was dispensed into a new microtiter plate containing 100 µL MALSTAT^{TM} reagent, and 20 µL of nitroblue tetrazolium and phenazine ethosulphate mixture.

Absorbance was measured with a plate reader at 630 nm. Percentage inhibition at each concentration was determined and the mean of EC_{50} values of parasite viability was calculated using probit analysis. EC_{50} or effective concentration is the extract concentration that kills 50% of malaria parasites. APM activity is effective if EC_{50}

value ≤ 10 µg/mL (Tran *et al.* 2003). The selectivity index (SI) for APM activity was calculated based on the ratio of cytotoxicity (IC_{50}) on HepG2 and MCF-7 cells to APM activity (EC_{50}) on chloroquine-resistant K1 strain.

3.7 ANALYSES OF PHYTOCHEMICAL CONTENTS

3.7.1 Total Alkaloid Content

Total alkaloid content (TAC) of crude and sequential leaf extracts of 10 Apocynaceae species was determined using the Dragendorff precipitation assay (Ribeiro *et al.* 2008). For each species, extracts (15 mg) were dissolved in 1 mL of distilled water that was acidified to pH 2.0–2.5 with 0.01 M HCl. Analysis was conducted in triplicate. Alkaloids were then precipitated with 0.4 mL of Dragendorff's reagent. Washed with 0.5 mL of distilled water to remove traces of the reagent, the precipitate was later treated with 0.4 mL of 1% sodium sulphide, resulting in a brownish-black precipitate. Precipitates formed at each stage were recovered by centrifugation at 14,000 rpm for 1 min. The resulting precipitate was dissolved in 0.2 mL of concentrated nitric acid and diluted to 1 mL with distilled water. Addition of 2.5 mL of 3% thiourea to 0.5 mL aliquots of this solution resulted in a yellow coloured complex.

Absorbance was measured at 435 nm and TAC was expressed as boldine equivalent in milligram per gram of extract. The calibration equation for boldine (Sigma) was y = 1.068x (R^2 = 0.9959) where y is absorbance and x is mg/mL of boldine.

Dragendorff's reagent was prepared by dissolving 0.8 g of bismuth nitrate (Sigma) in 40 mL of distilled water and 10 mL of glacial acetic acid. The resulting solution was mixed with 20 mL of 40% potassium iodide.

3.7.2 Total Phenolic Content

Total phenolic content (TPC) of crude and sequential leaf extracts of 10 Apocynaceae species, and fresh extracts of six Apocynaceae species was determined using the Folin-Ciocalteu (FC) assay (Kähkönen *et al.* 1999; Wong *et al.*

2009). TPC of fresh flower and stem extracts of *Vallaris glabra* was also analysed for comparison with that of leaves.

Extracts (300 µl in triplicate) were introduced into test tubes followed by 1.5 mL of FC reagent (Fluka) at 10 times dilution and 1.2 mL of sodium carbonate (Fluka) at 7.5% w/v. The tubes were allowed to stand for 30 min in the dark before absorbance was measured at 765 nm. TPC was expressed as gallic acid (GA) equivalent in milligram per gram of sample (fresh weight or dried extract). The calibration equation for GA (Fluka) was y = 0.0111x – 0.0148 (R^2 = 0.9998) where y is absorbance and x is mg/mL of GA.

3.7.3 Caffeoylquinic Acid Content

Caffeoylquinic acid content (CQAC) of fresh leaf extracts of six Apocynaceae species was quantified using the molybdate assay (Chan *et al.* 2009). CQAC of fresh flower and stem extracts of *Vallaris glabra* was also analysed for comparison with that of leaves.

Molybdate reagent was prepared by dissolving 16.5 g sodium molybdate, 8.0 g dipotassium hydrogen phosphate and 7.9 g potassium dihydrogen phosphate in 1 L water. The reagent (2.7 mL) was added to the plant extract (0.3 mL), mixed and incubated room temperature for 10 min before absorbance was measured at 370 nm against a sample blank consisting of 0.3 mL of the respective extracts with 2.7 mL of water. CQAC was expressed as mg chlorogenic acid equivalent (CGAE)/g of sample (fresh weight).

3.8 ANTIOXIDANT AND ANTITYROSINASE ASSAYS

3.8.1 Free Radical Scavenging Activity

Free radical scavenging (FRS) activity of crude and sequential leaf extracts of 10 Apocynaceae species, fresh leaf extracts of six Apocynaceae species, and fresh flower and stem extracts of *Vallaris glabra* was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay (Miliauskas *et al.* 2004; Wong *et al.* 2009).

Different dilutions of extracts (1 mL in triplicate) were added to 2 mL of DPPH (Sigma). The concentration of DPPH used was 5.9 mg in 100 mL of methanol. Absorbance was measured at 517 nm after 30 min. FRS activity was calculated as IC_{50}, the concentration of extract to scavenge 50% of the DPPH radical. FRS activity was then expressed as ascorbic acid (AA) equivalent antioxidant capacity (AEAC) in AA mg/g of sample (fresh weight or dried extract). FRS was calculated using the equation of AEAC = $IC_{50(ascorbate)}/IC_{50(sample)}$ x 10^3. IC_{50} of ascorbic acid used for calculation of AEAC was 0.00387 mg/mL.

3.8.2 Antityrosinase Activity

The antityrosinase (AT) activity of crude leaf extracts of ten Apocynaceae species was determined using the modified dopachrome method with L-3,4-dihydroxy phenylalanine (L-dopa) as substrate (Masuda *et al*. 2005).

Assays were conducted in a 96-well microtiter plate and a plate reader was used to measure absorbance at 475 nm, with 700 nm as reference. Samples were dissolved in 50% dimethyl sulphoxide (DMSO). Each well contained 40 µL of sample with 80 µL of phosphate buffer (0.1 M, pH 6.8), 40 µL of tyrosinase (31 units/mL) and 40 µL of L-dopa (2.5 mM). Each sample was accompanied by a blank that had all the components except L-dopa. Results were compared with a control consisting of 50% DMSO in place of sample. AT activity was calculated as $(A_{control} - A_{sample})/A_{control}$ x 100%. Leaves of *Psidium guajava* (guava) were used as positive control as they have been reported to have strong tyrosinase inhibition (Wong *et al*. 2010).

3.9 QUALITATIVE PHYTOCHEMICAL SCREENING

Qualitative phytochemical screening for saponins, tannins, cardenolides and terpenoids from sequential leaf extract of *Vallaris glabra* was carried out using standard phytochemical procedures (Harborne 1998; Houghton & Raman 1998). The frothing test was used for saponins, the ferric chloride test for tannins, the Keller-Killani test for cardenolides and the Salkowski test for terpenoids.

3.9.1 Frothing Test for Saponins

The procedure for frothing test for saponins described by Raaman (2006) was adopted. The extract (50 mg) was diluted with distilled water and made up to 20 mL. The solution was shaken in a graduated cylinder for 15 min. A layer of foam formed indicates the presence of saponins.

3.9.2 Ferric Chloride Test for Tannins

The procedure for ferric chloride test for tannins described by Raaman (2006) was adopted. The extract (50 mg) was dissolved in 5 mL of distilled water. A few drops of 5% ferric chloride solution were added. A dark green colour indicates the presence of tannins.

3.9.3 Keller-Killani Test for Cardenolides

The procedure for Keller-Killani test for cardenolides described by Edeoga *et al.* (2005) was adopted. The extract (5 mL) was treated with 2 mL of glacial acetic acid containing one drop of ferric chloride solution. Concentrated H_2SO_4 (1 mL) was then added. A brown ring formed at the interface indicates the presence of cardenolides. A violet ring may appear below the brown ring.

3.9.4 Salkowski Test for Terpenoids

The procedure for Salkowski test for terpenoids described by Edeoga *et al.* (2005) was adopted. The extract (5 mL) was mixed in 2 mL of $CHCl_3$ and concentrated H_2SO_4 (3 mL) was carefully added. A reddish brown colouration at the interface formed is a positive indication for the presence of terpenoids.

3.10 ISOLATION OF PHYTOCHEMICALS

3.10.1 Extraction for Isolation

Vallaris glabra was selected for isolation due to its potency with respect to antiplasmodial and antiproliferative activities. Fresh leaves (2 kg) were extracted successively three times with 5 L of Hex, DCM, DCM:MeOH (1:1) and MeOH. Extraction was performed three times for each solvent. Solvents were removed by evaporation and extracts were stored at -20^{o}C for later use in chromatography.

3.10.2 Isolation Procedures

a. Adsorbents used for isolation

Adsorbents used for isolating phytochemicals from MeOH and DCM leaf extracts of *Vallaris glabra* were MCI CHP-20P gel, Chromatorex C18, Diaion HP20SS, Silica gel 60 and Sephadex LH-20. Brief descriptions of these adsorbents are given in **Box 3.1**.

b. Column chromatography of MeOH leaf extract

The bioactivity-guided approach was adopted during the initial isolation steps. The MeOH sequential extract of *Vallaris glabra* that yielded the M series of fractions had the highest antioxidant activity of all the sequential extracts. However, as the results of **Table 4.3** show, the MeOH extract displayed antiproliferative activity against only two cell lines and was much less potent than the DCM sequential extract. Hence, steps were taken to isolate the major antioxidative constituents from the MeOH sequential extract. HPLC analysis showed that the UV spectra of major constituents corresponded to caffeoylquinic acids that are well-known antioxidants.

A schematic diagram of column chromatographic protocols for methanol leaf extract of *Vallaris glabra* is shown in **Figure 3.2**. Methanol leaf extract (40 g) of *Vallaris glabra* was chromatographed on a MCI CHP-20P gel column with gradient H_2O-MeOH (0→100% MeOH) to give eight fractions (M1 to M8).

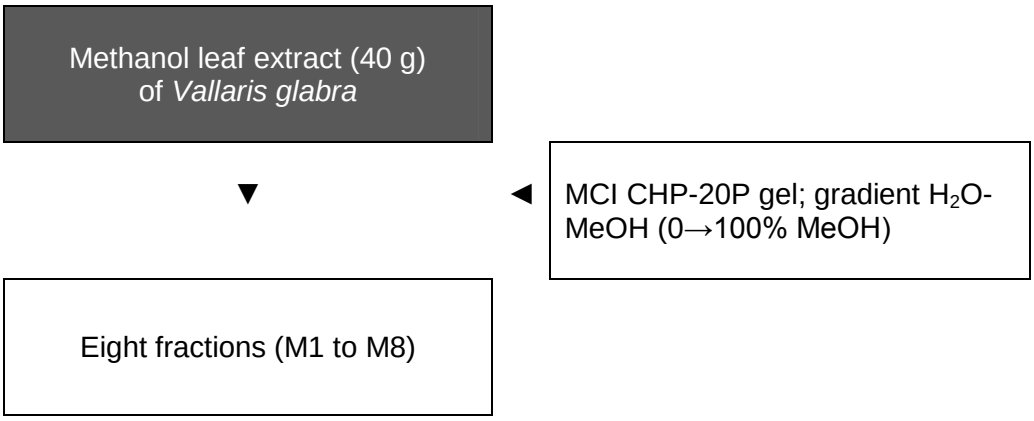


Figure 3.2 A schematic diagram of column chromatographic protocols for methanol leaf extract of *Vallaris glabra*

Fraction M4 (0.69 g) was then subjected to Chromatorex C18 with gradient H_2O-MeOH (0→100% MeOH) to give two sub-fractions (M4-1 and M4-2). Sub-fraction M4-1 (0.60 g) was passed through Silica gel 60 with gradient $CHCl_3$:MeOH:H_2O (10:0:0→6:4:1) to give eight sub-fractions (M4-1-1 to M4-1-8). Sub-fraction M4-1-6 (0.51 g) was then subjected to MCI CHP-20P gel with gradient H_2O-MeOH (0→100% MeOH) to obtain Compound **1** (9 mg), Compound **2** (38 mg) and Compound **3** (24 mg) [**Figure 3.3**].

Fraction M7 (1.15 g) was subjected to MCI CHP-20P gel with gradient H_2O-MeOH (0→100% MeOH) to give 21 sub-fractions (M7-1 to M7-21). Sub-fraction M7-11 (0.21 g) was then chromatographed with Chromatorex C18 of gradient H_2O-MeOH (20→100% MeOH) to give 10 sub-fractions (M7-11-1 to M7-11-10). Sub-fraction M7-11-5 (0.20 g) was then passed through Diaion HP20SS with gradient H_2O-MeOH (20→100% MeOH) to give 14 sub-fractions (M7-11-5-1 to M7-11-5-14). Sub-fraction M7-11-5-7 (0.19 g) was further chromatographed with Chromatorex C18 of gradient H_2O-MeOH (15→100% MeOH) to give nine sub-fractions (M7-11-5-7-1 to M7-11-5-7-9). Sub-fraction M7-11-5-7-7 (0.15 g) was subjected to Chromatorex C18 with gradient H_2O-MeOH (0→100% MeOH) to obtain Compound **4** (88 mg) [**Figure 3.4**].

Box 3.1 Adsorbents used in column chromatography

MCI CHP-20P gel

Particle size 75–150 µm, Mitsubishi (Supelco, USA)

MCI CHP-20P stationary phase is a poly-aromatic adsorbent resin made from styrene divinylbenzene. The resin, designed for biopharmaceutical separation, is covered with pores ranging 400–600 Å in size. It is useful for separating a wide range of compounds such as aromatic compounds, peptides and steroids. It is very good for reversed-phase chromatography applications, even with non-aqueous solvents.

Chromatorex C18 silica gel

Particle size 40–75 µm (Fuji Silysia, Japan)

Chromatorex C18 is an octadecyl derivatised silica stationary phase made from octadecyl groups (C18 hydrocarbon chain) chemically bonded to surface silanol groups (Si-O-H) of silica particles. It is a non-polar stationary phase commonly used in reversed-phase chromatography. It is perhaps the most widely used stationary phase as its physical and chemical properties make it suitable for a wide variety of analytical and preparative separations.

Diaion HP20SS

Particle size 75–150 µm (Supelco, USA)

Diaion HP20SS is a poly-aromatic adsorbent resin made from styrene divinylbenzene. They are chemically similar to MCI gel CHP-20P but have a smaller pore size of 260 Å. It provides excellent kinetics and capacity for small biomolecules at both preparative and process-scale. It offers a balance of pressure flow characteristics and chromatographic fractionation.

Silica gel 60

Particle size 40–63 µm (Merck, USA)

Silica gel 60 is used in normal phase chromatography. Because it is a polar stationary phase, polar components adsorb more strongly to silica gel and are eluted later compared to less polar ones. Silica particles of different pore sizes can be used to achieve optimal separation of certain molecular sizes. For natural products, silica particles with a pore size of 60 Å are often used.

Sephadex LH-20

Particle size 25–100 µm (Sigma-Aldrich, USA)

Sephadex LH-20, prepared by hydroxypropylation of Sephadex G-25, is a beaded dextran gel. It is specifically developed for filtration of natural products (e.g. steroids, terpenoids, lipids and low molecular weight peptides) in organic solvents.

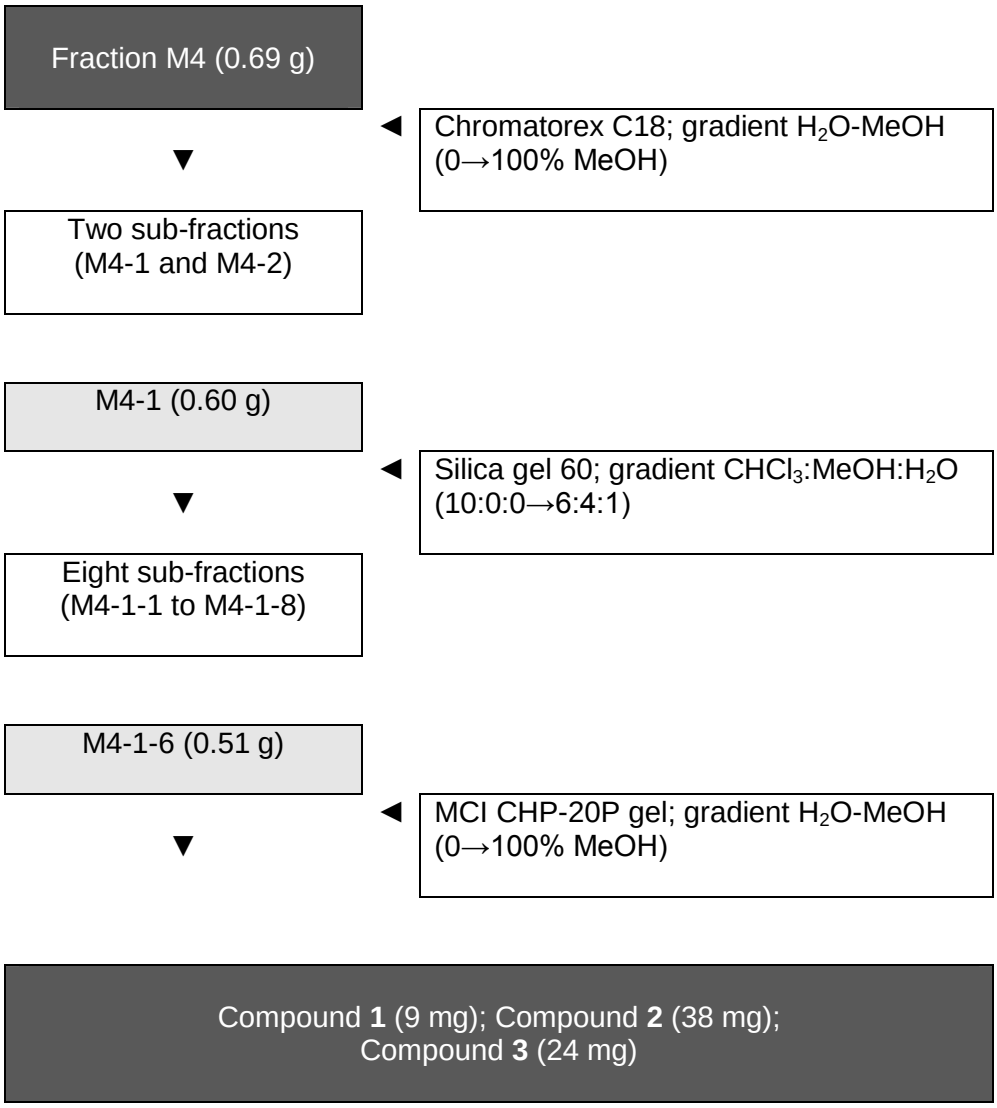


Figure 3.3 Schematic diagram of column chromatographic protocols for isolating Compounds **1**, **2** & **3** from methanol leaf extract of *Vallaris glabra*

Fractions were pooled according to their thin-layer chromatography (TLC) profile. TLC was carried out on Merck pre-coated silica gel F254 plates. Samples were spotted onto TLC plate and resolved using suitable mixtures of chloroform, methanol and water that ensure proper resolution of bands. Plates that were resolved were visualised under ultraviolet (UV) light of two wavelengths (254 and 365 nm) and then sprayed with 10% H_2SO_4 followed by heating.

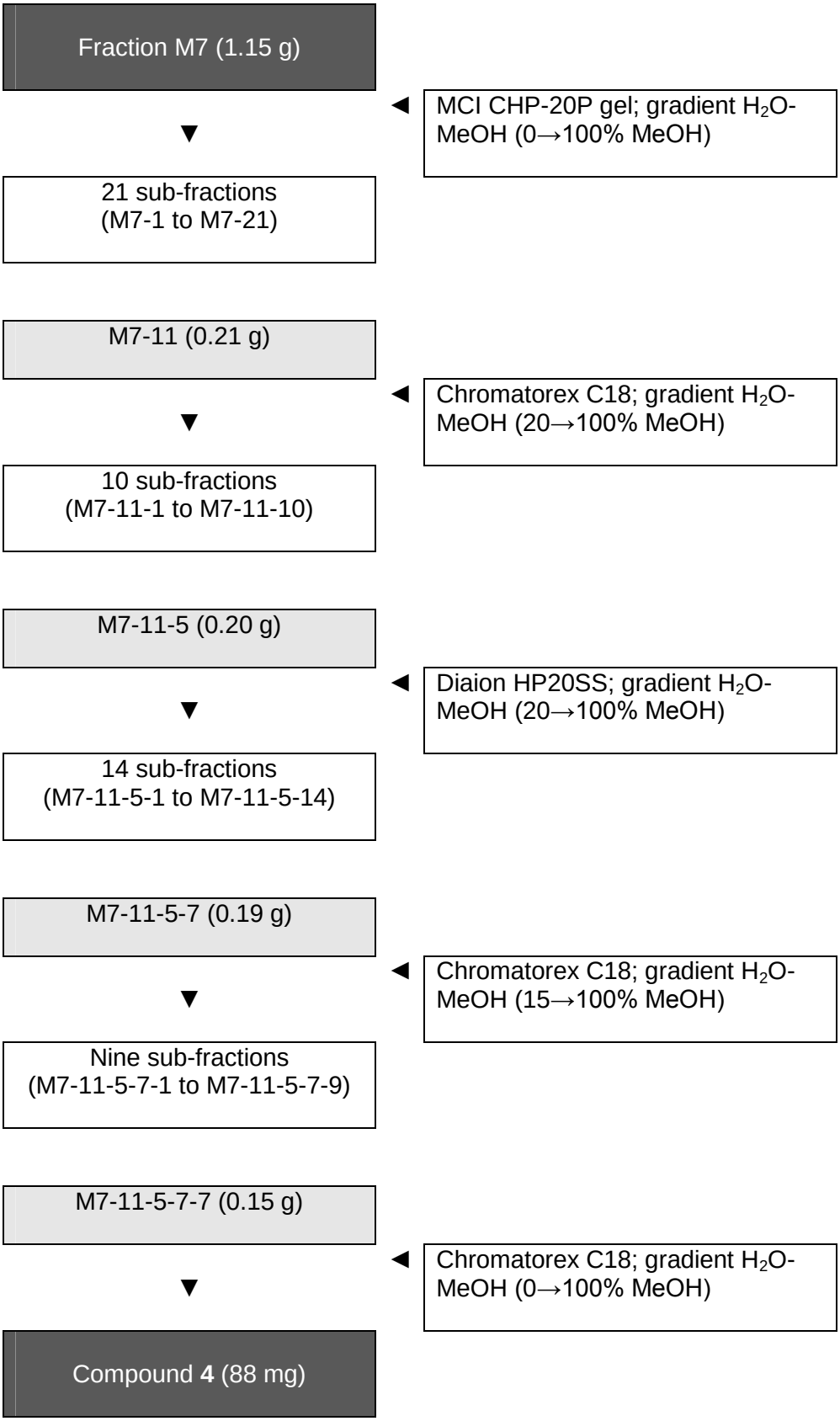


Figure 3.4 Schematic diagram of column chromatographic protocols for isolating Compound **4** from methanol leaf extract of *Vallaris glabra*

c. Column chromatography of DCM leaf extract

A schematic diagram of column chromatographic protocols for DCM leaf extract of *Vallaris glabra* is shown in **Figure 3.5**. DCM leaf extract (26 g) of *Vallaris glabra* was subjected to column chromatography over Silica gel 60. A solvent system with an increasing polarity gradient was used (Hex-EtAc and EtAc-MeOH) to give 13 fractions (D1 to D13).

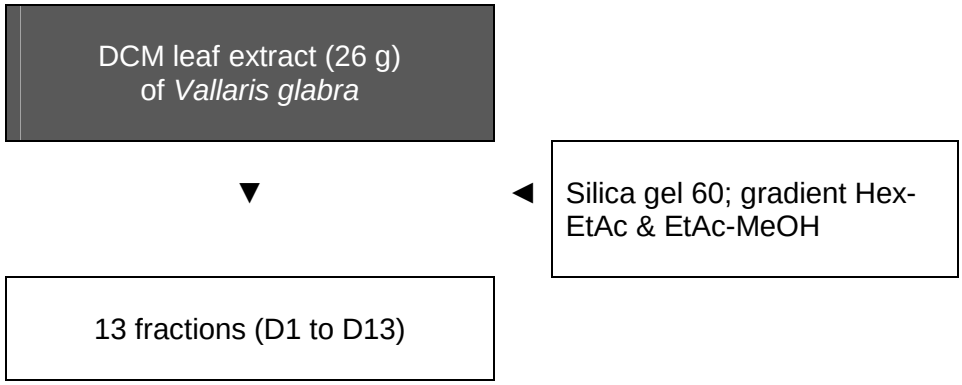


Figure 3.5 Schematic diagram of column chromatographic protocols for DCM leaf extract of *Vallaris glabra*

As fraction D11 (3.26 g) was found to have the strongest APF activity [**item 4.7.2**], it was passed through Sephadex LH-20 with gradient H_2O-MeOH (50→100% MeOH) to give five sub-fractions (D11-1 to D11-5). Sub-fraction D11-1 (1.38 g) was then subjected to Chromatorex C18 with gradient H_2O-MeOH (10→100% MeOH) to give six sub-fractions (D11-1-1 to D11-1-6). Sub-fraction D11-1-5 (0.27 g) was passed through Sephadex LH-20 with gradient $CHCl_3$-EtAc (0→100% EtAc) to give three sub-fractions (D11-1-5-1 to D11-1-5-3). Sub-fraction D11-1-5-2 (0.16 g) was passed through Sephadex LH-20 with gradient $CHCl_3$-EtAc (0→50% EtAc) to give six sub-fractions (D11-1-5-2-1 to D11-1-5-2-6). Sub-fraction D11-1-5-2-2 (86 mg) was subjected to Chromatorex C18 with gradient H_2O-MeOH (10→100% MeOH) to obtain Compound **5** (12 mg) [**Figure 3.6**].

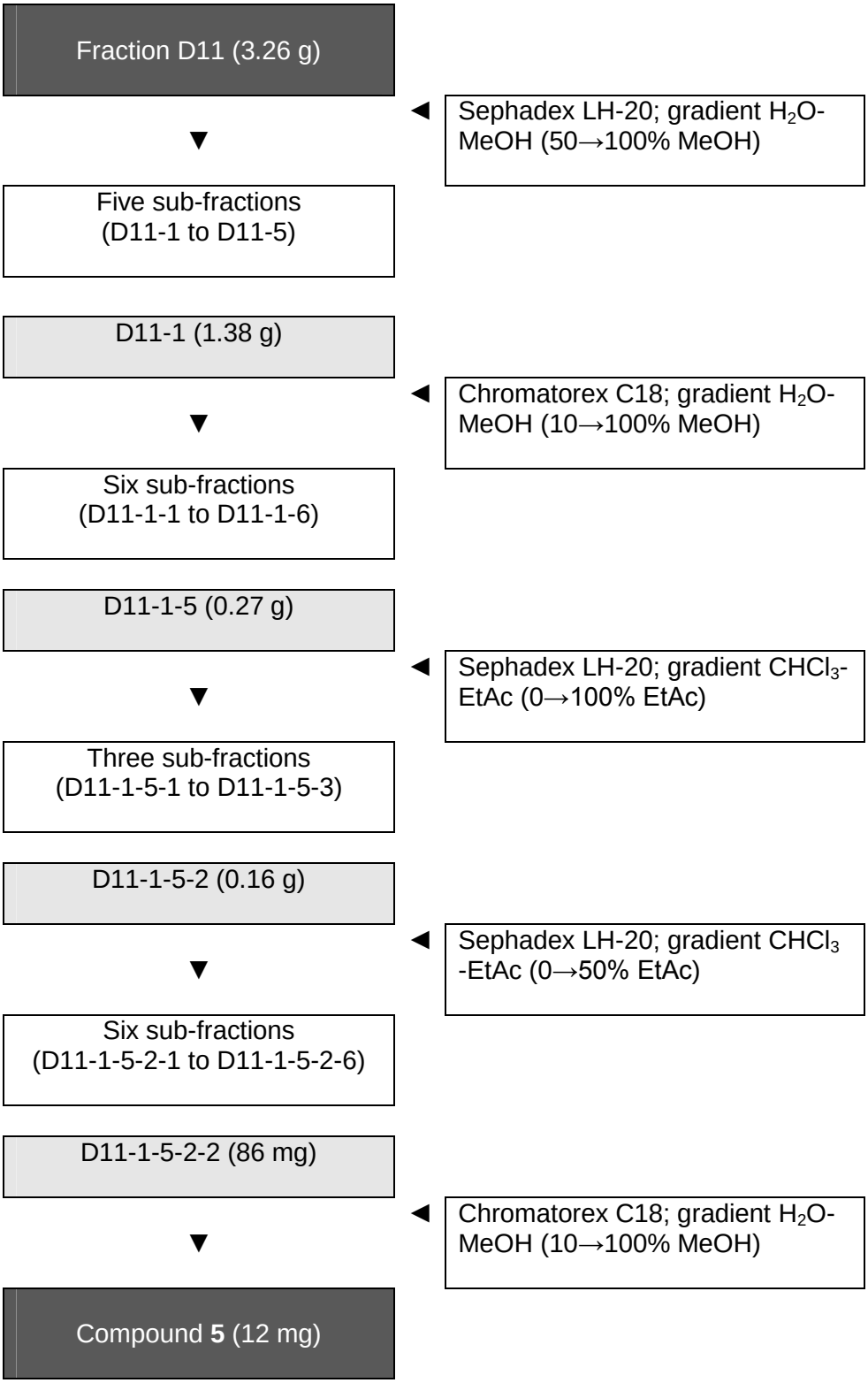


Figure 3.6 Schematic diagram of column chromatographic protocols for isolating Compound **5** from DCM leaf extract of *Vallaris glabra*

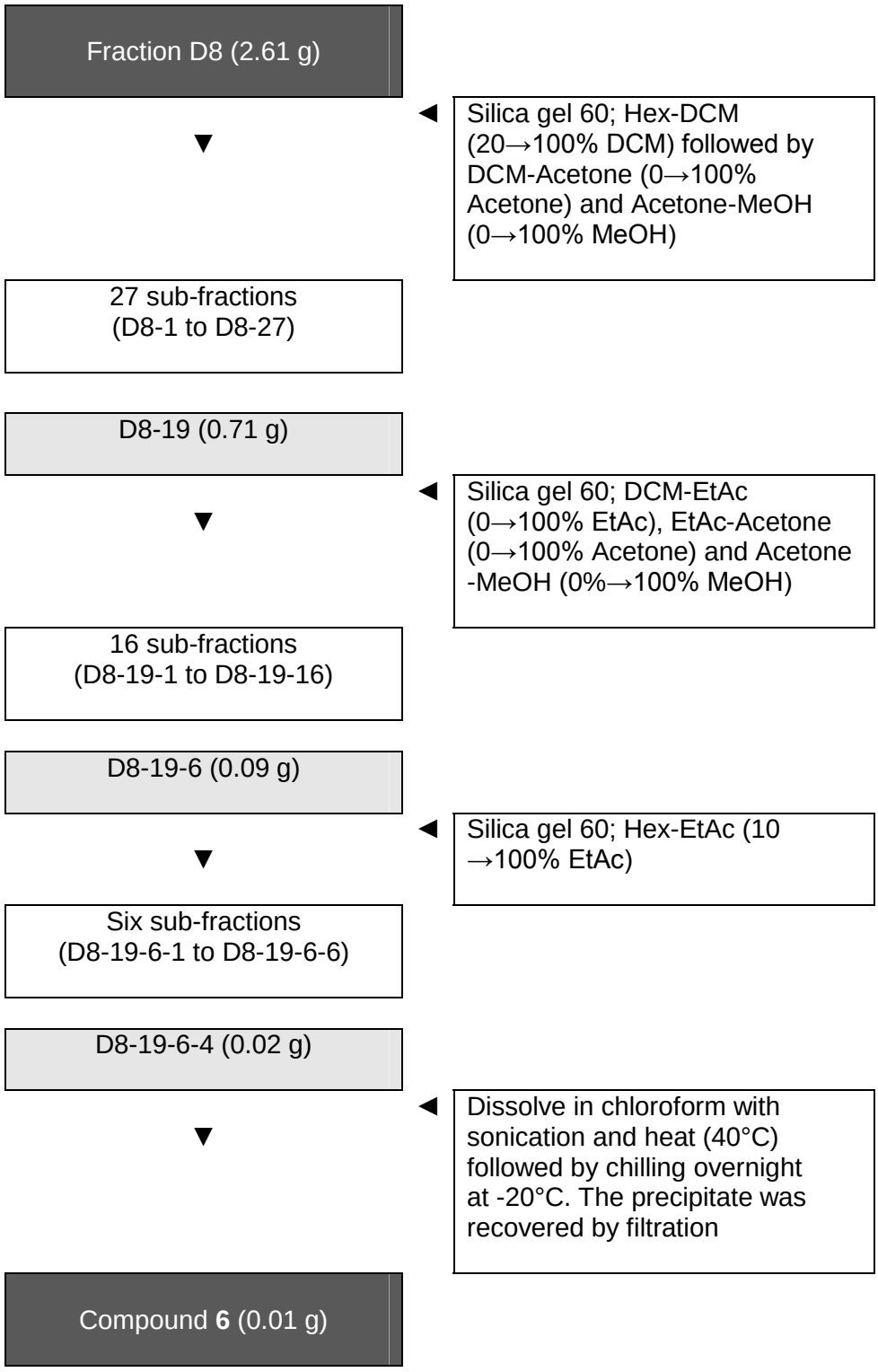


Figure 3.7 Schematic diagram of column chromatographic protocols for isolating Compound **6** from DCM leaf extract of *Vallaris glabra*

Compound **6** was obtained from the active Fraction D8 of the DCM leaf extract (2.61 g). Fraction D8 was passed through Silica gel 60 using a solvent system of Hex-DCM (20→100% DCM) followed by DCM-Acetone (0→100% Acetone) and finally Acetone-MeOH (0→100% MeOH) to give 27 sub-fractions (D8-1 to D8-27). Sub-fraction D8-19 (0.71 g) was subjected to Silica gel 60 chromatography by eluting with DCM-EtAc (0→100% EtAc), followed by EtAc-Acetone (0→100% Acetone) and finally Acetone-MeOH (0%→100% MeOH) to give 16 sub-fractions (D8-19-1 to D8-19-16). Sub-fraction D8-19-6 (0.09 g) was again chromatographed on Silica gel 60 with Hex-EtAc (10→100% EtAc) to obtain six sub-fractions (D8-19-6-1 to D8-19-6-6). Compound **6** was precipitated from sub-fraction D8-19-6-4 (0.02 g) by dissolving in chloroform with sonication and heat (40°C) followed by chilling overnight at -20°C. The precipitate was recovered by filtration and identified as Compound **6** (0.01 g) [**Figure 3.7**].

3.11 ANTIPROLIFERATIVE ACTIVITY OF FRACTIONS

Antiproliferative (APF) activity of the 13 fractions from DCM leaf extract of *Vallaris glabra* (25 µg/mL) was initially screened for inhibitory activity against four types of human cancer cells (HT-29, MCF-7, MDA-MB-231 and SKOV-3) using the sulphorhodamine B (SRB) assay (Vichai & Kirtikara 2006; Cheah *et al.* 2008). Following procedures described in **item 3.5.2**, the cells were seeded at appropriate densities into 96-well plates. The fractions were dissolved in 100% DMSO and diluted two-fold with cell culture media to the concentrations ranging from 3.13–50.0 µg/mL (final concentration 1.56–25.0 µg/mL). Diluted samples (100 µL) were added to the cells and incubated for 48 hours at 37°C in 5% CO_2. Inhibitory activity of the fractions were subsequently analysed by SRB assay.

Inhibitory activity with less than 50% cell density was considered positive while that with more than 50% cell density was considered negative (Vichai & Kirtikara 2006). APF activity of those fractions with positive growth inhibition were further analysed and quantified.

3.12 STRUCTURAL ELUCIDATION OF COMPOUNDS

Compounds isolated from leaves of *Vallaris glabra* were subjected to structural elucidation and identification by ^{1}H-NMR, ^{13}C-NMR and ESI-MS analyses.

3.12.1 Nuclear Magnetic Resonance Spectroscopy

Compounds were dissolved in a deuterated solvent and subjected to ^{1}H and ^{13}C NMR analysis using a Bruker DRX 300 MHz spectrometer (300 MHz for ^{1}H and 75.4 MHz for ^{13}C), and a Varian Unity Inova 500 MHz spectrometer (500 MHz for ^{1}H and 125.7 MHz for ^{13}C). Their chemical shifts were recorded in ppm (δ) using tetra-methylsilane (TMS) as internal standard.

3.12.2 Mass Spectrometry

Compounds were subjected to electrospray ionization mass spectrometry (ESI-MS) using a Perkin Elmer Flexar SQ 300 mass spectrometer or atmospheric pressure chemical ionisation mass spectrometry (APCI-MS) using a Thermo Finnigan LCQ Deca spectrometer. Mass spectra of ESI-MS and APCI-MS were acquired in negative ion mode $[M-H]^{-}$. EI-MS was carried out using a Thermo Finnigan Trace GC-PolarisQ system *via* direct probe analysis. Ion source temperature was programmed at 40°C with an increase to 300°C. Mass spectrum of EI-MS was acquired at 70 eV in positive mode $[M^{+}]$. Analytes were introduced into the mass spectrometer by direct infusion. Mass up to 3000 m/z was measured.

3.13 HPLC QUANTIFICATION OF CQA CONTENT

Fresh leaf extract of *Vallaris glabra* was analysed for its caffeoylquinic acid content (CQAC) using reversed-phase HPLC with comparison made to leaf extracts of five other Apocynaceae species. 5-caffeoylquinic acid (5-CQA or chlorogenic acid), and its isomeric forms of 3-caffeoylquinic acid (3-CQA or neochlorogenic acid) and 4-caffeoylquinic acid (4-CQA or cryptochlorogenic acid) isolated from the MeOH leaf extract of *Vallaris glabra* by column chromatography were used to identify and quantify the CQAC of the six species in the HPLC chromatogram. Flowers of

Lonicera japonica (Japanese honeysuckle), known to have high CQAC, were used as positive control.

HPLC (Agilent Technologies 1200 Series) instrument with Agilent Zorbax SBC-18 column (4.6 x 250 mm) were used in the HPLC analysis. A 15-min linear gradient from 5→100% MeOH was used to elute samples at 1.2 mL/min. Mobile phases were acidified with 0.1% trifluoroacetic acid (TFA) for better resolution. A 20-µL sample injection loop was used and elution was monitored at 280 nm. Commercially purchased HPLC standard of 5-CQA (chlorogenic acid) was used to construct the calibration curve. CQAC was determined using peak areas. The calibration equation of peak area (mAU*s) against concentration of CGA (mg/L) was y = 24.262x (R^2= 0.9998). CQAC was expressed as mg CGAE/100 g.

As 3-CQA, 4-CQA and 5-CQA all shared similar UV absorption pattern as the HPLC standard of 5-CQA, the amount of 3-CQA and 4-CQA present in the extracts can be inferred from the calibration curve of the HPLC standard of 5-CQA.

3.14 ANTIPROLIFERATIVE ACTIVITY OF COMPOUNDS

The isolated compounds, 3-CQA, 4-CQA, 5-CQA, quercetin 3-*O*-glucoside and stearic acid [**section 4.8**], were tested for their inhibitory activity against four types of human cancer cells (HT-29, MCF-7, MDA-MB-231 and SKOV-3). Cells were seeded into 96-well plate at appropriate densities following the procedures described in **item 3.5.2**. The compounds, dissolved in 100% DMSO, were diluted two-fold with cell culture media to a final concentration of 1.56–200 µg/mL. Diluted samples (100 µL) were added to the cell culture and treated for 48 hours with incubation at 37°C in 5% CO_2 followed by analysis with SRB assay.

3.15 HOECHST NUCLEAR STAINING ASSAY

Nuclear staining of cancer cells with Hoechst 33342 (Sigma Chemical Co.) [**Appendix IV**] was performed following the protocol described by Tee & Azimahtol (2005), Cheah *et al.* (2008), Zakaria *et al.* (2009) and Muhamad *et al.* (2011).

Visualization of nuclear morphology was performed using Hoechst 33342, a DNA-binding fluorescent dye. MDA-MB-231 breast cancer cells were cultured in 3.5 mm cell-culture dishes with a cover slip placed into each of the dishes and sterilised under UV radiation for 15 min. After culturing of cells overnight, they were treated for 48 hours with 1% DMSO as control and with varying concentrations of DCM leaf extracts of *Vallaris glabra* at 1.56, 3.13, 6.25, 12.5, 25.0 and 50.0 µg/mL. After removing the cell culture media, the dishes and cover slips were washed twice with 10% PBS followed by fixing the cells to the cover slips with 3.7% paraformaldehye (v/v) for 10 min. The fixing solution was removed and the dishes and cover slips were washed twice with 10% PBS. Hoechst 33342 (1 µg/mL) was added directly to the affixed cells and stained in the dark for 10 min before washing twice with 10% PBS. The cover slips were carefully lifted from the dishes, placed onto glass slides with 10 µL of Gel Mount aqueous mounting medium (Sigma) and sealed with nail varnish. The stains cells were visualised at 40x magnification using an inverted microscope (Olympus BX41) and a UV filter. Images of cells were photographed by a digital Nikon DS-Fi1c camera with NIS-Elements F software version 3.00.

3.16 CASPASE COLORIMETRIC ASSAY

3.16.1 Preparation of Cell Lysate

MDA-MB-231 cells were treated with varying concentrations (1.56–25.0 µg/mL) of DCM leaf extract of *Vallaris glabra*. The floating and trypsinised-adherent cells were collected and washed in cold phosphate-buffered saline (PBS). The cells were lysed by adding 50 µL chilled lysis buffer (BioVision, USA) and incubated on ice for 10 min. The suspension was then centrifuged for 1 min at 10,000 x g. The supernatant containing the cytosolic extract was transferred to a fresh tube and put on ice for immediate assay or stored in aliquots at -80°C for future use.

3.16.2 Quantification of Protein Content

The protein concentration was determined using the microassay procedure established by the manufacturer (Bio-Rad), based on the method of Bradford (1976). Protein standards (125–1500 µg/mL) using bovine serum albumin (BSA)

were prepared. The standards and samples of protein cell lysates in various dilutions (5 µL) were added into each well of a 96-well microtiter plate. Bio-Rad dye reagent concentrate (250 µL) was added into each well. Control consisted of water in place of the protein sample. The plate was incubated in the dark at room temperature for 30 min before reading absorbance at 595 nm.

3.16.3 Caspase-3, -6, -8 and -9 Colorimetric Assay

Activities of caspase-3, -6, -8 and -9 were determined using the Caspase Colorimetric Assay Kit (BioVision Research, Mountain View, CA, USA) [**Appendix V**] according to the manufacturer's instructions and following the procedures described by Cheah *et al.* (2008). Cytosolic protein of 200 µg was diluted to 50 µL cell lysis buffer for each assay in a 96-well plate.

To each well, 50 µL of reaction buffer containing 10 mM dithiothreitol (DTT) was added followed by 5 µL of substrate containing 4 mM Asp-Glu-Val-Asp (DEVD)-*p*NA substrate (caspase-3), 4 mM Val-Glu-Ile-Asp (VEID)-*p*NA substrate (caspase-6), 4 mM Ile-Glu-Thr-Asp (IETD)-*p*NA substrate (caspase-8), or 4 mM Leu-Glu-His-Asp (LEHD)-*p*NA substrate (caspase-9). The plate was then incubated at 37°C for 2 hours. At the end of incubation, the plate was read in a microtiter plate reader at 412 nm. Fold-increase in caspase activity was determined by comparing the results of treated samples with those of the control.

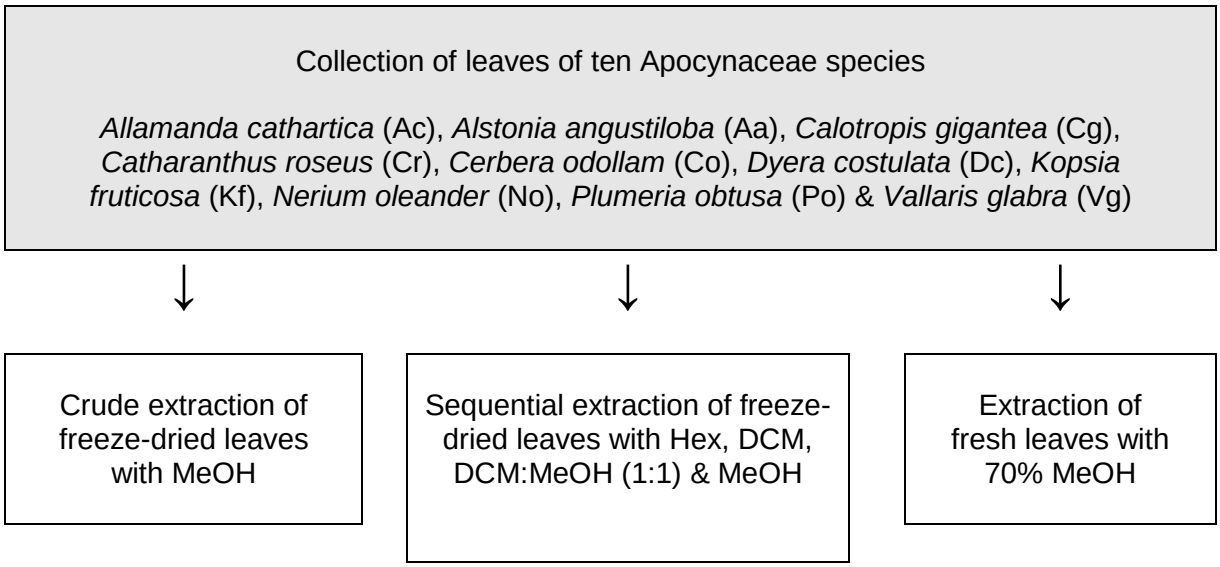
3.17 STATISTICAL ANALYSES

All analyses were carried out in triplicate (n = 3) and results were presented as means ± standard deviations (SD). For comparisons between samples, the Tukey Honestly Significant Difference (HSD) one-way analysis of variance (ANOVA) with significant level $P < 0.05$ was used to analyse variance. Correlation coefficient (R^2) was calculated and plotted using the Microsoft Excel 2007. R^2 is the percent of the data that is the closest to the line of best fit, represented by a regression equation.

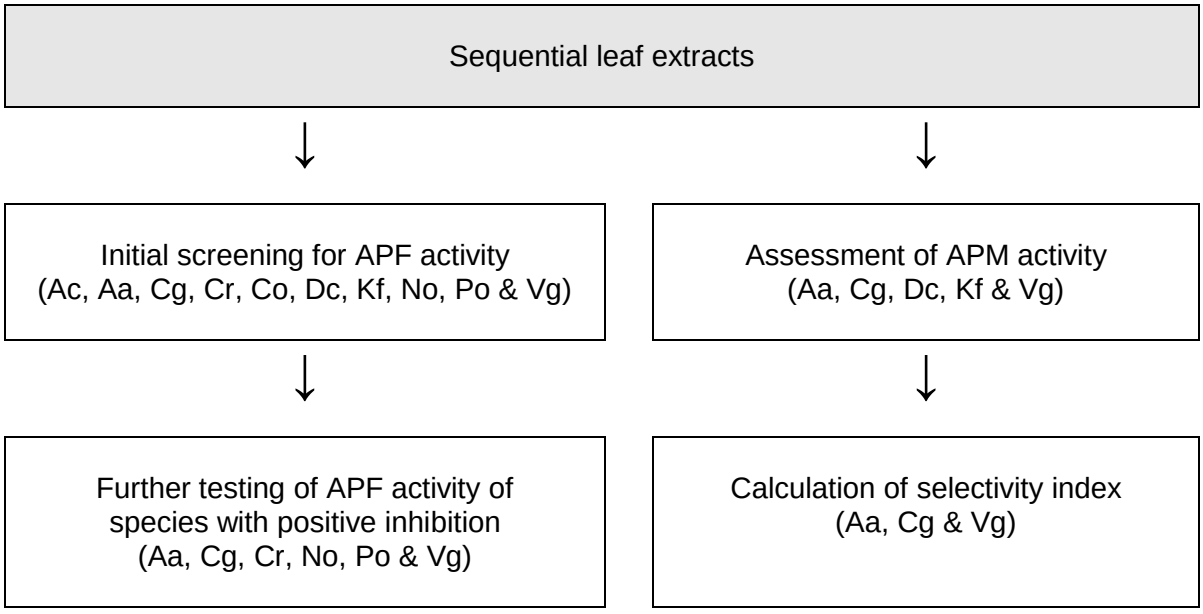
3.18 FLOW CHART OF RESEARCH ACTIVITIES

3.18.1 Apocynaceae Species

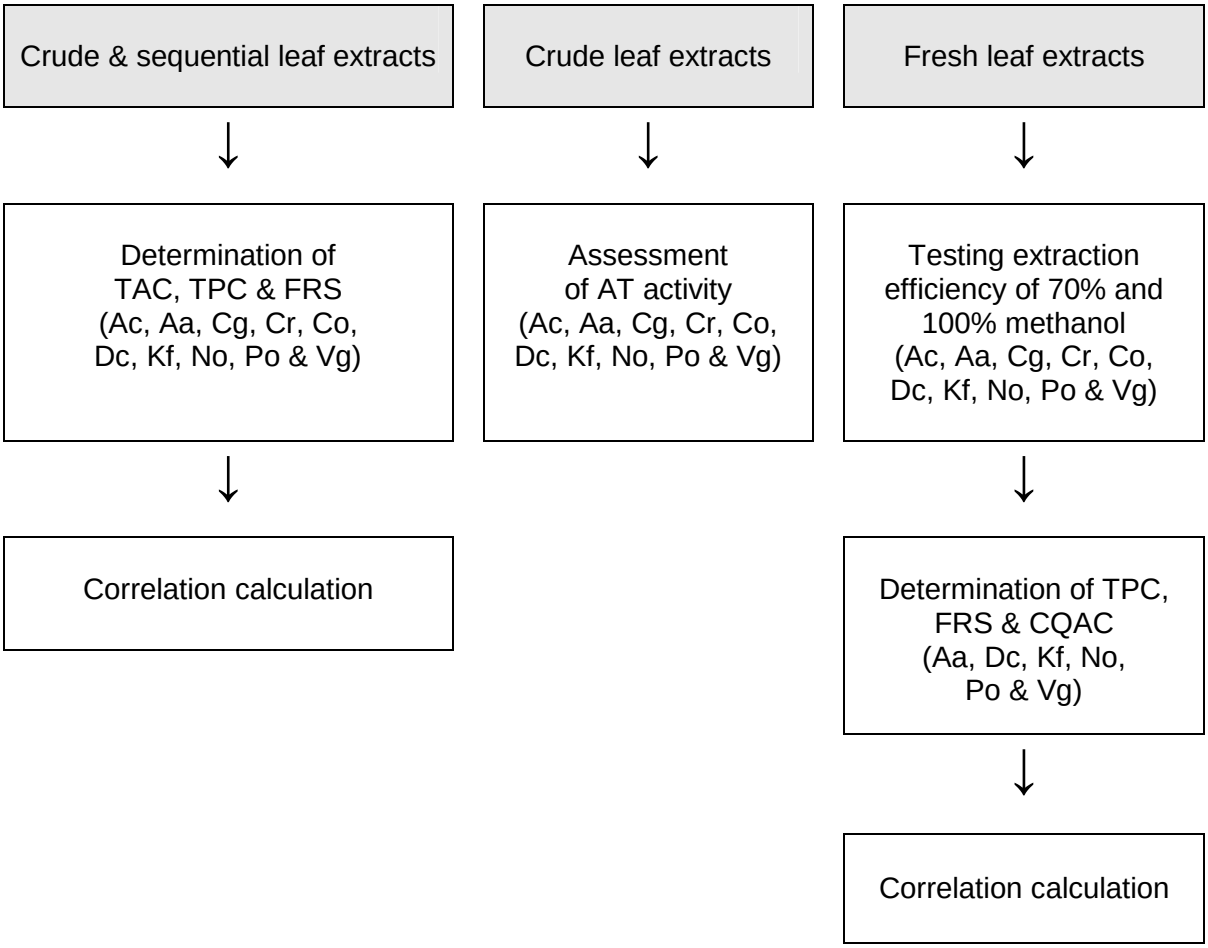
a. Leaf sampling and extraction



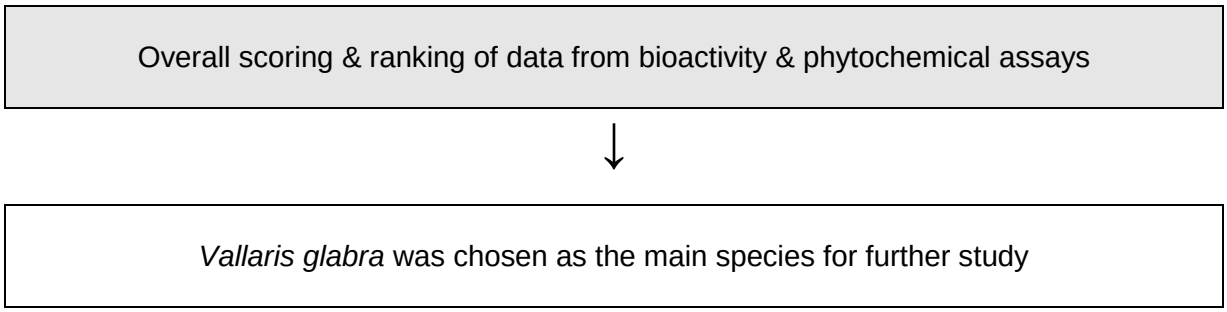
b. APF and APM activity of leaf extracts



c. Phytochemical content, and antioxidant and antityrosinase activity of leaf extracts

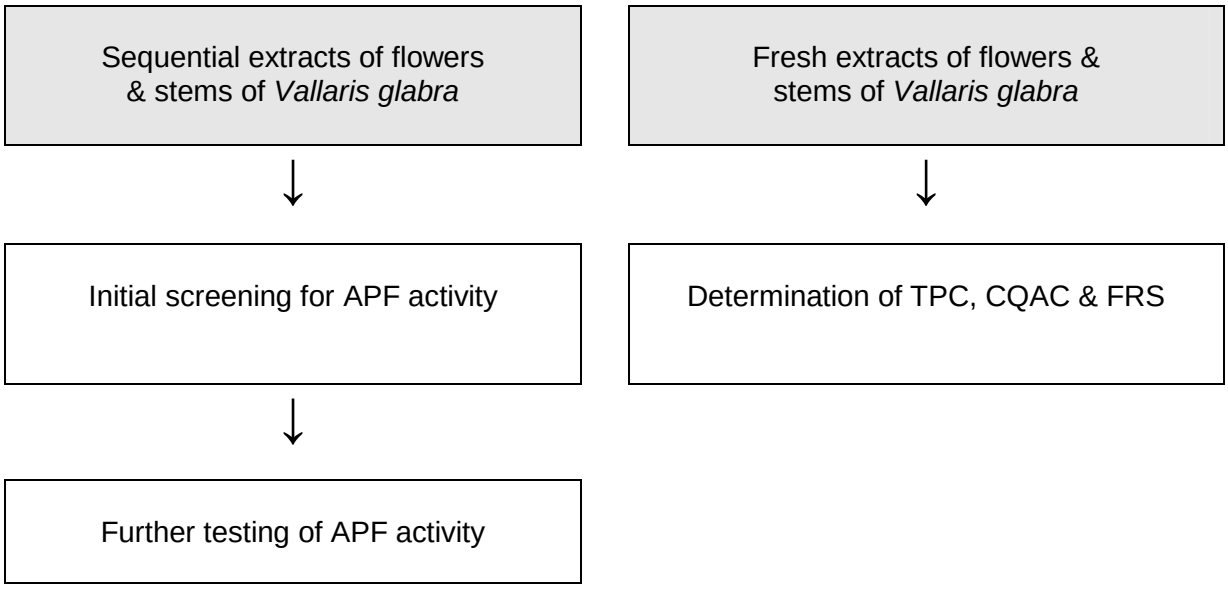


3.18.2 Choice of Species for Further Study

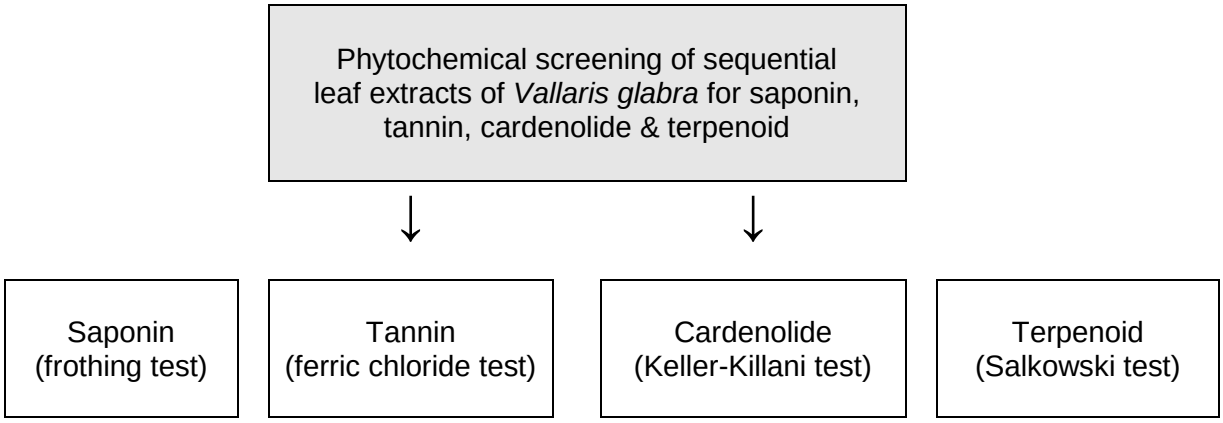


3.18.3 *Vallaris glabra*

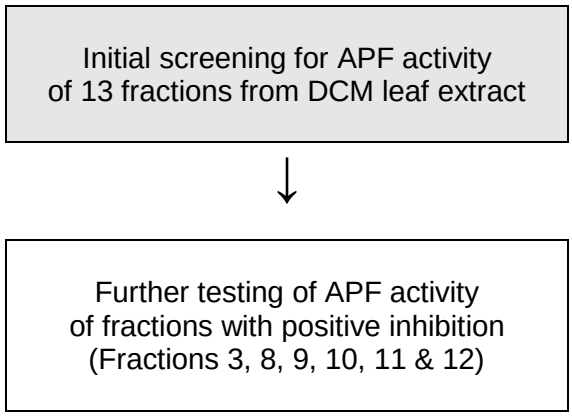
a. Other plant parts



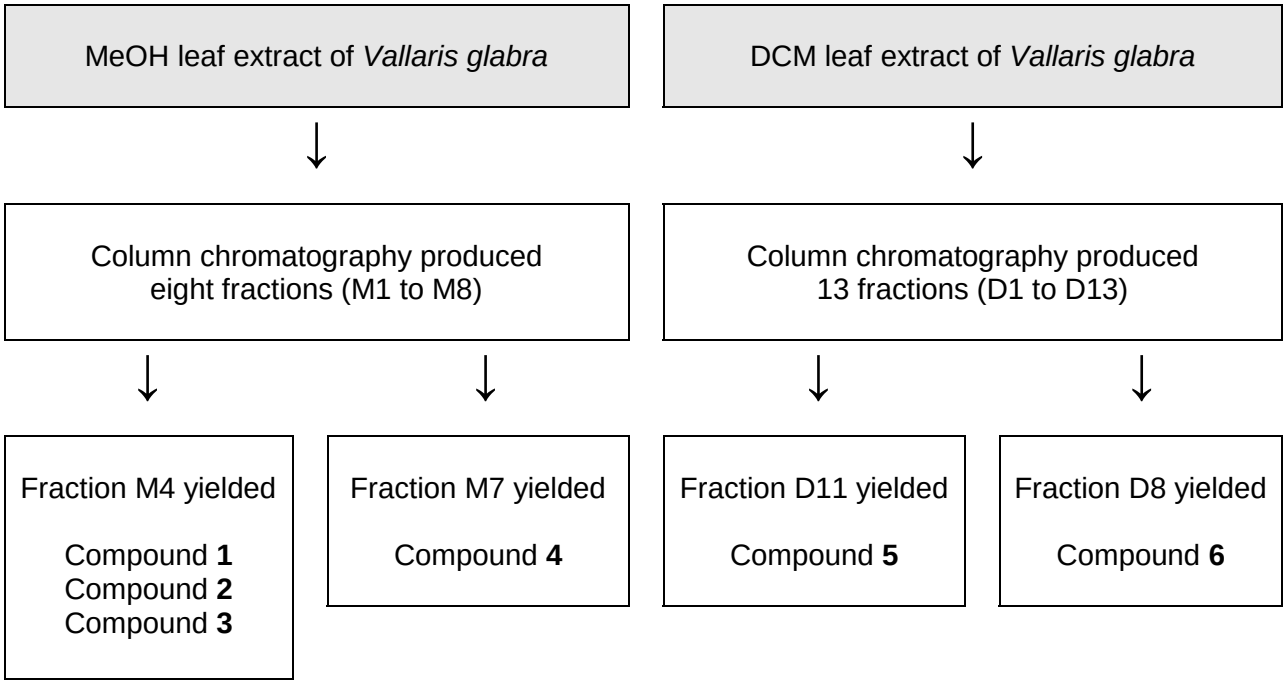
b. Phytochemical screening of leaf extracts



c. APF activity of fractions from DCM leaf extract



d. Isolation and structural elucidation of compounds



Compound **1** = 3-*O*-caffeoylquinic acid (3-CQA)

Compound **2** = 4-*O*-caffeoylquinic acid (4-CQA)

Compound **3** = 5-*O*-caffeoylquinic acid (5-CQA)

Compound **4** = quercetin 3-*O*-glucoside (QG)

Compound **5** = stearic acid (SA)

Compound **6** = ursolic acid (UA)

e. HPLC quantification of CQA content

With the isolation & identification of CQAs from MeOH leaf extract of *Vallaris glabra*

↓

CQA content of fresh leaf extract of *Vallaris glabra* was analysed using reversed-phase HPLC with comparison made to five other Apocynaceae species (Aa, Dc, Kf, No & Po)

f. Antiproliferative activity of compounds

Compounds isolated from methanol and DCM leaf extracts of *Vallaris glabra* were tested for their inhibitory activity

↓

3-CQA, 4-CQA, 5-CQA, quercetin 3-*O*-glucoside & stearic acid were tested against four types of human cancer cells (HT-29, MCF-7, MDA-MB-231 & SKOV-3)

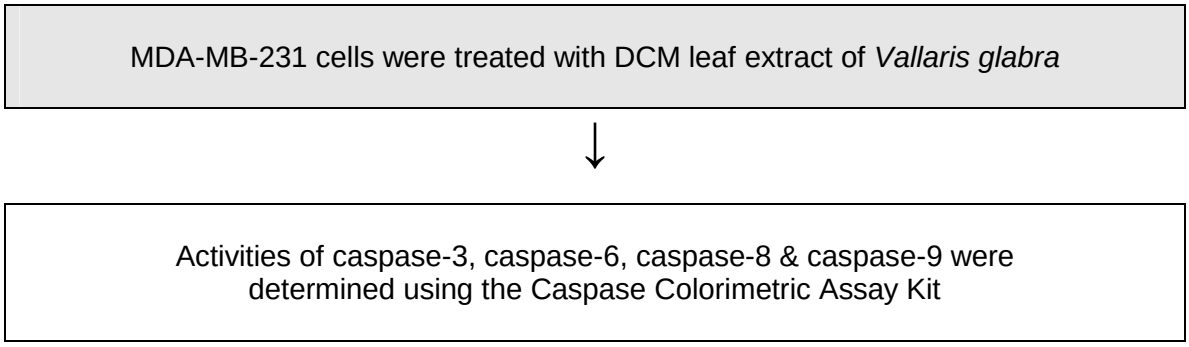
g. Hoechst nuclear staining

MDA-MB-231 cells were treated with DCM leaf extract of *Vallaris glabra*

↓

Treated cells were stained with Hoechst 33342 and their nuclear morphology visualised

h. Caspase colorimetry



Chapter IV

RESULTS AND DISCUSSION

IVa APOCYNACEAE SPECIES

4.1 ANTIPROLIFERATIVE ACTIVITY OF LEAF EXTRACTS

4.1.1 Initial Screening for Antiproliferative Activity

Initial screening for antiproliferative (APF) activity of sequential leaf extracts of ten Apocynaceae species against three human cancer cell lines (MCF-7, MDA-MB-231 and HeLa) was conducted using the sulphorhodamine B (SRB) assay. The purpose of the initial screening was to identify the extracts of species with and without APF activity. This would enable further testing of APF activity [**item 4.1.2**].

Results showed that the extracts of *Alstonia angustiloba*, *Calotropis gigantea*, *Catharanthus roseus*, *Nerium oleander*, *Plumeria obtusa* and *Vallaris glabra* displayed positive inhibition i.e. inhibition with less than 50% cell density [**Table 4.1**].

DCM extract of *Calotropis gigantea*, and DCM and DCM:MeOH extracts of *Nerium oleander* and *Vallaris glabra* inhibited all three cancer cell lines. All four extracts of *Nerium oleander* were effective against MCF-7 cells. Only DCM:MeOH extract of *Catharanthus roseus* was active against MCF-7 and HeLa cells. Hexane (Hex) extract of *Nerium oleander* inhibited MCF-7 cells while Hex extract of *Plumeria obtusa* inhibited MCF-7 and HeLa cells. MeOH extracts of *Nerium oleander* and *Vallaris glabra* inhibited MCF-7 cells. In general, DCM and DCM:MeOH extracts of these species were the most effective. All leaf extracts of *Allamanda cathartica*, *Cerbera odollam*, *Dyera costulata* and *Kopsia fruticosa* did not show any APF activity.

Table 4.1 Initial screening of sequential leaf extracts of six Apocynaceae species with positive inhibition of three human cancer cell lines

Species	Sequential leaf extract	Inhibition of cancer cells		
		MCF-7	MDA-MB-231	HeLa
Alstonia angustiloba	DCM	–	+	+
Calotropis gigantea	DCM	+	+	+
	DCM:MeOH	+	–	+
Catharanthus roseus	DCM:MeOH	+	–	+
Nerium oleander	Hex	+	–	–
	DCM	+	+	+
	DCM:MeOH	+	+	+
	MeOH	+	–	–
Plumeria obtusa	Hex	+	–	+
	DCM	–	–	+
Vallaris glabra	DCM	+	+	+
	DCM:MeOH	+	+	+
	MeOH	+	–	–

Inhibitory activity of less than 50% cell density is considered positive (+) while that of more than 50% cell density is considered negative (–). MCF-7 and MDA-MB-231 are human breast cancer cells, and HeLa are human cervical cancer cells. Abbreviations: DCM = dichloromethane, Hex = hexane and MeOH = methanol. Leaf extracts of *Allamanda cathartica*, *Cerbera odollam*, *Dyera costulata* and *Kopsia fruticosa* did not show any APF activity.

Contrary to findings of this study, cytotoxic activities have been reported in species of *Cerbera*, *Allamanda* and *Kopsia*. Methanol extract of leaves of *Cerbera odollam* strongly inhibited MCF-7 and T47D cells (Nurhanan *et al.* 2008). Cardenolides from seeds of *Cerbera odollam* had cytotoxic activity against KB, BC and NCI-H187 cells (Laphookhieo *et al.* 2004). Potent cytotoxic activity was reported in ethanol extracts of fruits and leaves of *Cerbera manghas* (Ali *et al.* 1996). Cardenolides from roots of *Cerbera manghas* also showed APF activity (Chang *et al.* 2000). Ethanol extracts of roots of *Allamanda schottii* and *Allamanda blanchetti* displayed stronger cytotoxicity against K-562 cells than leaf and stem extracts (Schmidt *et al.* 2006). Valparicine from the stem bark of *Kopsia arborea* showed cytotoxic effects against KB and Jurkat cells (Lim *et al.* 2007) while kopsimaline from leaves and stem bark of *Kopsia singapurensis* was found to inhibit KB cells (Subramaniam *et al.* 2008). The disparity

between these reported results and findings of this study might be due to differences in the choice of species, plant parts tested, extraction techniques and anti-proliferative assays used.

4.1.2 Further Testing of Antiproliferative Activity

Leaf extracts of the six Apocynaceae species with positive inhibition (i.e. *Alstonia angustiloba*, *Calotropis gigantea*, *Catharanthus roseus*, *Nerium oleander*, *Plumeria obtusa* and *Vallaris glabra*) as shown in **Table 4.1** were further tested against six human cancer cell lines (MCF-7, MDA-MB-231, HeLa, HT-29, SKOV-3 and HepG2). Data of the study are shown in **Table 4.2**.

Results showed that DCM extract of *Alstonia angustiloba* inhibited only MDA-MB-231, HeLa, and SKOV-3 cells with GI_{50} values of 20, 20 and 16 µg/mL, respectively. DCM extract of *Calotropis gigantea* strongly inhibited all cancer cell lines with GI_{50} values ranging from 1.3–3.3 µg/mL. Against MCF-7 and MDA-MB-231 cells, GI_{50} of DCM extract of *Calotropis gigantea* (1.9 and 1.3 µg/mL) was much stronger than those of xanthorrhizol (11 and 8.7 µg/mL), curcumin (4.1 and 8.7 µg/mL) and tamoxifen (8.3 and 4.6 µg/mL), respectively. Values of the standard drugs were those of Cheah *et al.* (2008) using the same technique.

DCM:MeOH extract of *Catharanthus roseus* strongly inhibited MCF-7 and HeLa cells with GI_{50} of 3.5 and 4.7 µg/mL, respectively. All four extracts of *Nerium oleander* were effective against MCF-7 cells with GI_{50} ranging from 3.7–12 µg/mL. DCM and DCM:MeOH extracts inhibited all cell lines except HepG2 cells. Hex extract of *Plumeria obtusa* was effective against MCF-7 and HeLa cells while its DCM extract was effective against HeLa cells. DCM and DCM:MeOH extracts of *Vallaris glabra* inhibited all cell lines with GI_{50} values ranging from 7.5–12 µg/mL and 5.8–13 µg/mL, respectively. In addition, MeOH extract of *Vallaris glabra* also inhibited the growth of MCF-7 and HepG2 cells. Against MCF-7 cells, GI_{50} of DCM and DCM:MeOH extracts of *Vallaris glabra* (7.7 and 7.0 µg/mL) was stronger than xanthorrhizol (11 µg/mL) and comparable to tamoxifen (8.3 µg/mL), respectively.

Table 4.2 Antiproliferative activity of sequential leaf extracts of six Apocynaceae species with positive growth inhibition against six human cancer cell lines

Species	Sequential leaf extract	Growth inhibition (GI_{50})					
		MCF-7	MDA-MB-231	HeLa	HT-29	SKOV-3	HepG2
Alstonia angustiloba	DCM	–	20 ± 1.7	20 ± 1.1	16 ± 1.4	–	–
Calotropis gigantea	DCM	1.9 ± 0.2	1.3 ± 0.3	2.5 ± 0.5	2.5 ± 0.2	3.3 ± 0.2	1.8 ± 1.7
	DCM:MeOH	13 ± 0.3	–	15 ± 1.0	20 ± 2.3	24 ± 0.7	16 ± 3.5
Catharanthus roseus	DCM:MeOH	3.5 ± 0.1	–	4.7 ± 0.6	–	–	–
Nerium oleander	Hex	11 ± 0.9	–	–	12 ± 2.8	–	–
	DCM	3.7 ± 0.1	5.2 ± 0.7	5.1 ± 0.7	4.5 ± 1.0	6.1 ± 0.9	–
	DCM:MeOH	4.3 ± 0.2	18 ± 2.3	6.8 ± 0.9	7.6 ± 2.1	9.2 ± 2.2	–
	MeOH	12 ± 1.1	–	–	–	–	–
Plumeria obtusa	Hex	5.7 ± 0.8	–	10 ± 1.4	–	–	–
	DCM	–	–	19 ± 4.0	–	–	–
Vallaris glabra	DCM	7.7 ± 1.3	12 ± 2.0	9.8 ± 1.5	7.5 ± 4.5	9.3 ± 2.0	7.6 ± 0.2
	DCM:MeOH	7.0 ± 2.5	13 ± 6.3	8.5 ± 2.9	7.7 ± 2.4	12 ± 1.2	5.8 ± 1.2
	MeOH	16 ± 2.1	–	–	–	–	19 ± 0.9
Standard drug							
Xanthorrhizol		11 ± 0.7	8.7 ± 0.8				
Curcumin		4.1 ± 0.9	8.7 ± 0.8				
Tamoxifen		8.3 ± 0.6	4.6 ± 0.5				

GI_{50} (µg/mL) is the concentration which causes 50% reduction in cell growth and inhibition is not effective (–) if values ≥ 20 µg/mL. MCF-7 and MDA-MB-231 are human breast cancer cells, HeLa and SKOV-3 are human cervical cancer cells, HT-29 are human colon cancer cells and HepG2 are human liver cancer cells. Values of standard drugs of xanthorrhizol, curcumin and tamoxifen against MCF-7 and MDA-MB-231 reported by Cheah *et al.* (2008) were used as positive controls. Abbreviations: Hex = hexane, MeOH = methanol and DCM = dichloromethane.

Against human breast cancer cells, 11 extracts of five species had positive GI_{50} against MCF-7 cells and six extracts of four species had positive GI_{50} against MDA-MB-231 cells [**Table 4.2**]. DCM and DCM:MeOH extracts of *Nerium oleander* and *Vallaris glabra* showed stronger inhibition against MCF-7 cells than MDA-MB-231 cells. In should be noted that MCF-7 cells are estrogen receptor (ER) positive while MDA-MB-231 cells are ER negative.

About 60–80% of human breast cancer cases are caused by ER positive cells (Hortobagyi *et al.* 2010). A study of the differential sensitivity of human breast cancer cell lines to the growth-inhibitory effects of tamoxifen by Reddel *et al.* (1985) reported that ER positive cells are generally more sensitive than ER negative cells to the growth inhibitory effects of tamoxifen. Growth inhibition and cytotoxicity of ER positive cells are accompanied by characteristic changes in cell cycle kinetic parameters. In contrast, different mechanisms may be involved in the effects of tamoxifen on ER negative cells.

To the best of my knowledge, this study represents the first report of APF activities from leaf extracts of *Alstonia angustiloba*, *Plumeria obtusa* and *Vallaris glabra*. Earlier studies have reported cytotoxic activity from the root bark of *Alstonia macrophylla* (Keawpradub *et al.* 1999a) and from the stem bark of *Alstonia scholaris* (Jagetia & Baliga 2006). Iridoids isolated from the bark of *Plumeria rubra* were cytotoxic (Kardono *et al.* 1990). A recent study reported potent cell growth inhibition of cardenolide glycosides isolated from *Vallaris solanacea* (Ahmed *et al.* 2010). The finding of strong APF activities from DCM and DCM:MeOH extracts of *Calotropis gigantea* from this study is supported by earlier reports that leaf and root extracts of *Calotropis gigantea* had strong inhibitory activity against cancer cells (Lhinhatrakool & Sutthivaiyakit 2006; Wang *et al.* 2008a & 2008b; Seeka & Sutthivaiyakit 2010). On the contrary, ethanolic leaf extract of *Calotropis gigantea* was reported to be non-cytotoxic to HeLa cell line (Ali *et al.* 1996). Isolated from leaves of *Nerium oleander*, pentacyclic triterpenoids were cytotoxic to KB cells (Siddiqui *et al.* 1995). Against HL60 and K562 cells, the stem extract of *Nerium oleander* displayed stronger cytotoxic activity than leaf and root extracts (Turan *et al.* 2006).

It is interesting to note that out of four leaf extracts of *Catharanthus roseus* tested against six cell lines, only DCM:MeOH extract inhibited MCF-7 and HeLa cells. The species is well known for its indole alkaloids notably vinblastine and vincristine which are used to treat Hodgkin's disease and acute leukaemia in children, respectively (Wiart 2006). It can be inferred that the APF activity of *Catharanthus roseus* may be cell line specific, unlike those of *Calotropis gigantea*, *Nerium oleander* and *Vallaris glabra*, which are wide spectrum, inhibiting most or all cell lines tested.

4.2 ANTIPLASMODIAL ACTIVITY OF LEAF EXTRACTS

4.2.1 Inhibition of K1 and 3D7 *Plasmodium falciparum*

The antiplasmodial activity of sequential leaf extracts of five Apocynaceae species (*Alstonia angustiloba*, *Calotropis gigantea*, *Dyera costulata*, *Kopsia fruticosa* and *Vallaris glabra*) is shown in **Table 4.3**.

Against chloroquine-resistant K1 strain of *Plasmodium falciparum*, leaves of *Vallaris glabra* were most effective as all four extracts had APM activity with EC_{50} less than 10 µg/mL. DCM leaf extract of *Vallaris glabra* was the strongest with EC_{50} of 0.85 µg/mL. Three extracts of *Alstonia angustiloba*, and two extracts of *Calotropis gigantea*, *Dyera costulata* and *Kopsia fruticosa* showed APM activity. It should be noted that DCM:MeOH extracts of all five species displayed APM activity with *Alstonia angustiloba* having the strongest activity (EC_{50} of 0.46 µg/mL).

Against chloroquine-sensitive 3D7 strain of *Plasmodium falciparum*, extracts of *Alstonia angustiloba* and *Vallaris glabra* showed no activity. Extracts of *Dyera costulata* were the exception in that DCM, DCM:MeOH and MeOH extracts showed positive APM activity with EC_{50} of 8.31, 2.13 and 3.56 µg/mL, respectively. Generally, extracts were less effective against 3D7 strain.

In general, EC_{50} values of leaf extracts against K1 and 3D7 strains of *Plasmodium falciparum* were much weaker than standard drugs of artemisinin and mefloquine.

Table 4.3 Antiplasmodial activity of sequential leaf extracts of five Apocynaceae species

Species	Sequential leaf extract	EC_{50} (µg/mL) K1	3D7
Alstonia angustiloba	Hex	7.81	–
	DCM	–	–
	DCM:MeOH	0.46	–
	MeOH	6.46	–
Calotropis gigantea	Hex	5.82	–
	DCM	–	–
	DCM:MeOH	0.97	3.29
	MeOH	–	–
Dyera costulata	Hex	–	–
	DCM	–	8.31
	DCM:MeOH	7.52	2.13
	MeOH	7.74	3.56
Kopsia fruticosa	Hex	–	–
	DCM	–	7.14
	DCM:MeOH	4.35	–
	MeOH	1.01	–
Vallaris glabra	Hex	1.00	–
	DCM	0.85	–
	DCM:MeOH	8.45	–
	MeOH	8.42	–
Standard drug			
Artemisinin		0.001	0.001
Mefloquine		0.008	0.018

EC_{50} or effective concentration (µg/mL) is the extract concentration that kills 50% of malaria parasites. Activity is not effective (–) if EC_{50} value > 10 µg/mL. K1 and 3D7 are chloroquine-resistant and chloroquine-sensitive strains of *Plasmodium falciparum*, respectively. Standard drugs of artemisinin and mefloquine were used as positive controls. Abbreviations: Hex = hexane, MeOH = methanol and DCM = dichloromethane.

This study is the first to report the APM activity of leaf extracts of *Alstonia angustiloba*, *Calotropis gigantea*, *Dyera costulata*, *Kopsia fruticosa* and *Vallaris glabra*. A notable finding is the APM activity of *Vallaris glabra* against K1 strain of *Plasmodium falciparum* in all leaf extracts. Other related species reported to have APM activity include *Alstonia macrophylla* (Keawpradub *et al.* 1999b) and *Calotropis procera* (Sharma & Sharma 1999 & 2000; Simonsen *et al*. 2001; Mudi & Bukar 2011).

4.2.2 Selectivity Index of APM Activity

The selectivity index (SI) of APM activity indicates the safety of an extract to be used for antimalarial therapy (Esmaeili *et al.* 2009). In this study, the index was calculated based on the ratio of cytotoxicity (IC_{50}) on HepG2 and MCF-7 cells to APM activity (EC_{50}). The SI was calculated for the most potent extracts against K1 strain of *Plasmodium falciparum* with $EC_{50} \leq 1.00$ µg/mL.

The most potent were DCM:MeOH extracts of *Alstonia angustiloba* (0.46 µg/mL) and *Calotropis gigantea* (0.97 µg/mL), and Hex and DCM extracts of *Vallaris glabra* (1.00 and 0.85 µg/mL), respectively [**Table 4.4**]. Against HepG2 cells, IC_{50} values were all > 25.0 µg/mL, and against MCF-7 cells, IC_{50} values were > 25.0, 16.8, > 25.0 and 12.0 µg/mL, respectively.

Against HepG2 cells, Hex and DCM extracts of *Vallaris glabra*, and DCM:MeOH extracts of *Alstonia angustiloba* and *Calotropis gigantea* were non-cytotoxic with SI values > 25.0. Against MCF-7 cells, the DCM extract of *Vallaris glabra* and the DCM:MeOH extract of *Calotropis gigantea* had SI values of 14.1 and 17.3, respectively. The Hex extract of *Vallaris glabra* and the DCM:MeOH extract of *Alstonia angustiloba* were non-cytotoxic with SI values > 25.0.

Table 4.4 Selectivity index of antiplasmodial activity of sequential leaf extracts of three Apocynaceae species

Species	Leaf extract	EC_{50} (µg/mL) of APM activity	IC_{50} (µg/mL) of APF activity		SI of APM activity	
		K1 strain	HepG2	MCF-7	HepG2	MCF-7
Alstonia angustiloba	DCM:MeOH	0.46	> 25.0	> 25.0	NC	NC
Calotropis gigantea	DCM:MeOH	0.97	> 25.0	16.8	NC	17.3
Vallaris glabra	Hex	1.00	> 25.0	> 25.0	NC	NC
	DCM	0.85	> 25.0	12.0	NC	14.1

EC_{50} or effective concentration (µg/mL) is the extract concentration that kills 50% of malaria parasites. Activity is not effective if EC_{50} value > 10 µg/mL. K1 is chloroquine-resistant strain of *Plasmodium falciparum*. IC_{50} of antiproliferative (APF) activity and selectivity index (SI) of APM activity against HepG2 and MCF-7 cells were calculated only for the most potent extracts against K1 strain with $EC_{50} \leq 1.00$ µg/mL. Abbreviations: Hex = hexane, MeOH = methanol, DCM = dichloromethane and NC = non-cytotoxic with selectivity index (SI) values > 25.0.

Recently, a study on antimalarial and cytotoxic activities of plants considered SI values of extracts > 10 as high (Lusakibanza *et al.* 2010). With SI values relatively higher than 10, the extracts of *Alstonia angustiloba*, *Calotropis gigantea* and *Vallaris glabra* are potentially safe for use to treat malaria.

4.3 PHYTOCHEMICAL CONTENTS AND BIOACTIVITIES

4.3.1 Extracts from Dried Leaves

a. Extraction yield

The yield of the methanol crude extracts ranged from 3.6–14 g per 100 g of fresh leaves [**Table 4.5**]. *Dyera costulata* had the highest yield while *Vallaris glabra* had moderate yield. Generally, the sum of the yield of the sequential extracts was comparable to those of methanol crude extracts of most species, with the exception of *Vallaris glabra* and *Alstonia angustiloba*. These plants had higher yield in their

hexane extracts compared to the other species, implying that they contain higher amounts of lipophilic compounds.

Table 4.5 Yield of crude and sequential extracts of Apocynaceae species studied

Species	Yield of crude extracts	Yield of sequential extracts			
		Hexane	DCM	DCM:MeOH	MeOH
Dyera costulata	14	1.0	1.2	6.9	5.2
Allamanda cathartica	5.9	0.9	1.0	2.6	1.6
Vallaris glabra	5.9	2.6	1.6	4.4	2.9
Kopsia fruticosa	5.5	0.7	0.8	2.1	2.2
Calotropis gigantea	3.6	0.6	0.5	1.9	0.8
Plumeria obtusa	5.8	0.5	0.8	2.5	0.9
Catharanthus roseus	5.1	0.5	0.9	2.1	2.2
Alstonia angustiloba	7.7	3.1	1.1	3.3	3.0
Cerbera odollum	11	1.6	0.9	6.0	4.3
Nerium oleander	9.9	1.8	1.9	4.7	3.0

Yield of extracts is weight of dried extract in g per 100 g of fresh leaves.

b. Total alkaloid content

The total alkaloid content (TAC) of crude and sequential leaf extracts of ten Apocynaceae species was determined using the Dragendorff precipitation assay. Results of their TAC are shown in **Table 4.6**.

Table 4.6 Total alkaloid content of crude and sequential leaf extracts of ten Apocynaceae species

Species	Crude leaf extract	Sequential leaf extract			
		Hex	DCM	DCM:MeOH	MeOH
Kopsia fruticosa	100 ± 4.2	63 ± 1.1	129 ± 4.0	99 ± 2.5	46 ± 1.6
Dyera costulata	58 ± 2.5	2.4 ± 0.6	11 ± 1.8	68 ± 2.7	41 ± 2.4
Catharanthus roseus	37 ± 2.8	18 ± 0.9	9.1 ± 0.8	76 ± 5.5	35 ± 3.3
Alstonia angustiloba	23 ± 0.3	13 ± 2.3	27 ± 3.1	58 ± 1.2	27 ± 1.0
Cerbera odollam	2.8 ± 0.9	3.6 ± 0.5	3.0 ± 0.5	5.0 ± 1.5	3.2 ± 0.4
Calotropis gigantea	2.7 ± 0.3	3.7 ± 1.0	8.6 ± 1.2	9.6 ± 1.9	9.2 ± 2.7
Vallaris glabra	2.7 ± 0.4	4.4 ± 0.8	8.9 ± 0.6	9.2 ± 0.2	8.7 ± 2.2
Plumeria obtusa	2.3 ± 0.3	2.3 ± 0.4	2.5 ± 0.1	4.3 ± 1.3	5.8 ± 1.7
Allamanda cathartica	2.0 ± 0.1	4.4 ± 1.3	8.7 ± 0.9	12 ± 1.0	8.7 ± 2.1
Nerium oleander	1.4 ± 0.7	1.6 ± 0.0	2.8 ± 0.8	3.9 ± 0.7	8.3 ± 0.5

Total alkaloid content (TAC) was expressed as mg BE/g of extract. Abbreviations: Hex = hexane, DCM = dichloromethane, MeOH = methanol and BE = boldine equivalent.

Of the ten species analysed, MeOH crude and DCM leaf extracts of *Kopsia fruticosa* had the highest TAC (100 mg BE/g and 129 mg BE/g of extract), respectively. Other species with moderately high TAC were *Dyera costulata*, *Catharanthus roseus* and *Alstonia angustiloba* with MeOH crude and DCM:MeOH extracts having values ranging from 23–58 mg BE/g and 58–68 mg BE/g of extract, respectively.

Based on TAC values of MeOH crude leaf extracts, the species can be ranked as high (*Kopsia fruticosa*), moderate (*Dyera costulata*, *Catharanthus roseus* and *Alstonia angustiloba*), and low (*Cerbera odollam*, *Calotropis gigantea*, *Vallaris glabra*, *Plumeria obtusa*, *Allamanda cathartica* and *Nerium oleander*). The classification used was ‘high’ for > 60 mg BE/g of extract, ‘moderate’ for 10–60 mg BE/g of extract and low for < 10 mg BE/g of extract.

The high TAC of leaf extracts of *Kopsia fruticosa* may be attributed to the presence of aspidofractinine alkaloids identified as kopsine, fruticosine and fruticosamine (Kam & Lim 2008). These heptacyclic bridged alkaloids are the predominant alkaloids in *Kopsia* species. The NMR spectral assignments of kopsine, fruticosine and fruticosamine are available for reference (Glover *et al.* 2005)

c. Phenolic content and antioxidant activity

Results on the total phenolic content (TPC) and free radical scavenging (FRS) activity of crude and sequential leaf extracts of ten Apocynaceae species are shown in **Table 4.7** and **Table 4.8**, respectively.

Table 4.7 Total phenolic content (TPC) of crude and sequential leaf extracts of ten Apocynaceae species

Species	Crude leaf extract	Sequential leaf extract			
		Hex	DCM	DCM:MeOH	MeOH
Dyera costulata	319 ± 5.5	21 ± 0.2	23 ± 0.1	354 ± 6.0	279 ± 3.0
Vallaris glabra	99 ± 3.8	15 ± 0.9	24 ± 0.5	134 ± 1.0	164 ± 13
Plumeria obtusa	85 ± 0.9	21 ± 1.7	52 ± 1.3	104 ± 4.0	134 ± 3.0
Kopsia fruticosa	83 ± 1.1	39 ± 0.5	20 ± 0.5	129 ± 1.0	84 ± 1.0
Alstonia angustiloba	68 ± 2.0	17 ± 0.6	24 ± 0.6	96 ± 1.1	94 ± 1.1
Nerium oleander	56 ± 3.8	18 ± 0.3	23 ± 0.1	57 ± 0.3	29 ± 0.3
Catharanthus roseus	53 ± 2.5	24 ± 0.5	46 ± 1.4	75 ± 0.7	61 ± 1.1
Allamanda cathartica	37 ± 0.8	11 ± 0.4	27 ± 0.2	39 ± 0.9	42 ± 0.5
Cerbera odollam	30 ± 4.1	19 ± 0.7	26 ± 0.5	30 ± 0.6	40 ± 0.9
Calotropis gigantea	28 ± 0.8	14 ± 0.6	44 ± 1.7	42 ± 0.8	33 ± 0.5

Values are expressed as mg GAE/g of extract and are means ± SD (n = 3). Abbreviations: Hex = hexane, DCM = dichloromethane, MeOH = methanol and GAE = gallic acid equivalent.

Extracts of *Dyera costulata* had the highest TPC and strongest FRS. Crude, DCM:MeOH and MeOH extracts yielded TPC values of 319, 354 and 279 mg GAE/g of extract, and FRS values of 377, 349 and 278 mg AA/g of extract, respectively. Compared to *Dyera costulata*, extracts of other species can be categorised as moderate to low.

Table 4.8 Free radical scavenging (FRS) activity of crude and sequential leaf extracts of ten Apocynaceae species

Species	Crude leaf extract	Sequential leaf extract			
		Hex	DCM	DCM:MeOH	MeOH
Dyera costulata	377 ± 25	15 ± 0.3	9.3 ± 0.5	349 ± 21	278 ± 5.4
Vallaris glabra	84 ± 0.5	6.0 ± 0.3	8.4 ± 0.4	77 ± 2.2	119 ± 6.8
Plumeria obtusa	66 ± 3.5	4.9 ± 0.2	10 ± 0.9	58 ± 0.1	115 ± 5.3
Kopsia fruticosa	63 ± 3.2	12 ± 1.4	7.5 ± 0.3	70 ± 2.0	48 ± 1.5
Nerium oleander	42 ± 1.3	6.4 ± 0.1	8.5 ± 0.6	48 ± 1.2	33 ± 0.2
Alstonia angustiloba	29 ± 0.9	10 ± 0.2	5.7 ± 0.7	50 ± 1.2	46 ± 2.2
Catharanthus roseus	24 ± 0.8	13 ± 0.5	21 ± 0.5	33 ± 1.1	31 ± 0.3
Allamanda cathartica	17 ± 1.1	11 ± 0.1	7.9 ± 0.8	18 ± 1.4	24 ± 2.0
Cerbera odollam	15 ± 1.0	16 ± 0.4	5.5 ± 0.1	14 ± 1.3	25 ± 0.7
Calotropis gigantea	7.6 ± 0.4	5.6 ± 0.3	6.1 ± 0.4	8.0 ± 0.6	14 ± 1.3

Values are expressed as mg AA/g of extract and are means ± SD (*n* = 3). Abbreviations: Hex = hexane, DCM = dichloromethane, MeOH = methanol and AA = ascorbic acid.

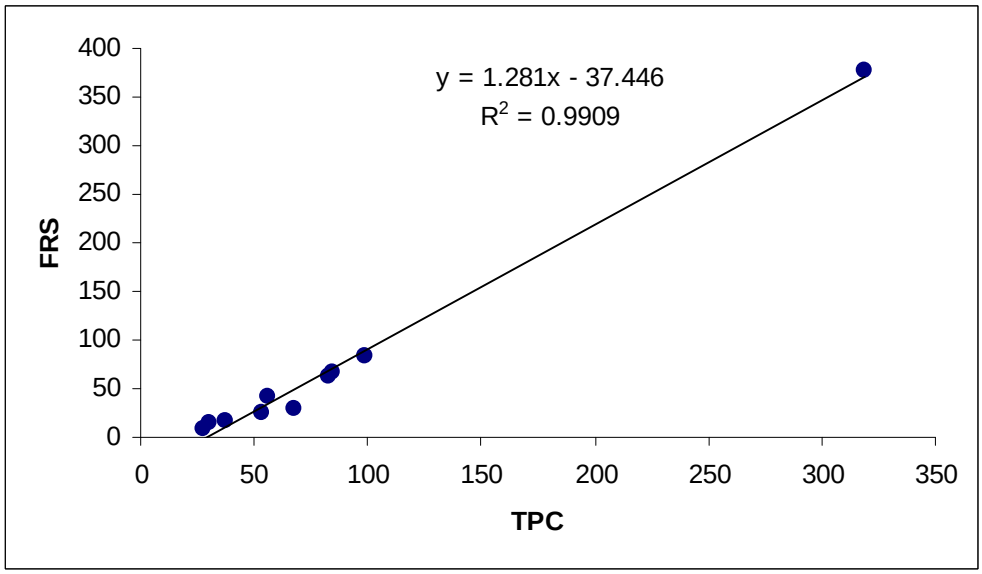
The presence of flavonols identified as 3,7-dimethoxyquercetin and quercetin-3-*O*-α-L-rhamnopyranoside (Subhadhirasakul *et al.* 2003) may contribute to the high TPC and FRS of leaf extracts of *Dyera costulata.* Based on DPPH radical scavenging activity, the IC_{50} values of quercetin-3-*O*-α-L-rhamnopyranoside was 9.37 ± 0.02 µM. Its antioxidative activity is therefore about eight times higher than that of butylated hydroxytoluene (BHT) with an IC_{50} value of 80.8 ± 0.01 µM. The crude chloroform extract and the crude n-butanol extract from the leaves of *Dyera costulata* exhibited

EC_{50} values for DPPH radical scavenging activity were 79.8 ± 0.2 and 12.0 ± 0.1 µg/mL, respectively.

It should be noted that ranking of the species based on TPC [**Table 4.6**] and FRS [**Table 4.7**] values of crude extracts was almost identical, suggesting the likelihood of a positive correlation between TPC and FRS.

TPC and FRS values of crude extracts were plotted to calculate the correlation coefficient. Overall, there was a strong correlation between TPC and FRS of extracts (R^2 = 0.991) but not between TPC and TAC (R^2 = 0.193), and not between TAC and FRS (R^2 = 0.168) [**Figure 4.1**]. Similarly, TPC and FRS values of sequential extracts were plotted, yielding an equally strong correlation (R^2 = 0.947) [**Figure 4.2**].

The strong correlation between TPC and FRS of extracts affirms that leaves of Apocynaceae species with higher concentration of phenolic compounds also have stronger radical scavenging capacity. This would mean that the presence of phenolic compounds contributes to their antioxidant potential.



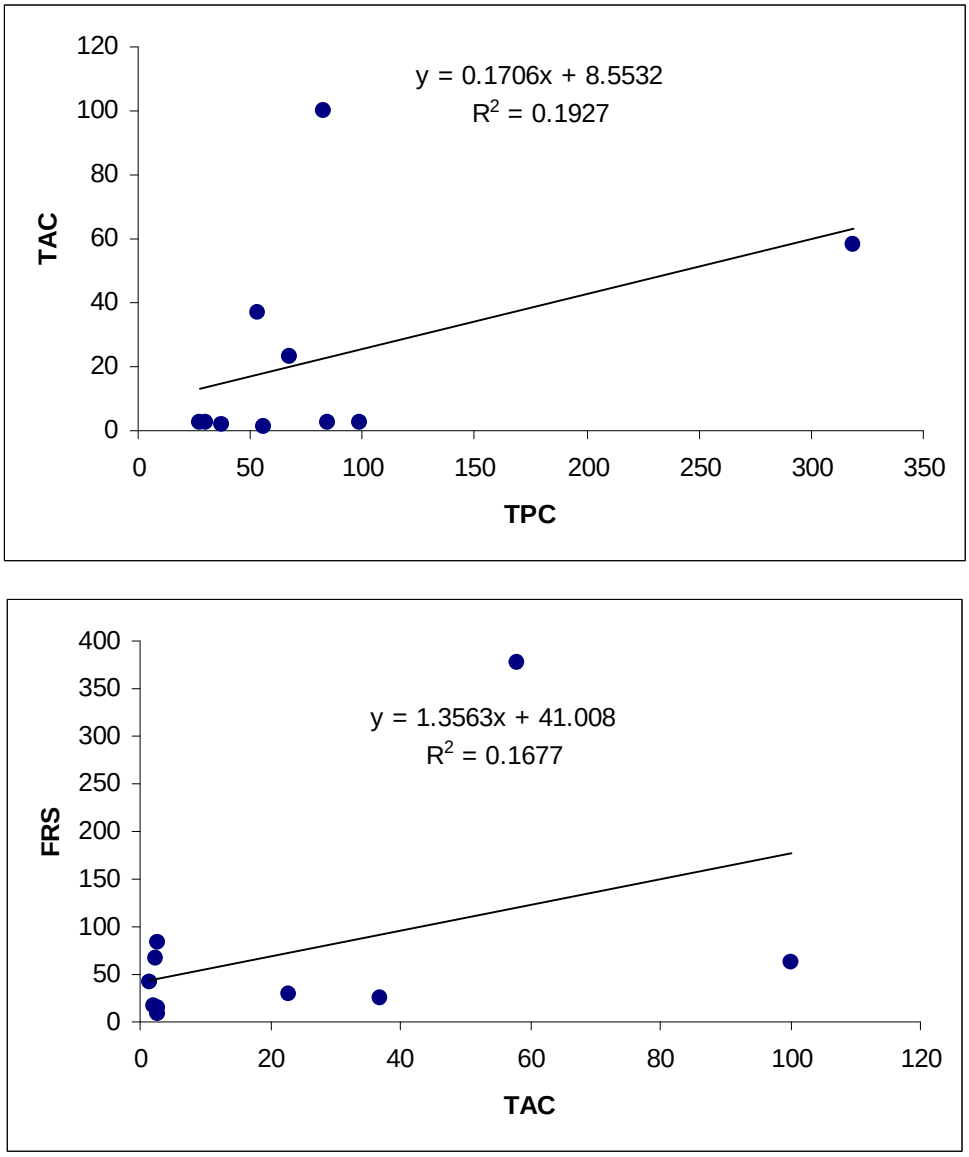


Figure 4.1 Correlation between free radical scavenging (FRS) activity and total phenolic content (TPC), between total alkaloid content (TAC) and TPC, and between FRS activity and TAC of crude leaf extracts of ten Apocynaceae species

Similar finding of positive correlations between TPC and FRS have been reported in medicinal plants and herbs (Guo *et al.* 2008; Dudonné *et al.* 2009; Akinmoladun *et al.* 2010; Raj *et al.* 2010), wild edible fruits (Lamien-Meda *et al.* 2008), and mushrooms (Cheung *et al.* 2003).

The correlation of results of phytochemical analysis with APF activities remains unclear. Extracts of *Calotropis gigantea*, *Nerium oleander* and *Vallaris glabra* which showed strong APF activities had low TAC, and moderate to low TPC and FRS.

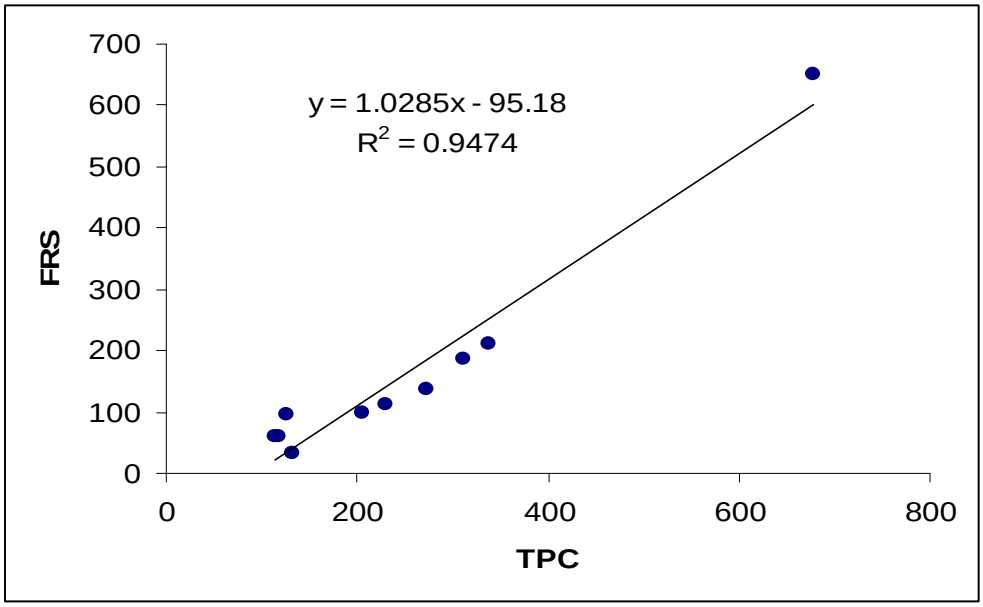


Figure 4.2 Correlation between free radical scavenging (FRS) activity and total phenolic content (TPC) of sequential leaf extracts of ten Apocynaceae species

c. Antityrosinase activity

The antityrosinase (AT) activity of crude leaf extracts of ten Apocynaceae species was determined using the modified dopachrome method with L-3,4-dihydroxyphenyl alanine (L-dopa) as substrate. Results are shown in **Table 4.9**.

Analysis showed that the AT activity of *Dyera costulata* (35 ± 4.0%) surpassed those of all other species and was comparable to that of *Psidium guajava* (41 ± 3.0%), the positive control. Values of the other species ranged from 2.0 ± 0.7% in *Vallaris glabra* to 19 ± 2.7% in *Catharanthus roseus* and 19 ± 2.8% in *Alstonia angustiloba*.

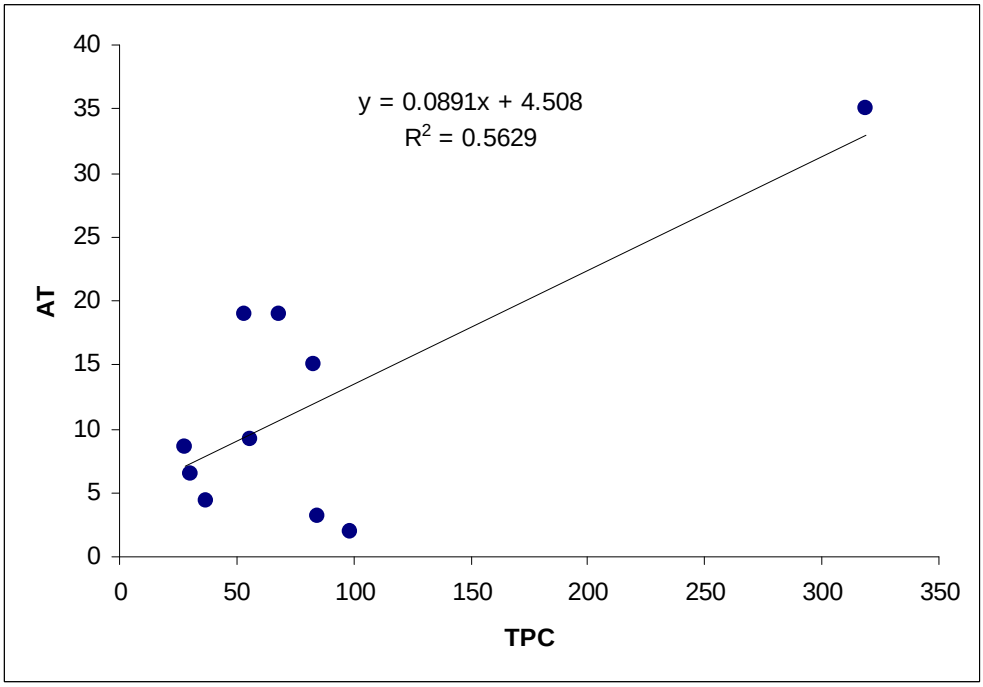
TPC and FRS values of crude extracts were plotted against AT values to calculate the correlation coefficient [**Figure 4.3**]. Overall, there was a weak correlation between TPC and AT of extracts (R^2 = 0.563) and between FRS and AT of extracts (R^2 = 0.535), suggesting that the AT activity in Apocynaceae species may not only be attributed to phenolic compounds with antioxidant properties.

Table 4.9 Antityrosinase activity of crude leaf extracts of ten Apocynaceae species

Species	Antityrosinase activity (%)
Dyera costulata	35 ± 4.0
Alstonia angustiloba	19 ± 2.8
Catharanthus roseus	19 ± 2.7
Kopsia fruticosa	15 ± 2.8
Nerium oleander	9.2 ± 2.9
Calotropis gigantea	8.5 ± 3.1
Cerbera odollam	6.4 ± 2.9
Allamanda cathartica	4.3 ± 1.6
Plumeria obtusa	3.1 ± 0.8
Vallaris glabra	2.0 ± 0.7
Psidium guajava	41 ± 3.0

Values are means ± SD (*n* = 3). Concentration of extracts used was 0.5 mg/mL. Leaves of *Psidium guajava* were used as positive control.

Earlier studies on the AT activity of leaves of five *Etlingera* gingers have reported values ranging from 22% in *Etlingera littoralis* to 55% in *Etlingera elatior* (Chan *et al.* 2008). Out of leaves of four *Hibiscus* species, Wong *et al.* (2010) reported values ranging from 5% in *Hibiscus sabdariffa* to 42% in *Hibiscus tiliaceus*.



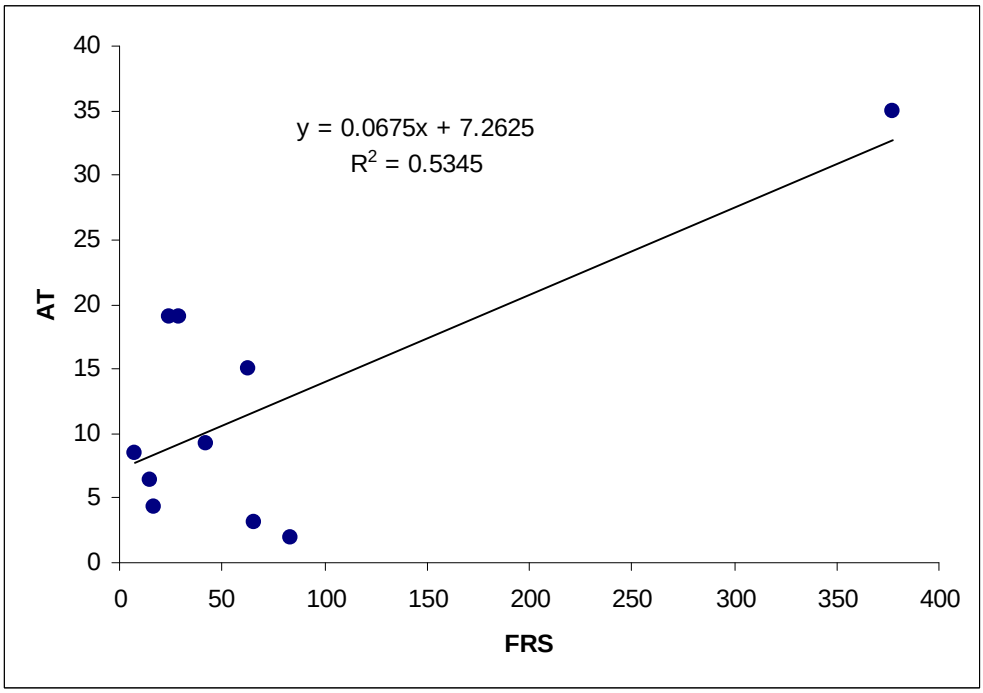


Figure 4.3 Correlation between antityrosinase (AT) activity and total phenolic content (TPC), and between AT activity and free radical scavenging (FRS) activity of crude leaf extracts of ten Apocynaceae species

4.3.2 Extracts from Fresh Leaves

a. Extraction efficiency of methanol

The extraction efficiency of 70% and 100% methanol based on total phenolic content (TPC) and free radical scavenging (FRS) activity of fresh leaves of six Apocynaceae species was compared [**Table 4.10**].

Table 4.10 Extraction efficiency of 70% and 100% methanol based on total phenolic content (TPC) and free radical scavenging (FRS) activity of fresh leaves of ten Apocynaceae species

Species	MeOH (%)	TPC	FRS
Dyera costulata	100	74 ± 1.0 [a]	70 ± 4.0 [a]
	70	61 ± 3.3 [b]	56 ± 1.4 [b]
Vallaris glabra	100	11 ± 2.1 [a]	7.3 ± 0.1 [b]
	70	13 ± 1.2 [a]	10 ± 1.0 [a]
Alstonia angustiloba	100	9.5 ± 0.5 [a]	4.0 ± 0.3 [b]
	70	10 ± 1.1 [a]	6.1 ± 0.4 [a]
Nerium oleander	100	8.8 ± 0.8 [b]	5.7 ± 0.1 [a]
	70	10 ± 0.4 [a]	6.0 ± 0.3 [a]
Plumeria obtusa	100	7.2 ± 0.6 [a]	4.7 ± 0.4 [a]
	70	6.7 ± 0.1 [a]	4.5 ± 0.6 [a]
Catharanthus roseus	100	5.8 ± 0.2 [b]	3.4 ± 0.1 [b]
	70	6.7 ± 0.0 [a]	4.1 ± 0.2 [a]
Allamanda cathartica	100	4.4 ± 0.1 [a]	2.6 ± 0.3 [a]
	70	4.7 ± 0.3 [a]	2.8 ± 0.4 [a]
Cerbera odollam	100	3.6 ± 0.0 [b]	1.6 ± 0.2 [b]
	70	5.4 ± 0.1 [a]	3.3 ± 0.0 [a]
Kopsia fruticosa	100	4.6 ± 0.2 [a]	2.4 ± 0.2 [a]
	70	4.3 ± 0.2 [a]	2.3 ± 0.1 [a]
Calotropis gigantea	100	1.6 ± 0.3 [a]	0.3 ± 0.0 [a]
	70	1.5 ± 0.1 [a]	0.4 ± 0.1 [a]

Total phenolic content (TPC) and free radical scavenging (FRS) activity were expressed as mg GAE/g and mg AA/g of samples (fresh weight), respectively. For the column of each species, values followed by the same superscript (a–b) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test. ANOVA does not apply between species. Values are means ± SD ($n = 3$).

Results showed that values of TPC and/or FRS of 70% methanol were significantly higher than 100% methanol for *Vallaris glabra*, *Alstonia angustiloba*, *Nerium oleander*, *Catharanthus roseus* and *Cerbera odollam*. Values were comparable for *Plumeria obtusa*, *Allamanda cathartica*, *Kopsia fruticosa* and *Calotropis gigantea*. *Dyera costulata* was the only exception where values of 100% methanol extraction were significantly higher than those of 70% methanol.

A trial was also carried out to test the extraction efficiency of 50%, 70% and 100% methanol based on TPC and FRS activity of fresh leaves of *Vallaris glabra*. Results showed TPC and FRS values of 70% methanol were comparable to those of 50% methanol [**Table 4.11**]. Both their values were significantly higher than 100% methanol.

Table 4.11 Extraction efficiency of 50%, 70% and 100% methanol based on total phenolic content and free radical scavenging activity of fresh leaves of *Vallaris glabra*

Methanol (%)	TPC	FRS
50	12 ± 1.3 ^{ab}	9.6 ± 1.3 ^{ab}
70	13 ± 1.2 ^{a}	10 ± 1.0 ^{a}
100	10 ± 1.3 ^{b}	7.3 ± 1.2 ^{b}

Total phenolic content (TPC) and free radical scavenging (FRS) activity were expressed as mg GAE/g and mg AA/g of samples (fresh weight), respectively. Values are means ± SD ($n = 3$). For each column, values followed by the same superscript (a–b) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test.

Based on the results obtained, 70% methanol was used for extraction to determine the phenolic content and antioxidant activity of fresh leaves. Aqueous methanol is commonly used to extract herbs and herbal teas (Atoui *et al.* 2005; Turkmen *et al.* 2006; Chan *et al.* 2012). Methanol and aqueous methanol are the most suitable

solvent for extracting phenolic compounds from plants due to their ability to inhibit the action of polyphenol oxidases (PPO) that cause the oxidation of phenolic compounds and their ease of evaporation compared to water (Waterman & Mole 1994; Arts & Hollman 1998; Bravo & Mateos 2008). Methanol has higher extraction efficiency than ethanol, and aqueous methanol has been used for extracting hydroxycinnamic acids and flavonoids from plants (Tsao & Deng 2004). Methanol extracts more catechins from fresh leaf shoots of *Camellia sinensis* than hot water (Yao *et al*. 2004b).

b. Phenolic content and antioxidant activity

In **item 4.3.1c**, total phenolic content and antioxidant activity were determined using extracts from dried leaves. In this section, antioxidant properties are assessed using extract solutions from fresh leaves. Results of antioxidant properties based on fresh leaves extracted with 70% methanol are shown in **Table 4.12.**

Table 4.12 Total phenolic content, free radical scavenging activity and caffeoylquinic acid content of fresh leaf extracts of six species of Apocynaceae

Species	TPC	FRS	CQAC
Dyera costulata	67 ± 4.5 ^{a}	102 ± 9.8 ^{a}	8.9 ± 1.0 ^{a}
Vallaris glabra	13 ± 1.2 ^{b}	10 ± 1.0 ^{b}	3.8 ± 0.5 ^{b}
Alstonia angustiloba	9.6 ± 0.4 ^{c}	6.1 ± 1.1 ^{cd}	2.1 ± 0.3 ^{c}
Nerium oleander	9.7 ± 1.7 ^{c}	6.0 ± 0.3 ^{d}	8.2 ± 1.8 ^{a}
Kopsia fruticosa	7.5 ± 0.5 ^{d}	6.3 ± 1.0 ^{cd}	4.2 ± 0.4 ^{b}
Plumeria obtusa	6.6 ± 0.5 ^{d}	4.5 ± 0.6 ^{e}	4.2 ± 0.4 ^{b}
Lonicera japonica	5.3 ± 0.4 ^{e}	7.5 ± 1.0 ^{c}	2.5 ± 0.5 ^{c}

Total phenolic content (TPC), free radical scavenging (FRS) activity and caffeoylquinic acid content (CQAC) were expressed as mg GAE/g, mg AA/g and mg CGAE/g of samples (fresh weight), respectively. Values are means ± SD (n = 3). For each column, values followed by the same superscript (a–e) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test. Flowers of *L. japonica* are the commercial source of CGA.

Leaves of *Dyera costulata* yielded the highest values of TPC (67 ± 4.5 mg GAE/g) and FRS (102 ± 9.8 mg AA/g). Highest CQAC values were those of *Dyera costulata* (8.9 ± 1.0 mg CGAE/g) and *Nerium oleander* (8.2 ± 1.8 mg CGAE/g).

Dyera costulata had the highest TPC, FRS and CQAC among the six Apocynaceae species studied. *Nerium oleander*, despite having a lower TPC, had a CQAC comparable to that of *Dyera costulata*. Leaves of all the Apocynaceae species studied had higher TPC than flowers of *Lonicera japonica*, the commercial source of chlorogenic acid.

TPC and FRS values of fresh leaf extracts of the six Apocynaceae species were plotted to calculate the correlation coefficient (R^2). Results showed a strong correlation between TPC and FRS of extracts (R^2 = 0.997) [**Figure 4.4**].

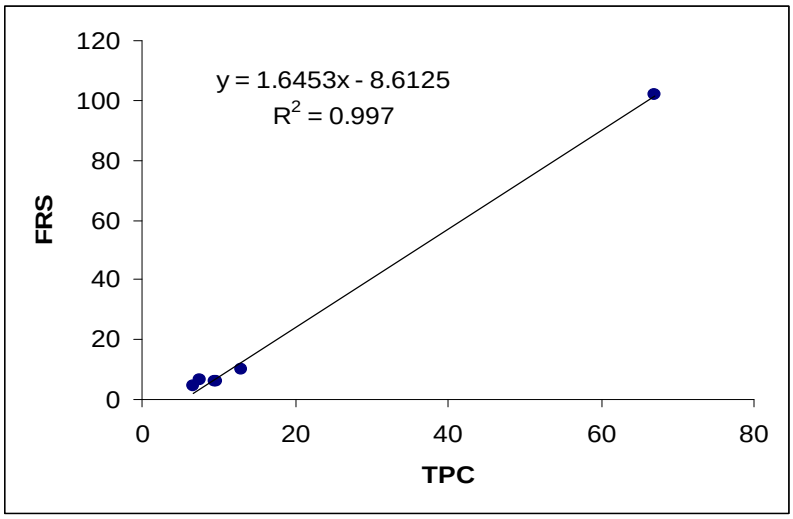


Figure 4.4 Correlation between free radical scavenging (FRS) activity and total phenolic content (TPC) of fresh leaf extracts of six Apocynaceae species

The leaves of *Vallaris glabra* extracted showed relatively high amount of caffeoylquinic acid (CQA) of 3.8 ± 0.5 mg CGAE/g compared to flowers of *Lonicera japonica* which had 2.5 ± 0.5 mg CGAE/g as reported by Chan *et al.* (2009). Several CQA derivatives reported by Iwai *et al.* (2004) have demonstrated potent APF activity against human cancer cell lines (U937, MCF-7 and KB) as well as normal human diploid lung fibroblast WI38 and SV40 virally transformed WI38 (WI38VA) with IC_{50} ranging from 0.10–5.99 mM. Because *Vallaris glabra* leaves contain good amount of CQA, it is worthwhile to identify the caffeoylquinic acids present.

4.4 CHOICE OF SPECIES FOR FURTHER STUDY

Overall score and ranking of Apocynaceae species to the full range of bioactivity and phytochemical assays are shown in **Table 4.13**. Ranking was of the order: *Dyera costulata* > *Vallaris glabra* ~ *Alstonia angustiloba* > *Kopsia fruticosa* > *Calotropis gigantea*.

Table 4.13 Overall score and ranking of crude and sequential leaf extracts of Apocynaceae species based on bioactivity and phytochemical assays

Species	TAC	TPC	FRS	AT	APF	APM	Overall score	Overall ranking
Alstonia angustiloba	3 (58)	2 (68)	2 (29)	4 (19)	3 (16)	5 (0.5)	19	2
Calotropis gigantea	2 (2.7)	1 (28)	1 (7.6)	2 (8.5)	5 (1.3)	3 (1.0)	14	5
Dyera costulata	4 (58)	5 (319)	5 (377)	5 (35)	0 (ND)	1 (7.5)	20	1
Kopsia fruticosa	5 (100)	3 (83)	3 (63)	3 (15)	0 (ND)	3 (1.0)	17	4
Vallaris glabra	2 (2.7)	4 (99)	4 (84)	1 (2.0)	4 (5.8)	4 (0.9)	19	2

Species were those subjected to the full range of bioactivity and phytochemical assays. Figures within brackets are approximate actual values obtained from the assays. Highest or strongest values are given a score of five while lowest or weakest values are given a score of one. Abbreviations: TAC = total alkaloid content (mg BE/g of extract), TPC = total phenolic content (mg GAE/g of extract), FRS = free radical scavenging activity (mg AA/g of extract), AT = antityrosinase activity (%), APF = antiproliferative activity based on strongest GI_{50} (µg/mL) of extract, APM = antiplasmodial activity based on strongest EC_{50} (µg/mL) of extract and ND = not detected.

Dyera costulata had the highest overall score (20) and ranking (1), but scored poorly in APF and APM activities. *Alstonia angustiloba* and *Vallaris glabra* had the second highest overall score (19) and ranking (2).

Vallaris glabra was chosen as the main species for further study as it displayed outstanding APF and APM activities, and the literature on the bioactivity and phytochemistry of the plant is scarce.

IVb *VALLARIS GLABRA*

4.5 PHYTOCHEMICAL SCREENING OF LEAF EXTRACTS

Phytochemical screening of sequential leaf extracts of *Vallaris glabra* showed the presence of terpenoids in all extracts except MeOH extract. Its presence was strong in the Hex extract and moderate in DCM and DCM:MeOH extracts.

Saponins and tannins were present in DCM and DCM:MeOH extracts, and in DCM:MeOH and MeOH extracts, respectively [**Table 4.14**]. Cardenolides were not detected.

Table 4.14 Phytochemical screening of sequential leaf extracts of *Vallaris glabra*

Sequential leaf extract	Qualitative test			
	Saponin	Tannin	Cardenolide	Terpenoid
Hex	–	–	–	+++
DCM	+	–	–	++
DCM:MeOH	++	++	–	++
MeOH	–	++	–	–

Presence or absence was categorised as strong (+++), moderate (++), weak (+) or absent (–). Classification was based on colour intensity and amount of precipitate. Saponins, tannins, cardenolides and terpenoids were screened using the frothing, ferric chloride, Kedde and vanillin-H_2SO_4 tests. Abbreviations: Hex = hexane, DCM = dichloromethane and MeOH = methanol.

Terpenoids are the most numerous and structurally diverse plant natural products with many functional roles and applications (Zwenger & Basu 2008). They are used for the treatment of human diseases such as cancer and malaria, and infectious diseases caused by virus and bacterial (Wang *et al.* 2005). Taxol and artimesinin are renowned terpenoid-based anticancer and antimalarial drugs, respectively.

Tannins are water-soluble polymeric polyphenols. They are water-soluble phenolic compounds having molecular weights between 500 and 3000 Da (Koleckar *et al.* 2008; Serrano *et al.* 2009). On the basis of their structures, tannins are classified into two major groups, i.e. hydrolysable tannins and condensed tannins.

Literature on the effects of tannins on human health is vast and sometimes conflicting (Chung *et al.* 1998). Incidences of esophageal cancer have been attributed to consumption of tannin-rich foods such as herbal teas, suggesting that tannins might be carcinogenic. However, reports have indicated a negative association between consumption of tea and the incidence of cancer. Teas with high tannin content have been suggested to be anticarcinogenic and antimutagenic which may be related to their antioxidative property in protecting cellular oxidative damage against lipid peroxidation and superoxide radicals.

Tannins have been reported to be more potent antioxidants than are simple monomeric phenolics (Hagerman *et al.* 1998). The antioxidant activity of tannins was 15–30 times more effective than simple phenolics. The antimicrobial activities of tannins are well documented (Chung *et al.* 1998; Koleckar *et al.* 2008). As tannins have high molecular weight and they form insoluble complexes with proteins, they are not expected to be absorbed in the human body (Koleckar *et al.* 2008). Though some studies have confirmed the absorption of tannins in the body, there are still questions about their bioavailability.

Saponins are naturally occurring glycosides with a distinctive foaming characteristic and bitter taste (Shi *et al.* 2004; Desai *et al.* 2009). They have a wide range of properties, which include both beneficial and detrimental effects on human health. Saponins affect the immune system in ways that help to protect the human body against cancers, and also lower cholesterol levels. They decrease blood lipids, lower cancer risks and lower blood glucose response.

4.6 EXTRACTS OF OTHER PLANT PARTS

4.6.1 Initial Screening for Antiproliferative Activity

Other plant parts (stems and flowers) of *Vallaris glabra* that were initially screened for APF activity showed that DCM extract of stems inhibited three cell lines of HT-29, MCF-7 and SKOV-3 [**Table 4.15**]. The other three sequential extracts of the stems seemed to be selectively toxic towards MCF-7 and SKOV-3 cells, with no inhibition against HT-29 and MDA-MB-231.

Table 4.15 Sequential flower and stem extracts of *Vallaris glabra* with positive inhibition of four human cancer cell lines

Plant part	Sequential extract	Inhibition of cancer cells			
		HT-29	MCF-7	MDA-MB-231	SKOV-3
Stems	Hex	–	+	–	+
	DCM	+	+	–	+
	DCM:MeOH	–	+	–	+
	MeOH	–	+	–	+
Flowers	Hex	+	+	–	–
	DCM	+	+	+	+
	DCM:MeOH	+	+	–	+
	MeOH	–	–	–	+

Inhibitory activity of less than 50% cell density is considered positive (+) while that of more than 50% cell density is considered negative (–). MCF-7 and MDA-MB-231 are human breast cancer cells, HT-29 are human colon cancer cells, and SKOV-3 are human ovarian cancer cells. Abbreviations: DCM = dichloromethane, Hex = hexane and MeOH = methanol.

On the contrary, DCM extract of flowers exhibited broad-spectrum APF activity against all four cell lines. Hex flower extract showed positive inhibition against HT-29 and MCF-7 cells, suggesting selective inhibition. DCM:MeOH extract was ineffective against MDA-MB-231 cells, but showed inhibition against the other three cell lines of HT-29, MCF-7 and SKOV-3. Interestingly, MeOH flower extract showed inhibition against SKOV-3 cells only.

In comparison, DCM and DCM:MeOH leaf extracts of *Vallaris glabra* had positive inhibition against all three human cancer cell lines of MCF-7, MDA-MB-231 and HeLa [**Table 4.1**]. Hex leaf extract was not active while MeOH leaf extract inhibited only MCF-7 cells.

4.6.2 Further Testing of Antiproliferative Activity

APF activity of sequential flower and stem extracts of *Vallaris glabra* with positive growth inhibition against HT-29, MCF-7, MDA-MB-231 and SKOV-3 cancer cells was further tested [**Table 4.16**].

Table 4.16 Antiproliferative activity of sequential flower and stem extracts of *Vallaris glabra* with positive growth inhibition of four human cancer cell lines

Plant part	Sequential extract	Growth inhibition (GI_{50})			
		HT-29	MCF-7	MDA-MB-231	SKOV-3
Stems	Hex	–	7.0 ± 0.6	–	1.8 ± 0.3
	DCM	1.7 ± 0.5	1.4 ± 0.2	–	1.2 ± 0.1
	DCM:MeOH	–	3.9 ± 0.7	–	1.7 ± 0.3
	MeOH	–	5.4 ± 1.6	–	1.8 ± 0.2
Flowers	Hex	5.9 ± 2.0	3.3 ± 1.0	–	–
	DCM	1.4 ± 0.3	1.4 ± 0.2	4.4 ± 1.2	1.6 ± 0.2
	DCM:MeOH	6.5 ± 1.1	4.6 ± 1.1	–	3.1 ± 0.7
	MeOH	–	–	–	7.4 ± 0.8
Xanthorrhizol			11 ± 0.7	8.7 ± 0.8	
Curcumin			4.1 ± 0.9	8.7 ± 0.8	
Tamoxifen			8.3 ± 0.6	4.6 ± 0.5	

GI_{50} (µg/mL) is the concentration which causes 50% reduction in cell growth and inhibition is not effective (–) if values ≥ 20 µg/mL.HT-29 are human colon cancer cells, MCF-7 and MDA-MB-231 are human breast cancer cells, and SKOV-3 are human cervical cancer cells. Values of standard drugs of xanthorrhizol, curcumin and tamoxifen against MCF-7 and MDA-MB-231 reported by Cheah *et al.* (2008) were used as positive controls. Abbreviations: Hex = hexane, MeOH = methanol and DCM = dichloromethane.

APF activity of stems and flowers of *Vallaris glabra* has not been reported before. DCM extract of both stems and flowers were the most potent against MCF-7 cells compared to the other three sequential extracts. GI_{50} of DCM extract of stems and flowers (both 1.4 ± 0.2 µg/mL) for MCF-7 cells was lower than that of the positive controls. Against MCF-7 cells, GI_{50} of Hex extract of stems (7.0 ± 0.6 µg/mL) and flowers (3.3 ± 1.0 µg/mL) was more potent than GI_{50} of tamoxifen (8.3 ± 0.6 µg/mL) and xanthorrhizol (11 ± 0.7 µg/mL). Hex flower extract was as effective as curcumin (4.1 ± 0.9 µg/mL).

Overall, the inhibition of stem extracts was more specific to MCF-7 and SKOV-3 cells. Similar to leaves, flower extracts displayed more broad-spectrum APF activity against all four cancer cell lines.

4.6.3 Phenolic Content and Antioxidant Activity

Phenolic content (TPC and CQAC) and antioxidant activity (FRS) of different parts of *Vallaris glabra* were compared. Results showed that TPC, CQAC and FRS values of leaves were significantly higher than those of flowers and stems [**Table 4.17**]. The only exception was CQAC values of flowers and stems which were comparable. Ranking based on TPC, CQAC and FRS activity was of the order: leaves > flowers > stems.

Table 4.17 Total phenolic content, caffeoylquinic acid content and free radical scavenging activity of fresh leaf, flower and stem extracts of *Vallaris glabra*

Plant part	TPC	CQAC	FRS
Leaves	8.1 ± 0.4 [a]	3.7 ± 0.5 [a]	7.7 ± 0.6 [a]
Flowers	4.1 ± 0.2 [b]	0.9 ± 0.1 [b]	3.9 ± 0.1 [b]
Stems	2.8 ± 0.2 [c]	1.2 ± 0.3 [b]	2.1 ± 0.3 [c]

Total phenolic content (TPC), caffeoylquinic acid content (CQAC) and free radical scavenging (FRS) activity were expressed as mg GAE/g, mg AA/g and mg CGAE/g of samples (fresh weight), respectively. Values are means ± SD (n = 3). For each column, values followed by the same superscript (a–c) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test.

4.7 ANTIPROLIFERATIVE ACTIVITY OF FRACTIONS

4.7.1 Initial Screening for Antiproliferative Activity

Isolation of DCM leaf extract of *Vallaris glabra*, using silica gel 60 column chromatography with Hex-EtAc solvent system, resulted in 13 fractions. Eluates from column chromatography were collected in test tubes and each of the tubes was subjected to TLC chromatography. Tubes with the same TLC profiles were pooled to obtain the various fractions. The fractions were initially screened for APF activity against colon (HT-29), breast (MCF-7 and MDA-MB-231), and ovarian (SKOV-3) human cancer cell lines. Six fractions i.e. 3, 8, 9, 10, 11 and 12 displayed APF activity with positive inhibition of < 50% against at least three of the cell lines [**Table 4.18**]. Fractions 8, 10, 11 and 12 inhibited all four cell lines.

4.7.2 Further Testing of Antiproliferative Activity

Fractions with positive inhibitory activity were further tested against human cancer cell lines of HT-29, MCF-7, MDA-MB-231 and SKOV-3 [**Table 4.19** and **Table 4.20**].

Table 4.18 Fractions from DCM leaf extract of *Vallaris glabra* with positive inhibition of four human cancer cell lines

Fraction No.	Inhibition of cancer cells			
	HT-29	MCF-7	MDA-MB-231	SKOV-3
3	+	+	+	–
8	+	+	+	+
9	+	+	–	+
10	+	+	+	+
11	+	+	+	+
12	+	+	+	+

Inhibitory activity of less than 50% cell density is considered positive (+) while that of more than 50% cell density is considered negative (–). HT-29 are human colon cancer cells, MCF-7 and MDA-MB-231 are human breast cancer cells, and SKOV-3 are human ovarian cancer cells.

Table 4.19 Antiproliferative activity of fractions from DCM leaf extract of *Vallaris glabra* with positive growth inhibition of HT-29 and MCF-7 cancer cells

Fraction No.	HT-29		MCF-7	
	IC_{50}	GI_{50}	IC_{50}	GI_{50}
3	12 ± 1.7 [b]	5.2 ± 0.5 [c]	7.2 ± 0.8 [b]	3.4 ± 0.7 [b]
8	11 ± 1.4 [b]	5.3 ± 0.6 [c]	8.2 ± 0.7 [b]	3.8 ± 0.7 [b]
9	17 ± 3.8 [c]	5.4 ± 0.9 [c]	13 ± 2.7 [c]	4.2 ± 0.6 [c]
10	9.6 ± 2.5 [b]	3.5 ± 0.8 [b]	6.9 ± 1.4 [b]	2.1 ± 0.2 [a]
11	1.4 ± 0.2 [a]	2.1 ± 0.1 [a]	4.8 ± 0.9 [a]	2.3 ± 0.2 [a]
12	19 ± 4.0 [c]	12 ± 2.2 [d]	11 ± 0.9 [c]	5.1 ± 0.7 [c]
Standard drug				
Xanthorrhizol				11 ± 0.7
Curcumin				4.1 ± 0.9
Tamoxifen				8.3 ± 0.6

IC_{50} (µg/mL) is the concentration which causes 50% inhibition of cell density while GI_{50} (µg/mL) is the concentration which causes 50% reduction in cell growth. Values are means ± SD. For each column, values followed by the same superscript (a–d) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test. Values of standard drugs of xanthorrhizol, curcumin and tamoxifen against MCF-7 and MDA-MB-231 reported by Cheah *et al.* (2008) were used as positive controls.

Table 4.20 Antiproliferative activity of fractions from DCM leaf extract of *Vallaris glabra* with positive growth inhibition of MDA-MB-231 and SKOV-3 cancer cells

Fraction No.	MDA-MB-231		SKOV-3	
	IC_{50}	GI_{50}	IC_{50}	GI_{50}
3	12 ± 2.0 [b]	5.4 ± 1.1 [d]	24 ± 5.1 [c]	4.0 ± 0.7 [d]
8	12 ± 3.5 [b]	3.5 ± 0.5 [b]	13 ± 0.8 [b]	4.6 ± 0.3 [d]
9	37 ± 6.5 [c]	10 ± 3.3 [e]	24 ± 6.0 [c]	2.7 ± 0.7 [c]
10	16 ± 2.4 [b]	5.2 ± 1.8 [d]	11 ± 0.6 [b]	1.3 ± 0.1 [b]
11	5.5 ± 1.1 [a]	1.4 ± 0.4 [a]	2.4 ± 0.6 [a]	1.2 ± 0.0 [a]
12	13 ± 2.6 [b]	3.9 ± 0.2 [c]	13 ± 1.3 [b]	3.8 ± 0.9 [cd]
Standard drug				
Xanthorrhizol		8.7 ± 0.8		
Curcumin		8.7 ± 0.8		
Tamoxifen		4.6 ± 0.5		

IC_{50} (µg/mL) is the concentration which causes 50% inhibition of cell density while GI_{50} (µg/mL) is the concentration which causes 50% reduction in cell growth. Values are means ± SD. For each column, values followed by the same superscript (a–d) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test. Values of standard drugs of xanthorrhizol, curcumin and tamoxifen against MCF-7 and MDA-MB-231 reported by Cheah *et al.* (2008) were used as positive controls.

Results showed that the strongest APF activity was observed in Fractions 10 and 11. Their respective GI_{50} values were 3.5 ± 0.8 and 2.1 ± 0.1 µg/mL against HT-29 cells, and 2.1 ± 0.2 and 2.3 ± 0.2 µg/mL against MCF-7 cells, respectively [**Table 4.19**]. Their GI_{50} values were 5.2 ± 1.8 and 1.4 ± 0.4 µg/mL against MDA-MB-231 cells, and 1.3 ± 0.1 and 1.2 ± 0.0 µg/mL against SKOV-3 cells, respectively [**Table 4.20**].

Ranking of fractions based on IC_{50} and GI_{50} of APF activity against the four human cancer cell lines was of the order: Fraction 11 > Fraction 10 > Fraction 8 > Fraction 3 > Fraction 12 > Fraction 9.

Against MCF-7 cells, GI_{50} of Fractions 10 and 11 (2.1 ± 0.2 and 2.3 ± 0.2 µg/mL) was significantly stronger than xanthorrhizol (11 ± 0.7 µg/mL), curcumin (4.1 ± 0.9 µg/mL) and tamoxifen (8.3 ± 0.6 µg/mL) [**Table 4.19**]. Against MDA-MB-231 cells, GI_{50} of Fraction 11 (1.4 ± 0.2 µg/mL) was significantly stronger than xanthorrhizol (8.7 ± 0.8 µg/mL), curcumin (8.7 ± 0.8 µg/mL) and tamoxifen (4.6 ± 0.5 µg/mL) [**Table 4.20**]. GI_{50} of Fraction 10 (5.2 ± 1.8 µg/mL) was stronger than xanthorrhizol and curcumin, but comparable to tamoxifen.

4.8 STRUCTURAL ELUCIDATION OF COMPOUNDS

4.8.1 Structural Elucidation

Chemical constituents of leaves of *Vallaris glabra* were analysed because they displayed outstanding antiproliferative and antiplasmodial activity. The species is cultivated as an ornamental plant for its white flowers with a scent characteristic of pandan leaves or fragrant rice. Leaves are available in large quantities and are currently of no commercial value.

The M and D series of fractions were isolated from the MeOH and DCM leaf extracts of *Vallaris glabra*, respectively. Therefore, most of the consituents of the M fractions are somewhat polar and the D fractions had much less polar constituents.

From this study, four compounds were isolated from the methanol extract of leaves of *Vallaris glabra*. They were three caffeoylquinic acids (Compound **1**, Compound **2** and Compound **3**) from Fraction M4 [**Figure 3.3**] and one flavonol (Compound **4**) from Fraction M7 [**Figure 3.4**]. The compounds were numbered according to their order of elution. Two compounds were isolated from the DCM extract of the leaves. They were one fatty acid (Compound **5**) from Fraction D11 [**Figure 3.6**] and one triterpenoid (Compound **6**) from Fraction D8 [**Figure 3.7**].

4.8.2 Caffeoylquinic Acids (3-CQA, 4-CQA and 5-CQA)

a. ^{1}H and ^{13}C NMR spectroscopy

Caffeoylquinic acids (CQA) isolated from leaves of *Vallaris glabra* were identified based on NMR analysis, and on comparison of ^{1}H and ^{13}C NMR data with literature values.

Compound **1** was identified as 3-*O*-caffeoylquinic acid (3-CQA) or neochlorogenic acid (Nakatani *et al.* 2000). The ^{1}H and ^{13}C NMR spectra and spectral data of 3-CQA, are shown in **Figure 4.5** and **Table 4.21**, respectively.

Compound **2** was identified as 4-*O*-caffeoylquinic acid (4-CQA) or cryptochlorogenic acid (Rechner 2003). The ^{1}H and ^{13}C NMR spectra and spectral data of 4-CQA and are shown in **Figure 4.6** and **Table 4.22**, respectively.

Compound **3** was identified as 5-*O*-caffeoylquinic acid (5-CQA) or chlorogenic acid (CGA) (Naidu *et al*. 2008). The ^{1}H and ^{13}C NMR spectra and spectral data of 5-CQA are shown in **Figure 4.7** and **Table 4.23**, respectively.

Compound **1** (3-CQA) has a caffeoyl group attached to carbon 3 and OH groups at carbons 1, 4 and 5 [**Figure 4.8**]. Compound **2** (4-CQA) has a caffeoyl group at carbon 4 and OH groups at carbons 1, 3 and 5. Compound **3** (5-CQA or CGA) has a caffeoyl group at carbon 5 and OH groups at carbons 1, 3 and 4.

All three compounds displayed similar ^{1}H and ^{13}C NMR spectra and have a molecular formula of $C_{16}H_{18}O_9$. Their ^{1}H NMR showed 10 signals, corresponding to the 10 non-OH protons. They have an ABX spin system at around 6.9, 6.8 and 6.7 ppm, and trans-conjugation at around 7.5 ppm (J = 15.9 Hz) and 6.2 ppm (J = 15.9 Hz) derived from the caffeoyl group. ^{13}C NMR spectra of 3-CQA, 4-CQA and 5-CQA exhibited 16 signals. [Note: The apparent discrepancy of 0.12–0.16 ppm in ^{1}H chemical shifts between this work and that reported by Chan *et al.* (2009) could be due to non-homogeneity of the magnetic field].

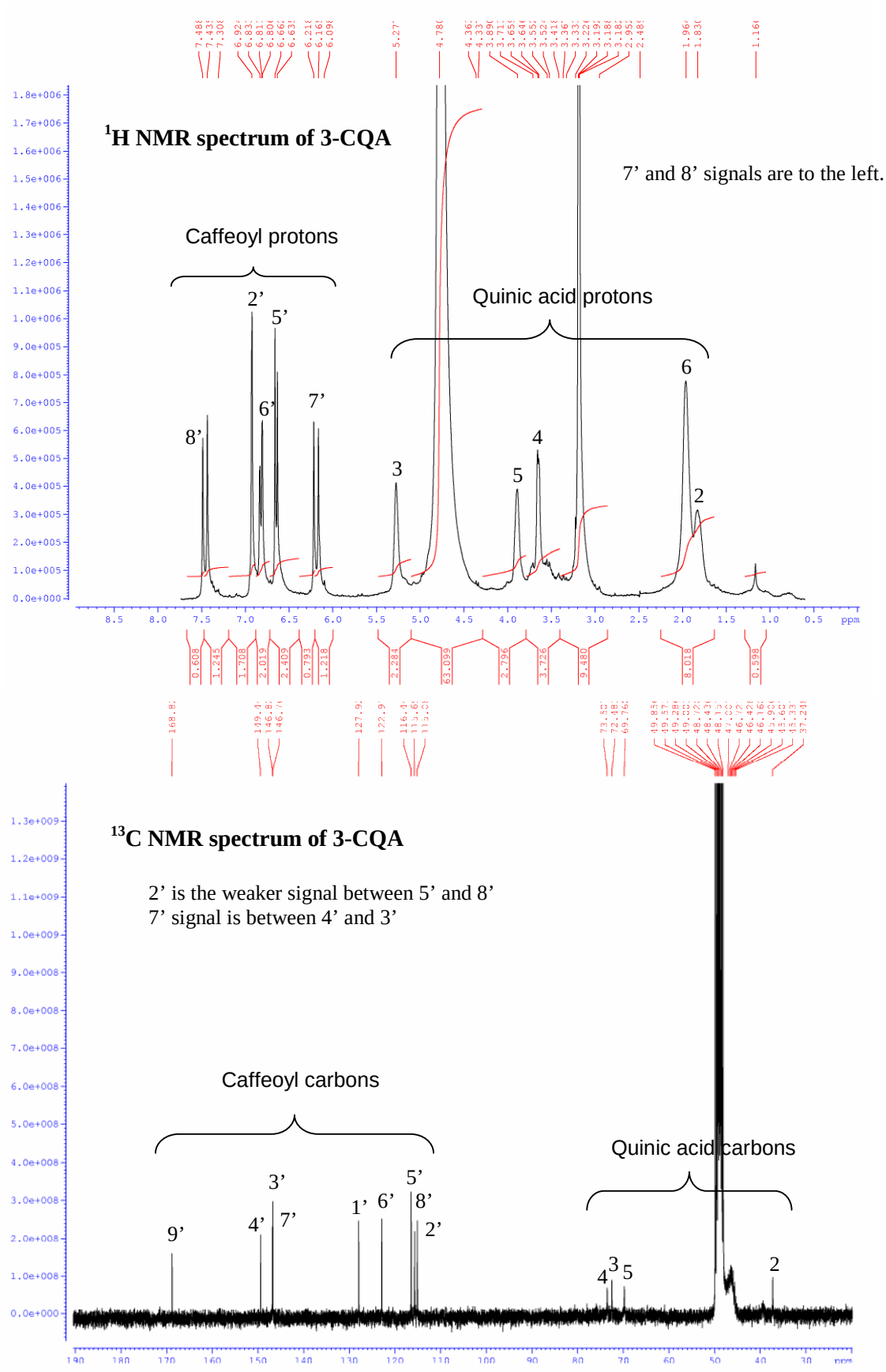
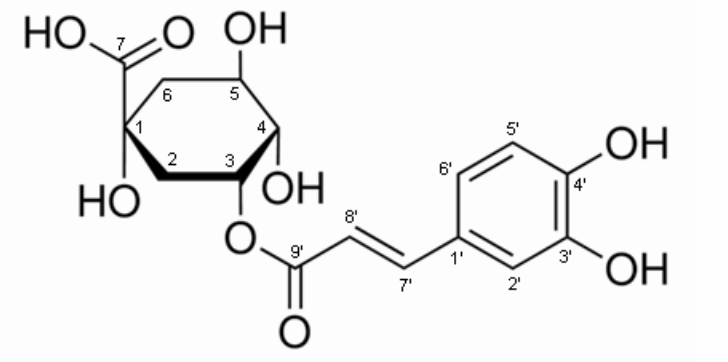


Figure 4.5 ^{1}H and ^{13}C NMR spectra of 3-*O*-caffeoylquinic acid in deuterated methanol

Table 4.21 ^{1}H and ^{13}C NMR spectral data, and molecular structure of 3-*O*-caffeoylquinic acid from leaves of *Vallaris glabra*

Atom	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
	Thesis data (2013)		Chan *et al*. (2009)		Nakatani *et al.* (2000)
Quinic moiety					
1			76.0		75.4
2	37.3	1.83 (*m*)	37.3	1.91 (*m*)	36.7
3	72.5	5.28 *(m)*	72.4	5.42 *(m)*	73.0
4	73.5	3.66 (*m*)	73.3	3.82 (*m*)	74.8
5	69.8	3.89 (*m*)	70.0	4.02 *(m)*	68.3
6	40.0	1.94 (*m*)	39.1	2.10 (*m*)	41.5
Caffeoyl moiety					
1’	127.9		127.9		127.9
2’	115.1	6.92 (*m*)	115.1	7.07 (*d* 1.7)	115.1
3’	146.8		146.7		146.8
4’	149.4		149.4		149.4
5’	116.4	6.66 (*d* 8.1)	116.5	6.79 (*d* 8.2)	116.4
6’	122.9	6.83 (*dd* 1.5,	122.9	6.96 (*dd* 1.7,	122.9
7’	146.8	8.1)	146.8	8.2)	146.8
8’	115.7	6.22 (*d* 15.9)	115.7	6.33 (*d* 15.9)	115.8
9’	168.8	7.49 (*d* 15.9)	168.8	7.60 (*d* 15.9)	169.0
COO^{-}			182.1		178.3



Structure of 3-CQA with numbering system

Values of ^{1}H (300 MHz) and ^{13}C (75.4 MHz) are in ppm (δ), and of *J* (bracketed) are in Hz. Values matched the ^{1}H and ^{13}C data of Chan *et al.* (2009), and the ^{13}C data of Nakatani *et al.* (2000). Carbon 1 and the COO^{-} carbon were not visible as their signals were relatively weak.

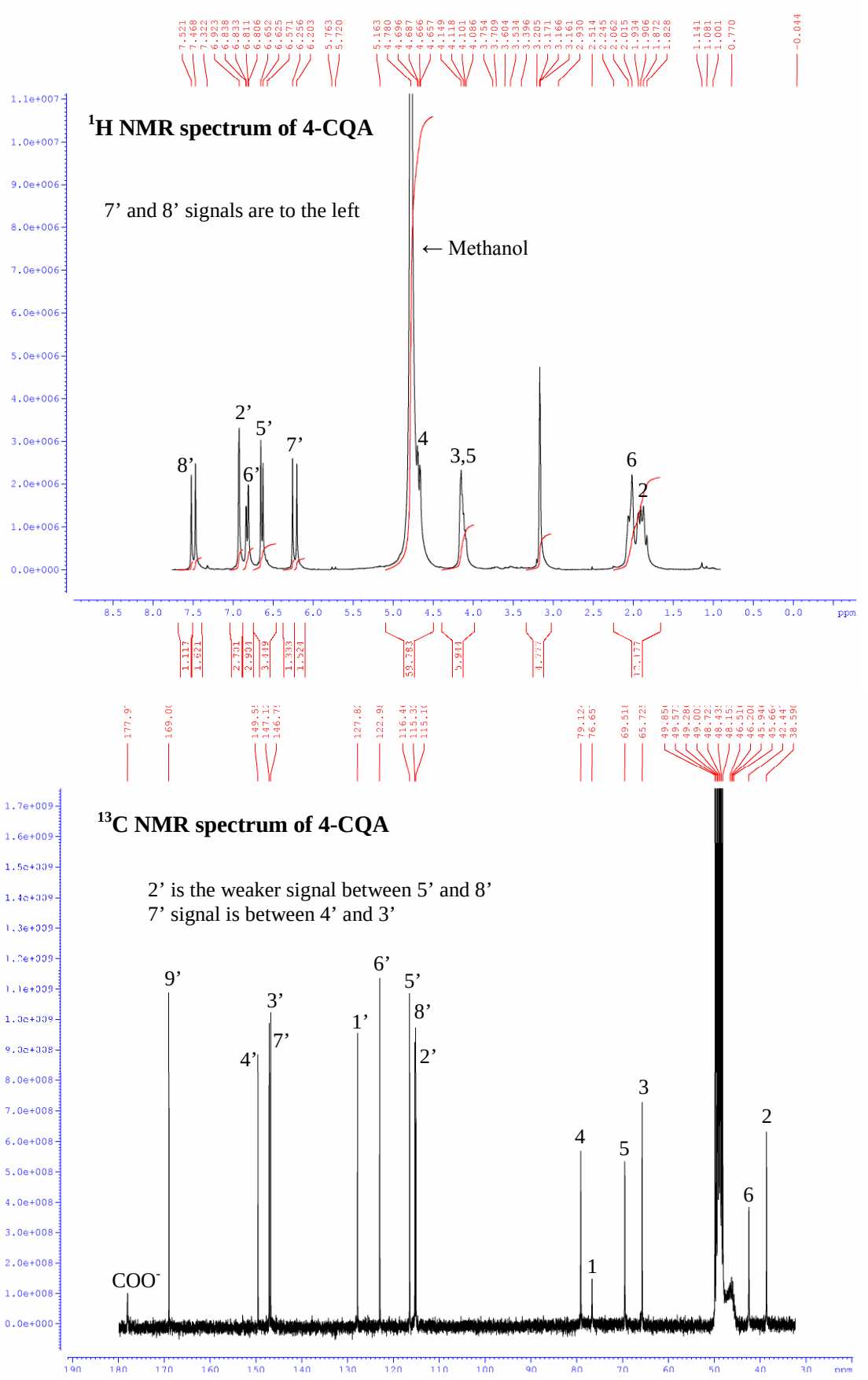
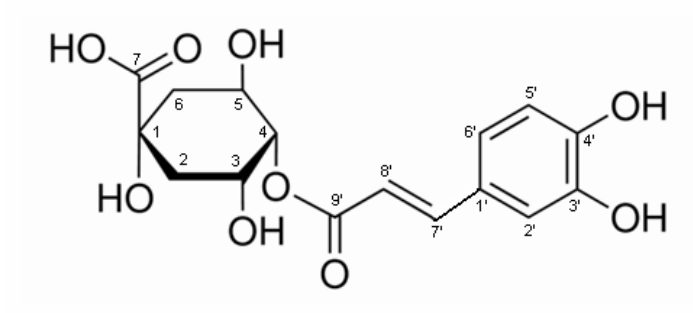


Figure 4.6 ^{1}H and ^{13}C NMR spectra of 4-*O*-caffeoylquinic acid in deuterated methanol

Table 4.22 ^{1}H and ^{13}C NMR spectral data, and molecular structure of 4-*O*-caffeoylquinic acid from leaves of *Vallaris glabra*

Atom	^{13}C	^{1}H	^{13}C
	Thesis data (2013)		Nakatani *et al.* (2000)
Quinic moiety			
1	76.6		76.6
2	38.6	1.91 (*m*)	38.4
3	69.5	4.15 (*m*)	69.6
4	79.1	4.70 (*m*)	79.3
5	65.7	4.09 (*m*)	65.5
6	42.5	2.02 (*m*)	42.7
Caffeoyl moiety			
1’	127.8		127.8
2’	115.1	6.92 (*m*)	115.1
3’	147.1		146.8
4’	149.6		149.6
5’	116.4	6.65 (*d* 8.1)	116.5
6’	122.9	6.83 (*dd* 1.5, 8.1)	123.0
7’	146.8	6.22 (*d* 15.9)	147.1
8’	115.3	7.52 (*d* 15.9)	115.4
9’	169.0		169.0
COO^{-}	178.0		177.3



Structure of 4-CQA with numbering system

Values of ^{1}H (300 MHz) and ^{13}C (75.4 MHz) are in ppm (δ), and *J* (bracketed) are in Hz. ^{13}C data matched those of Nakatani *et al.* (2000).

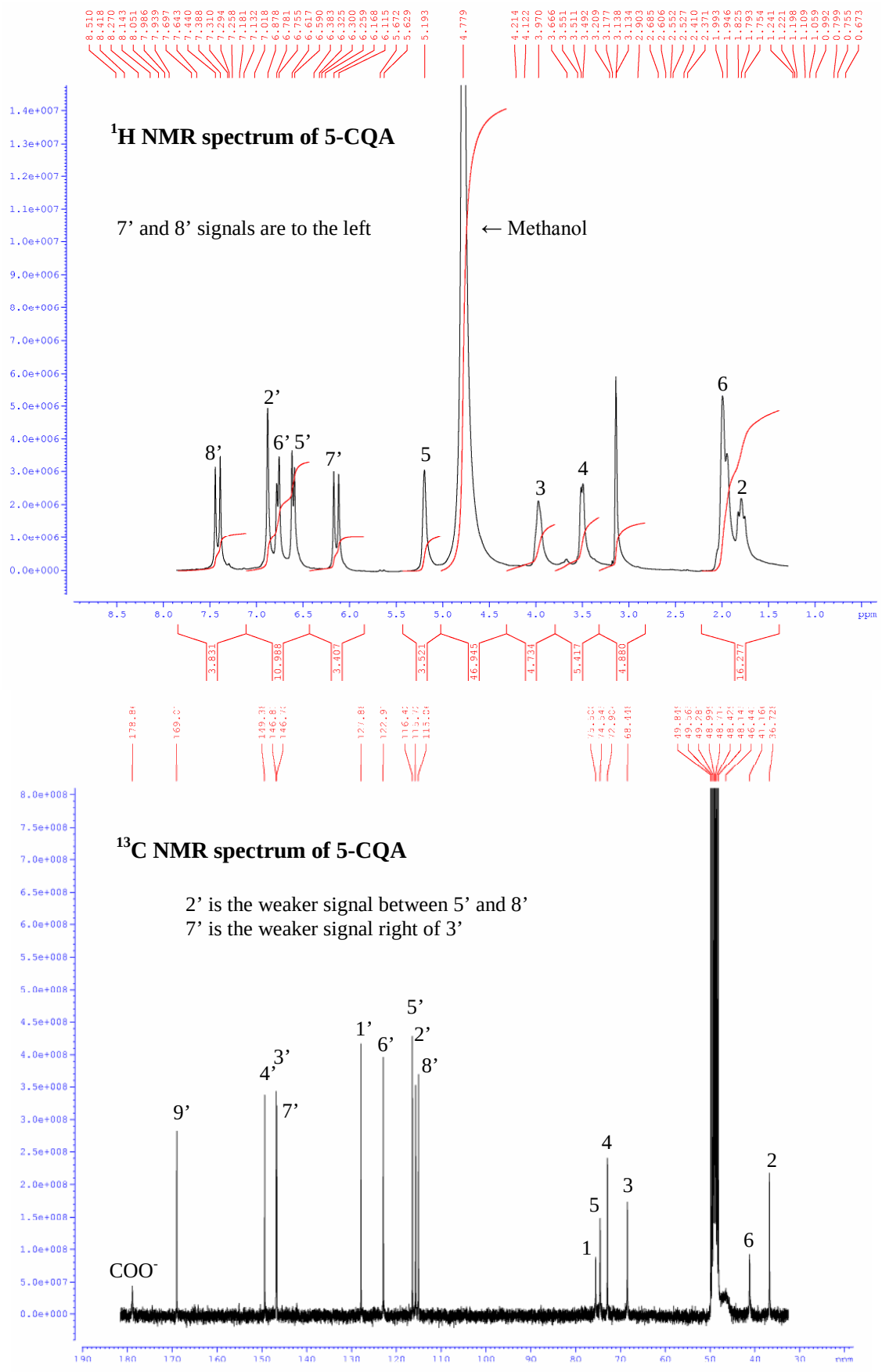
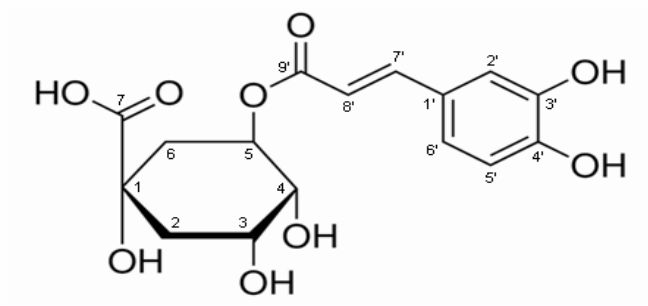


Figure 4.7 ^{1}H and ^{13}C NMR spectra of 5-*O*-caffeoylquinic acid in deuterated methanol

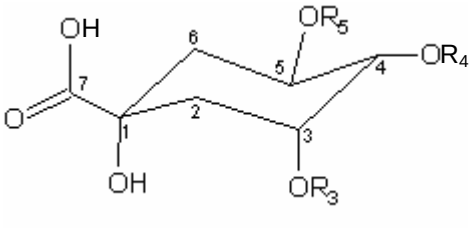
Table 4.23 ^{1}H and ^{13}C NMR spectral data, and molecular structure of 5-*O*-caffeoylquinic acid from leaves of *Vallaris glabra*

Atom	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
	Thesis data (2013)		Chan (2009)		Lin *et al.* (2002)
Quinic moiety					
1	75.5		76.1		76.3
2	36.7	1.75–1.83 (*m*)	38.2	1.92–1.98 (*m*)	38.9
3	68.4	3.97 (*m*)	71.3	4.10 (*m*)	71.4
4	72.9	3.51 (*m*)	72.0	3.67 (*m*)	72.0
5	74.5	5.19 (*m*)	73.5	5.36 (*m*)	73.6
6	41.2	1.99 (*m*)	38.8	2.11 (*m*)	38.9
Caffeoyl moiety					
1’	127.9		127.8		127.8
2’	115.7	6.88 (*d* 1.7)	115.2	7.04 (*d* 1.81)	115.3
3’	146.8		147.1		146.8
4’	149.4		149.6		149.6
5’	116.4	6.62 (*d* 8.1)	116.5	6.76 (*d* 8.16)	116.5
6’	123.0	6.78 (*dd* 1.7, 7.8)	123.0	6.94 (*dd* 1.82, 8.12)	123.0
7’	146.7	6.17 (*d* 15.9)	146.8	6.28 (*d* 15.9)	147.1
8’	115.1	7.44 (*d* 15.6)	115.1	7.56 (*d* 15.9)	115.2
9’	169.0		168.7		168.7
COO^{-}	178.9		177.0		177.3

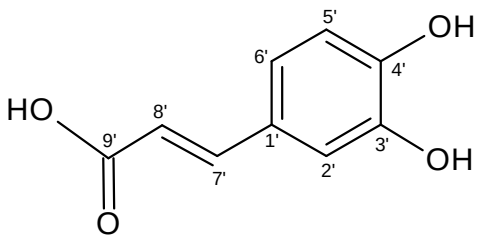


Structure of 5-CQA with numbering system

Values of ^{1}H (300 MHz) and ^{13}C (75.4 MHz) are in ppm (δ), and of *J* (bracketed) are in Hz. Values matched the ^{1}H and ^{13}C data of Chan (2009), and the ^{13}C data of Lin *et al.* (2002).

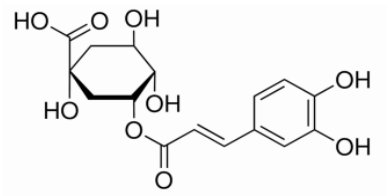


Quinic acid

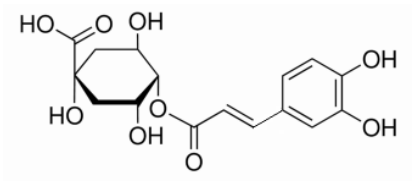


Caffeic acid

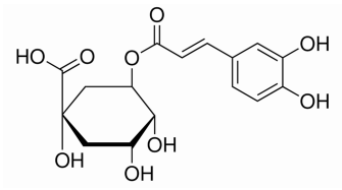
Compound	Identity	R_3	R_4	R_5
1	3-CQA	caffeoyl	H	H
2	4-CQA	H	caffeoyl	H
3	5-CQA (CGA)	H	H	caffeoyl



3-CQA



4-CQA



5-CQA

Figure 4.8 Structural relationship among 3-*O*-caffeoylquinic acid (3-CQA), 4-*O*-caffeoylquinic acid (4-CQA) and 5-*O*-caffeoylquinic acid (5-CQA)

b. ESI-MS spectrometry

Following NMR analysis, the identity of these compounds was reaffirmed using ESI-MS. The mass spectra of 3-CQA, 4-CQA and 5-CQA or CGA are shown as **Figure 4.9**. All showed a strong peak at 353.08 which corresponds to the $[M-H]^-$ molecular ion. Other peaks were observed at 354, 390, 446, 731 and 840 m/z for 3-CQA, at 354, 715, 788 and 1703 m/z for 4-CQA, and at 354, 707, 716 and 1858 m/z for 5-CQA. Some of these peaks could be due to the formation of dimers and of adducts to formic acid present in the solvent system.

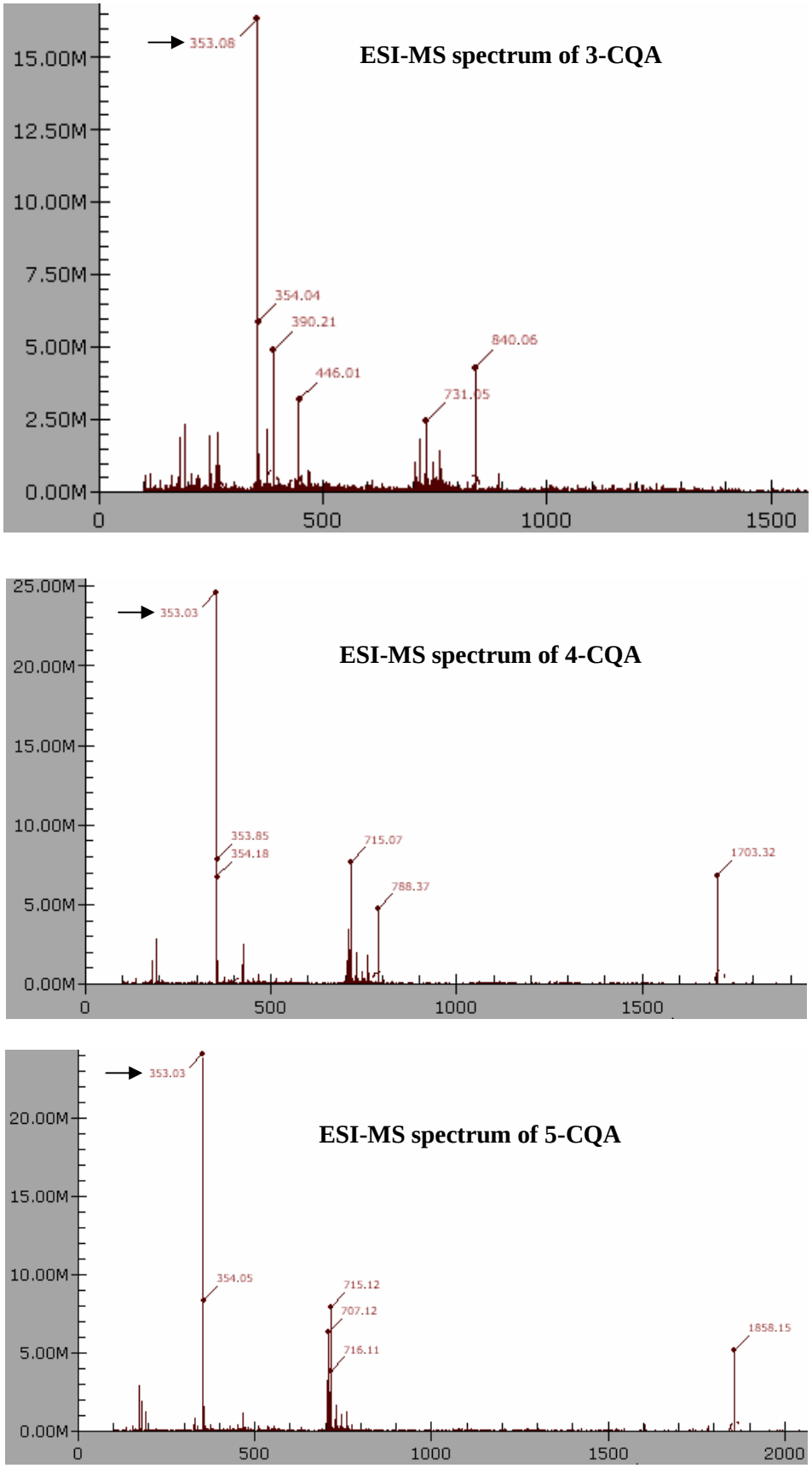


Figure 4.9 ESI-MS spectra of 3-CQA, 4-CQA and 5-CQA in negative mode $[M-H]^-$

c. 3-CQA, 4-CQA and 5-CQA

3-*O*-caffeoylquinic acid (3-CQA) or neochlorogenic acid is (1R,3R,4S,5R)-3-[(E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy-1,4,5-trihydroxycyclohexane-1-carboxylic acid (IUPAC name). The compound has a molecular formula of $C_{16}H_{18}O_9$ and molecular weight of 354.31. 3-CQA is found in fruits e.g. prunes, plums, cherries and apricots (Nakatani *et al*. 2000).

4-*O*-caffeoylquinic acid (4-CQA) or cryptochlorogenic acid is synonymous to 4-*O*-(3, 4-dihydroxycinnamoyl)-D-quinic acid. The compound has a molecular formula of $C_{16}H_{18}O_9$ and molecular weight of 354.31. 4-CQA is found in fruits such as prunes and plums (Nakatani *et al*. 2000; Fleuriet & Macheix 2003).

5-*O*-caffeoylquinic acid (5-CQA) is (1S,3R,4R,5R)-3-[(E)-3-(3,4-dihydroxyphenyl) prop-2-enoyl]oxy-1,4,5-trihydroxycyclohexane-1-carboxylic acid (IUPAC name). Also known as chlorogenic acid (CGA), the compound has a molecular formula of $C_{16}H_{18}O_9$ and a mass of 354.31. It has the caffeoyl moiety at carbon 5 of the quinic molecule.

CGA is the most common CQA. Coffee beans are one of the richest dietary sources of CGA. A single cup of coffee may contain up to 350 mg CGA (Manach *et al.* 2004). It is also found in fruits e.g. cherries, blueberries, prunes, plums apples and pears, and in brassica vegetables e.g. kale, cabbage and brussel sprout (Clifford 1999, 2000; Nakatani *et al*. 2000; Fleuriet & Macheix 2003).

d. Caffeoylquinic acids

The isolation of 3-CQA, 4-CQA and 5-CQA from leaves of *Vallaris glabra* represents the first report of CQAs in the genus *Vallaris*. Earlier studies have documented the occurrence of CQAs in Apocynaceae species. 5-CQA had been reported in leaves of *Catharanthus roseus* (Choi *et al.* 2004). In the same species, Ferreres *et al.* (2008) isolated 3-CQA and 5-CQA from stems and leaves, and 4-CQA from petals. CGA was reported in leaves of *Vinca major* (Sakushima & Nishibe 1988), and in stems and leaves of *Trachelospermum jasminoides* (Sheu *et al.* 2009). The content of 4-

CQA and 5-CQA identified from leaves of *Apocynum venetum* had been reported to be 0.0–0.7% and 2.1–3.9%, respectively (Yin *et al*. 2012).

Caffeoylquinic acids (CQAs) are esters of caffeic and quinic acids. Their occurrence and contents in fruits and vegetables have been compiled by Kuhnert *et al.* (2012). Plants rich in CQAs include flowers of *Lonicera japonica* (Japanese honeysuckle), the commercial source of CGA (Xiang & Ning 2008), leaves of *Ipomoea batatas* (sweet potato) (Zheng & Clifford 2008) and heads of *Cynara scolymus* (artichoke) (Schutz *et al.* 2004).

Recently, leaves of some *Etlingera* gingers were reported to have significantly higher contents of CQAs and CGA than flowers of *Lonicera japonica* and leaves of *Ipomoea batatas* (Chan *et al.* 2009). Contents of CQAs and CGA of *Etlingera elatior* (320 mg CGAE/100 g and 294 mg CGA/100 g) and *Etlingera fulgens* (326 mg CGAE/100 g and 219 mg CGA/100 g) were significantly higher than flowers of *Lonicera japonica* (250 mg CGAE/100 g and 173 mg CGA/100 g) and leaves of *Ipomoea batatas* (125 mg CGAE/100 g and 115 mg CGA/100 g), respectively. CQAs including 3-CQA and 5-CQA isolated from leaves of *Etlingera elatior* were reported to be non-cytotoxic to normal human liver (WRL-68) and African green monkey kidney (Vero) cells.

In prunes, the contents of 3-CQA, 4-CQA and 5-CQA were of the ratio 79:18:4 (Nakatani *et al.* 2000). In plums, their contents were 541, 9 and 73 mg/kg, respectively (Herrmann 1989). The contents of CQA in three Chinese traditional herbs were investigated (Wang & Clifford 2008). 5-CQA was dominant in leaves of *Eucommia ulmoides* and flowers of *Lonicera japonica*. 3-CQA and 4-CQA dominated the CQAs in leaves of *Houttuynla cordata.*

The antioxidant properties of CQAs are widely recognised, with those of CGA most studied. They have the ability to inhibit human low density lipoprotein (LDL) oxidation (Nardini *et al.* 1995; Rice-Evans *et al.* 1996), to scavenge free radicals such as reactive oxygen and nitrogen species (Kono *et al.* 1997 & 1998), to inhibit to lipid peroxidation (Marinova *et al.* 2009), to chelate iron in iron-induced lipid peroxidation (Kono *et al.* 1998), and to protect against DNA breakage caused by monochloramine (Shibata *et al.* 1999). In terms of *in vitro* peroxidation of human LDL, both CGA and

caffeic acid are equally effective antioxidants, with stronger activity than sinapic acid, ferulic acid and *p*-coumaric acid (Cheng *et al.* 2007).

The antioxidant activity of CQA is higher than those of vitamin C and vitamin E, based on the Trolox equivalent antioxidant activity (TEAC) (Rice-Evans *et al.* 1997). The high antioxidant activity of *Prunus domestica* (prunes) has been attributed to CQA (Nakatani *et al.* 2000; Kayano *et al.* 2002). 3-CQA, 4-CQA and 5-CQA had strong scavenging activity on superoxide anion radicals (O_2^-) and inhibitory effect against oxidation of methyl linoleate (Nakatani *et al.* 2000). The oxygen radical absorbance capacity (ORAC) values of 3-CQA (5.27 units/mg) and 4-CQA (5.42 units/mg) were slightly higher than 5-CQA (4.60 units/mg) (Kayano *et al.* 2002).

It was reported that the number of caffeoyl groups contributes to the scavenging activity of DPPH and superoxide radicals rather than the linkage positions of caffeoyl groups to the quinic moiety (Iwai *et al.* 2004). This implies that diCQA would have stronger antioxidant activity than CQA.

Besides antioxidant properties, studies have shown that CQA display diverse bioactivities. CGA is known to have strong antimicrobial properties (Almeida *et al*. 2006), and is an effective anti-inflammatory, analgesic and antipyretic agent (Jin *et al*. 2006; dos Santos *et al*. 2006). Other bioactivities include anti-skin aging, anti-hypercholesterolemia and anti-hyperglycaemia activities (Harrison *et al.* 2008). CGA has been reported to be cytotoxic to oral tumour cell lines of human oral squamous cell carcinoma (HSC-2) and salivary gland tumour (HSG) cell lines (Jiang *et al.* 2000). CGA isolated from stems of *Euonymus alatus* has been reported to inhibit metallo-proteinase-9, suggesting its chemopreventive properties against cancer (Jin *et al.* 2005).

It has been demonstrated that diCQA exhibited more potent tyrosinase inhibition that is 2.0–2.2 times compared to CQA, arbutin and ascorbic acid (Iwai *et al.* 2004). CQA and diCQA exhibited antiproliferative activities against U937, KB and MCF-7 cancer cells. IC_{50} of 3-CQA, 4-CQA and 5-CQA ranged from 0.18–1.20, 0.16–0.92 and 0.14–1.54 mM, respectively. They were found to be non-cytotoxic to WI38 normal cells with IC_{50} values of 5.87, 3.56 and 5.87 mM, respectively.

CQA and derivatives can be used to treat or prevent neurodegenerative diseases by reducing reactive oxygen species (ROS) (Nakajima *et al.* 2007). CGA inhibits carcinogenesis in rats (Tanaka *et al.* 1990 & 1993) and protects against paraquat-induced oxidative stress in rats (Tsuchiya *et al.* 1996). Recently, the role of CQA as natural anticonvulsants has been reviewed (Kim & Oh 2012).

4.8.3 Flavonol (quercetin 3-*O*-glucoside)

a. ^{1}H and ^{13}C NMR spectroscopy

Compound **4** isolated from leaves of *Vallaris glabra* was identified as quercetin 3-*O*-glucoside (QG) or isoquercitrin. Its ^{1}H and ^{13}C NMR spectra and spectral data are shown in **Figure 4.10** and **Table 4.24**.

QG had an ABX spin system at the B-ring and this was observable at 7.67, 7.65 and 6.82 ppm. It showed a signal at 5.46 ppm, corresponding to the 1'' proton of the glucoside group. Because QG was analysed in deuterated DMSO, an OH signal was observed at 12.6 ppm.

b. ESI-MS spectrometry

Following NMR analysis, the identity of QG was reaffirmed using ESI-MS. The mass spectrum showed a $[M-H]^-$ molecular ion peak at 463.01 m/z with other peaks at 464, 608, 609, 927 and 928 m/z [**Figure 4.11**].

c. Quercetin 3-*O*-glucoside

Quercetin 3-*O*-glucoside (QG) or 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[(2S,3R, 4S,5R,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-chromen-4-one (IUPAC name) has a molecular formula of $C_{21}H_{20}O_{12}$ and a mass of 464.38. It has hydroxyl groups at carbons 3', 4', 5 and 7 with a glucoside moiety as the sugar group attached to carbon 3 [**Figure 4.12**].

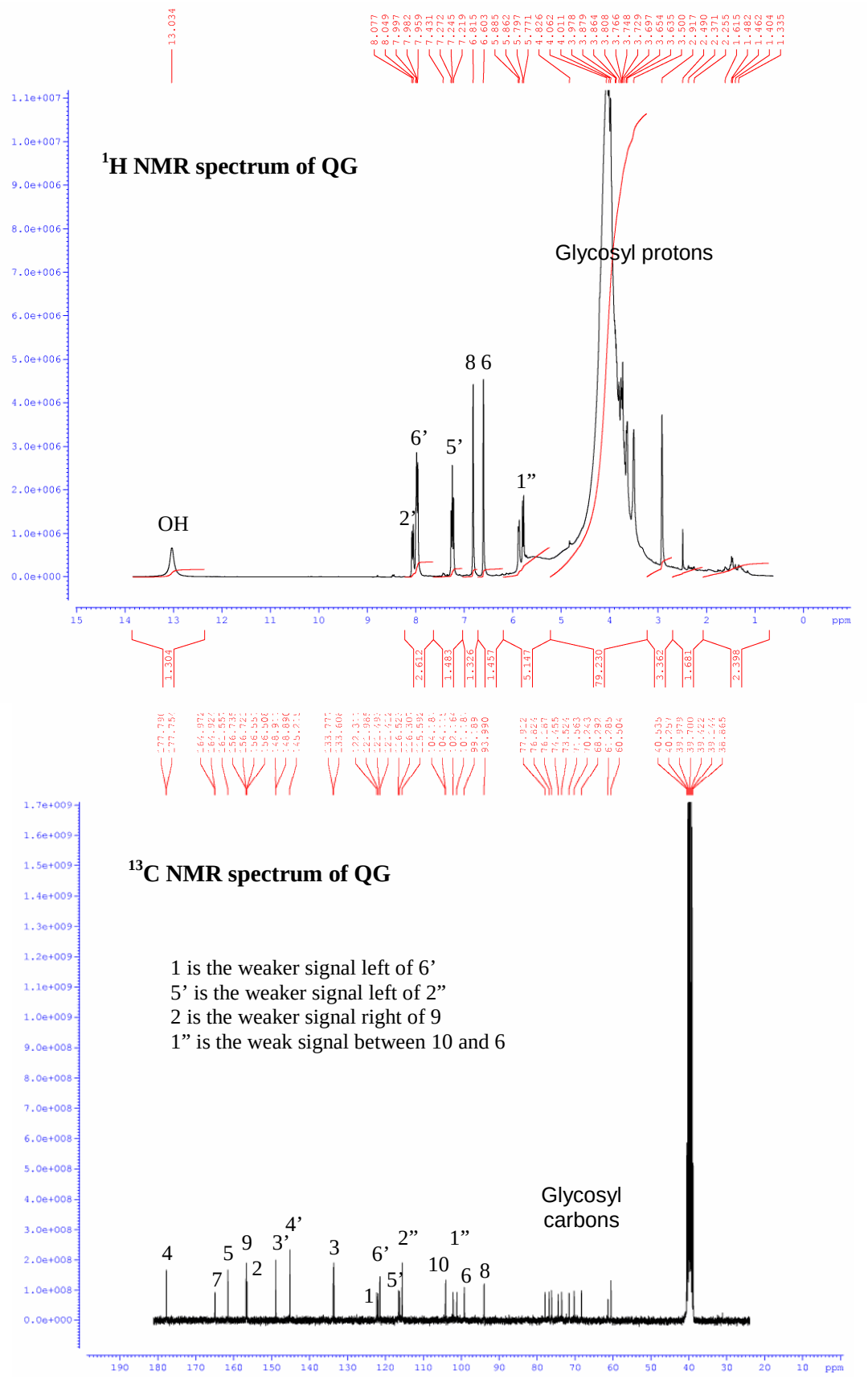


Figure 4.10 ^{1}H and ^{13}C NMR spectra of quercetin 3-*O*-glucoside (QG) in deuterated DMSO

Table 4.24 ^{1}H and ^{13}C NMR spectral data of quercetin 3-*O*-glucoside from leaves of *Vallaris glabra*

Atom	^{13}C	^{1}H	^{13}C	^{1}H	
	Thesis data (2013)		Awaad *et al*. (2008)	Bennini *et al.* (1992)	Chan (2009)
Aglycone					
2	156.4		156.6		
3	133.5		136.2		
4	177.7		176.3		
5	161.5		161.2		
6	98.97	6.19 (*d* 2.0)	98.80	6.20 (*d* 1.9)	6.19 (*d* 1.8)
7	164.6		164.5		
8	93.80	6.39 (*d* 2.0)	93.70	6.40 (*d* 1.9)	6.40 (*d* 1.8)
1’	121.9		120.4		
2’	115.5	7.67 (*d* 2.0)	116.1	7.52 (*d* 2.1)	7.58 (*d* 2.1)
3’	148.7		147.2		
4’	145.1		145.5		
5’	116.4	6.82 (*d* 8.8)	115.5	6.81 (*d* 8.5)	6.81 (*d* 14.1)
6’	121.4	7.65 (*dd* 8.0, 2.0)	122.4	7.65 (*dd* 8.5, 2.1)	7.54 (*dd* 2.1, 14.1)
5-OH		12.6 (*s*)		12.6 (*s*)	12.6 (*s*)
Glycosyl					
1”	101.1	5.45 (*d* 7.4)	103.4	5.38 (*d* 7.6)	5.46 (*d* 7.2)
2”	74.31	3.63–3.07 (*m*, 6 H of glucose + H_2O)	75.05		
3”	76.69		78.11		
4”	70.13		71.34		
5”	77.84		78.40		
6”	61.17		62.74		

Values of ^{1}H (300 MHz) and ^{13}C (75.4 MHz) are in ppm (δ), and of *J* (bracketed) are in Hz. Values of ^{13}C matched those of Awaad *et al.* (2008), and ^{1}H matched those of Bennini *et al.* (1992) and Chan (2009).

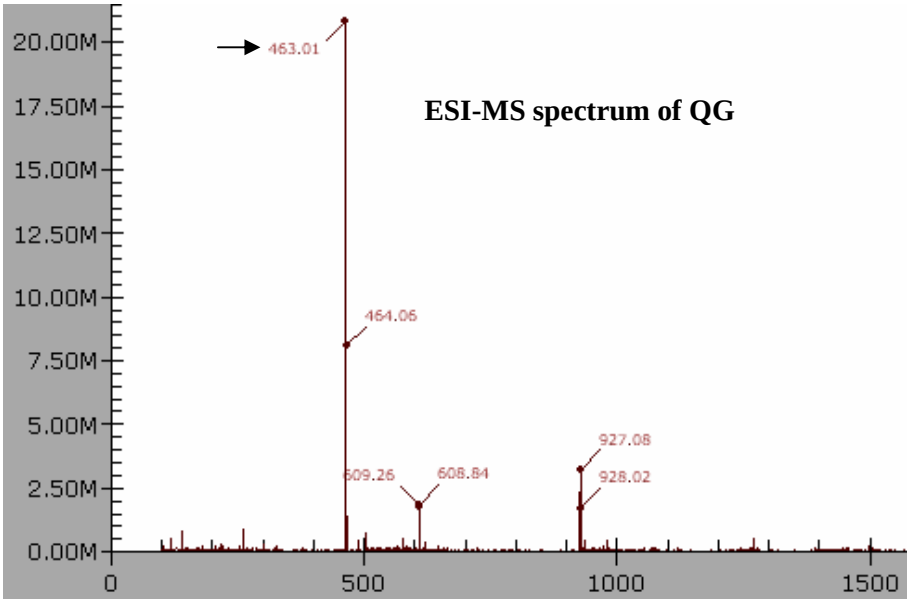


Figure 4.11 ESI-MS spectra of quercetin 3-*O*-glucoside (QG) in negative mode $[M-H]^-$

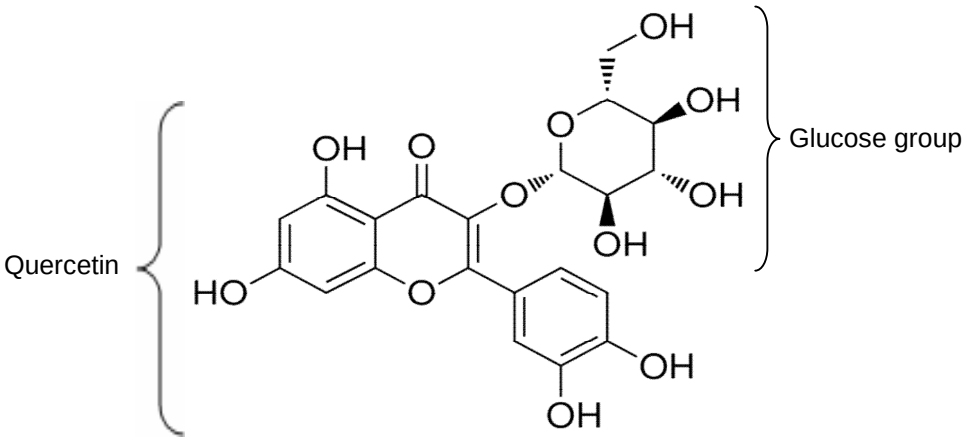


Figure 4.12 Molecular structure of quercetin 3-*O*-glucoside

QG (flavonol group of flavonoids) isolated from leaves of *Vallaris glabra* has also been reported earlier in stems and leaves of *Trachelospermum jasminoides* (Fu *et al.* 2008; Sheu *et al.* 2009). Analysed using HPLC, the content of QG in aqueous leaf extract of *Apocynum venetum* was 2.7% (Butterweck *et al.* 2001) and 0.5–0.9% (Yin *et al*. 2012). Glucosides of quercetin and kaempferol were also reported in leaves of *Vinca minor* (Szostak & Kowalewski 1975).

QG is commonly found in coffee beans (4.3 g/100 g) and blueberries (3.5 g/100 g) (Pietta *et al.* 2003). It has been reported in leaves of *Salix matsudana* (Li *et al.* 2008), in leaves, flowers and dietary supplement of *Hypericum perforatum* (St. John's wort) (Liu *et al.* 2000; Wach *et al*. 2007), in flowers of *Erica cinerea* (Bennini *et al.* 1992), in whole plant of *Diodia teres* (Lee *et al.* 2004), in aerial parts of *Desmostachya bipinnata* (Awaad *et al.* 2008) and in whole plant of *Eupatorium perfoliatum* (Maas *et al.* 2009). The content of QG in *Corchorus olitorius* has been reported to be 377 mg/100 g (Azuma *et al.* 1999), which is about twice that of onion, a major source of quercetin glycosides (Price & Rhodes 1997).

The antioxidant properties of QG have been documented. In terms of DPPH radical scavenging ability, the IC_{50} of QG (12 ± 1.1 mg/mL) was significantly stronger than that of quercetin (66 ± 0.6 mg/mL) (Silva *et al.* 2009). QG isolated from aerial parts of *Hyptis fasciculata* showed outstanding antioxidant activity in yeast cells by reducing intracellular lipid and protein oxidation levels, and by inducing superoxide dismutase (SOD) activity.

QG has been reported to inhibit the formation of advanced glycation end-products (AGE) which accumulate in the body with age especially of individuals with diabetes mellitus (Kim *et al.* 2011a). At 50 µM concentration, inhibitory activity of QG was 90%. QG isolated from leaves of *Salix matsudana* had moderate inhibitory activity against cyclooxygenase COX-1 (Li *et al.* 2008). QG isolated from *Annona squamosa* leaves has the potential to ameliorate diabetes mellitus and tissue lipid peroxidation (Panda & Kar 2007). The anti-diabetic effect is possibly mediated through its insulin stimulating and/or free radical scavenging properties. QG has been reported to have the ability to protect human neuroblastoma cells (SH-SY5Y), human embryonic kidney cells (HEK-293) and human breast cancer cells (MCF-7) against hydrogen peroxide-induced oxidative stress (Soundararajan *et al.* 2008).

4.8.4 Fatty Acid (stearic acid)

a. ^{1}H and ^{13}C NMR spectroscopy

The fractionation of the most potent fraction, Fraction 11 of the DCM leaf extract of *Vallaris glabra*, led to the isolation of Compound **5**, identified as stearic acid (SA). ^{1}H and ^{13}C NMR spectra and spectral data are shown in **Figure 4.13** and **Table 4.25**. NMR spectra matched those reported by Dinda & Guha (1988) and Li e*t al.* (2010). The ^{1}H NMR spectrum of SA (authentic sample) in deuterated chloroform published by Knothe (2006) is shown in **Figure 4.14**.

According to Dinda & Guha (1988), the ^{1}H NMR spectrum of SA indicated terminal methyl protons at δ 0.84, CH_2 protons at δ 1.23, and CH_2 protons adjacent to carboxylic group at δ 2.3. This was supported by signals at δ 180.1 (C1) and δ 14.30 (C18) in the ^{13}C NMR spectrum.

b. EI-MS spectrometry

Following NMR analysis, the identity of SA was reaffirmed using EI-MS. The EI-MS spectrum of SA has a molecular mass of 284.2 m/z $[M]^+$ which corresponds to the molecular formula of $C_{18}H_{36}O_2$ [**Figure 4.15**].

Fragmentation peaks which occurred from mz = 255–115 [**Figure 4.15**] represented the loss of methylene groups. These peaks were observed at 255 m/z $[M\text{-}CH_2CH_3]^+$, 241 m/z $[M\text{-}(CH_2)_2CH_3]^+$, 227 m/z $[M\text{-}(CH_2)_3CH_3]^+$, 213 m/z $[M\text{-}(CH_2)_4CH_3]^+$, 199 m/z $[M\text{-}(CH_2)_5CH_3]^+$, 185 m/z $[M\text{-}(CH_2)_6CH_3]^+$, 171 m/z $[M\text{-}(CH_2)_7CH_3]^+$, 157 m/z $[M\text{-}(CH_2)_8CH_3]^+$, 143 m/z $[M\text{-}(CH_2)_9CH_3]^+$, 129 m/z $[M\text{-}(CH_2)_{10}CH_3]^+$, and 115 m/z $[M\text{-}(CH_2)_{11}CH_3]^+$. The most abundant ion or the base peak was observed at 87 m/z which corresponds to $[M\text{-}(CH_2)_{13}CH_3]^+$.

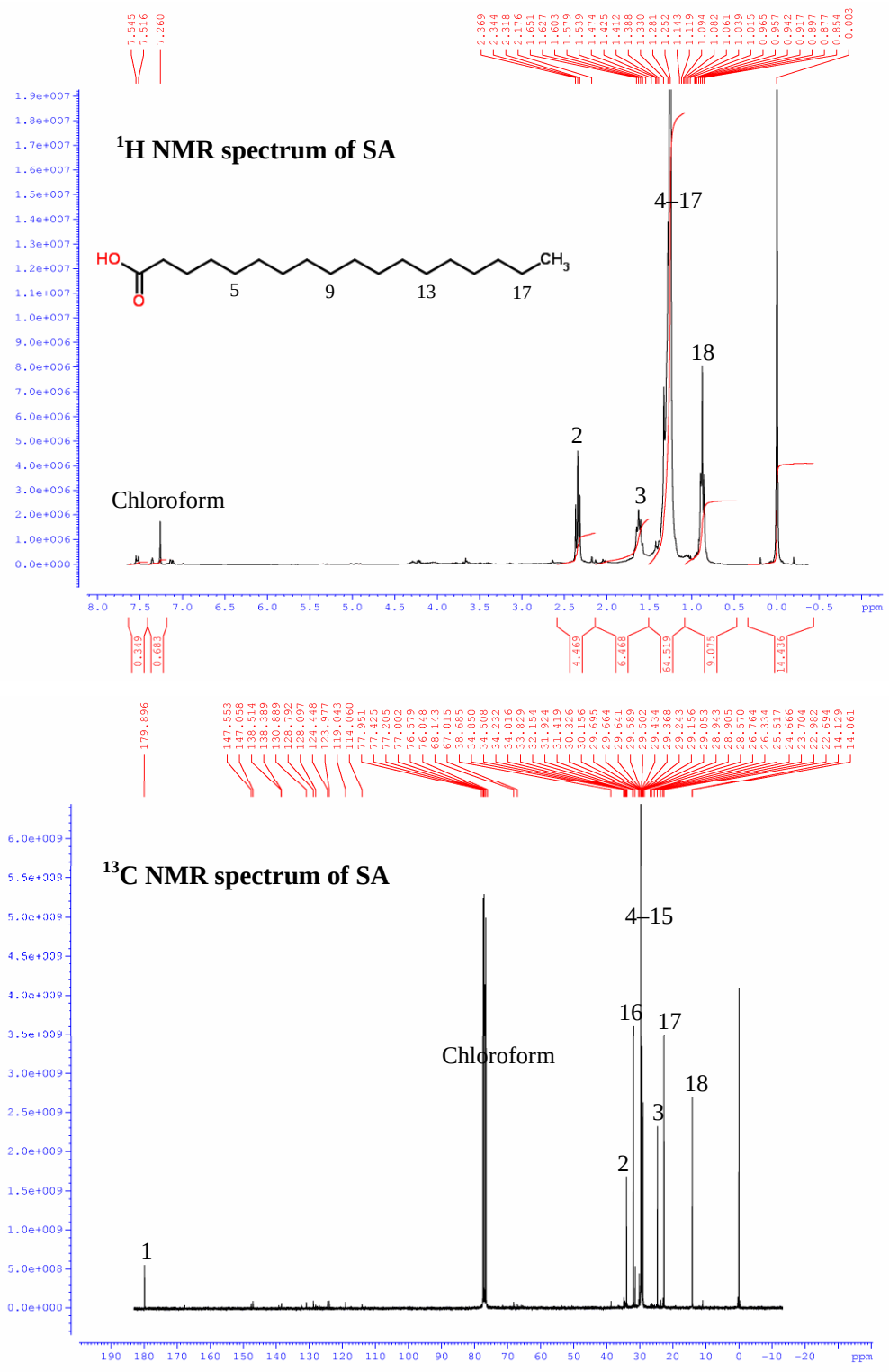


Figure 4.13 ^{1}H and ^{13}C NMR spectra of stearic acid (SA) in deuterated chloroform

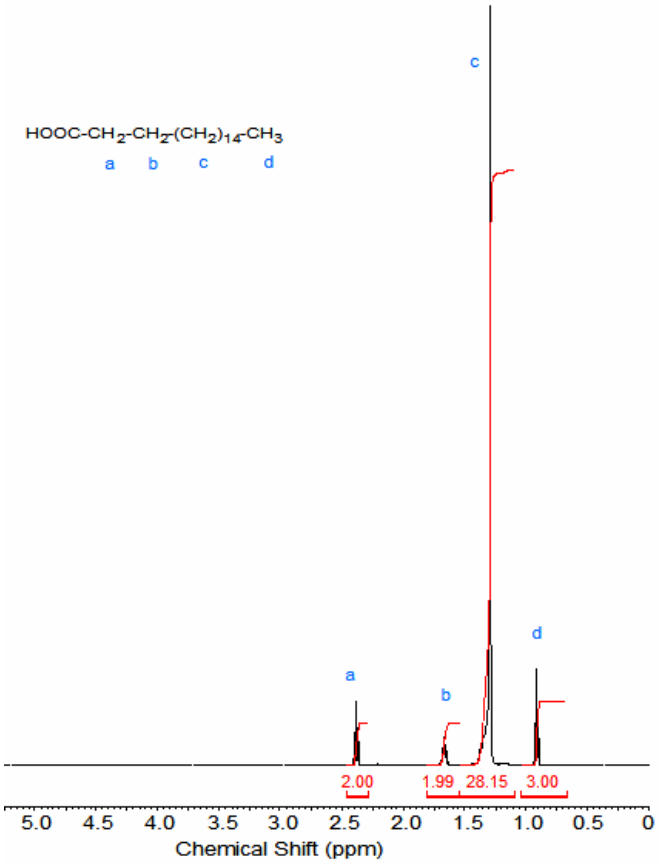


Figure 4.14 ^{1}H NMR spectrum of stearic acid (authentic sample) in deuterated chloroform (Knothe 2006)

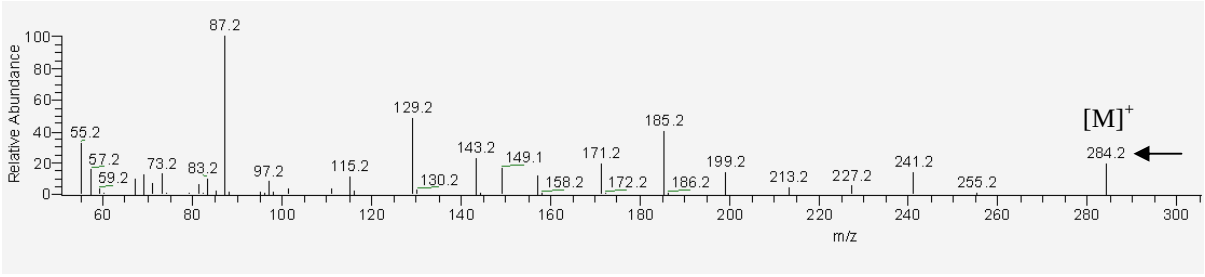


Figure 4.15 EI-MS spectrum of stearic acid (SA) in positive mode [M^{+}]

c. Stearic acid

Stearic acid (SA) isolated from leaves of *Vallaris glabra* is a major fatty acid found in Apocynaceae species. The content of SA in *Catharanthus roseus* was 1.5% in leaves and 0.4% in flowers (Pandey-Rai *et al*. 2006). Seed oils of *Rauvolfia serpentine*, *Rauvolfia tetraphylla* and *Vinca rosea* have 4.9%, 10.3% and 6.8% SA

content, respectively (Daulatabad & Ankalgi 1985). Oils from aerial parts of eight species of Apocynaceae from the Western Ghats in Tamil Nadu, India, contained SA ranging from 35–159 g/kg (Augustus & Seiler 2011). Ethanol extract of leaves of *Alstonia boonei* yielded 10.3% content of SA (Okwu & Ighodaro 2010).

Table 4.25 ^{1}H and ^{13}C NMR spectral data of stearic acid from leaves of *Vallaris glabra*

Atom	Thesis data (2013)	Dinda & Guha (1988)	Li *et al.* (2010)
	^{13}C		
1	179.9 (COO)	173.7 (COO)	179.6 (COO)
2	34.23 (CH_2)	34.20 (CH_2)	34.00 (CH_2)
3	24.67 (CH_2)	24.60 (CH_2)	24.70 (CH_2)
4	29.43 (CH_2)	29.40 (CH_2)	31.90 (CH_2)
5-16	29.16–32.15 (CH_2)	29.10–32.30 (CH_2)	27.20–29.70 (CH_2)
17	22.69 (CH_2 α to CH_3)	23.00 (CH_2 α to CH_3)	22.70 (CH_2 α to CH_3)
18	14.13 (CH_3)	14.30 (CH_3)	14.10 (CH_3)
	^{1}H		
2	2.37 (*t*, 2H, CH_2, *J* = 7.5 Hz at C2)	2.30 (*t*, 2H, *J* = 7.4 Hz at C2)	2.35 (*t*, 2H, *J* = 7.2 Hz at C2)
3	1.54 (*m*, 2H, at C3)	1.59 (*m*, 2H, at C3)	1.63 (*m*, 2H, at C3)
4-17	1.25 (*m*, 14 × CH_2, 28H)	1.23 (*br s*, 14 x CH_2, 28H)	1.25 (*m*, 14 x CH_2, 28H)
18	0.854 (*t*, 3H, *J* = 6.9 Hz, terminal methyl)	0.84 (*t*, 3H, *J* = 6.21 Hz, terminal methyl)	0.88 (*t*, 3H, *J* = 6.8 Hz, terminal methyl)

Values of ^{1}H (300 MHz) and ^{13}C (75.4 MHz) are in ppm (δ). Values of ^{13}C and ^{1}H matched those of Dinda & Guha (1988) and Li *et al.* (2010).

SA is a saturated fatty acid found in animal fats and vegetable oils. Food rich in SA include cocoa butter, lard, beef tallow and butter, with cocoa butter having 34% content (Kris-Etherton *et al.* 2005). Milk fats have 5–15% of SA content while lard, cocoa butter and butter contain 10–35% (Sampath & Ntambi 2005).

Fatty acids can be grouped as saturated or unsaturated based on the presence of double or triple bonds (Lobb & Chow 2007). Most saturated fatty acids have unbranched structures with an even number of carbon atoms. They have a general formula R-COOH, in which the R group is a straight-chain hydrocarbon. Saturated fatty acids are also functionally divided into short- and long-chain acids, and are most widely known by their common names.

Studies have been conducted on the metabolic and health effects of increasing dietary intake of saturated fatty acids. In the U.S., the average intake of SA is 5.2 g per day for women and 8.1 g per day for men, representing 9.2% and 9.4% of total fat for women and men (Kris-Etherton *et al.* 2005). SA is generally well-absorbed as other fatty acids. Unlike other long-chain fatty acids, it does not raise the serum cholesterol concentration in cultured hamster hepatocytes (Bruce & Salter 1996). Rather than poor absorption, it is likely that this may be due to its metabolism within the body i.e. SA is more rapidly converted into oleic acid than other fatty acids.

Studies have also shown that SA displayed cytotoxic activity. SA has been reported to inhibit rat and human tumour development (Habib *et al.* 1987). It significantly inhibited the colony-forming ability of the tumour cell lines in a dose-related manner. At 10 µg/mL, SA significantly inhibited colony formation in four rat hepatoma cell lines of D23, D261, D262A and D262B. For human tumour cells lines, doses of SA needed to cause 70% inhibition (ID_{70}) were 2.8, 3.2 and 8.6 µg/mL for the 833K, RT112 and HFL human tumour cells lines, respectively.

Fatty acids including palmitic acid and SA induced apoptosis in human granulosa cells, not directly but through acyl-CoA, an effect that may cause reproductive abnormalities, observed in obese women (Mu *et al.* 2001). At doses ranging from 50–300 µM (14–85 µg/mL), SA induced apoptosis in a dose- and time-dependent manner. The cell survival was less than 20% after exposure to 300 µM (85 µg/mL) for three days. SA induced endoplasmic reticulum stress, and caused apoptotic and necrotic cell death in the primary rat hepatocytes (Zhang *et al.* 2011). Incubation of the hepatocyte cultures with 250 µM (71 µg/mL) of SA for 16 hours resulted in a significant loss of cell viability. The SA-mediated apoptosis of primary rat hepatocytes coincided with a significant increase in caspase-3 activation.

SA at 100 μM (28 μg/mL) significantly reduced cell viability of cardiomyocytes cultured from chick embryos (Kong & Rabkin 2002). A study of the effects of dietary fatty acids on the proliferation of normal human urothelial cells reported that SA had no effect on cell proliferation over a 1–100 μM concentration range (Southgate et al. 1996).

Given that the results in my study showed that SA inhibited HT-29, MCF-7, MDA-MB-231 and SKOV-3 cancer cells with IC_{50} ranging from 59–102 μg/mL, one could postulate that a 70 kg adult would have to consume 4.1–7.1 g of SA daily to observe a cytostatic effect. However, this postulation is based on *in vitro* data and it would be imprudent to draw any meaningful conclusions until further work has been undertaken to study the metabolism of SA in the body. Indeed, there have been no such studies on the relationship between dietary intake of SA and its role in inhibiting tumour growth. However, there is a disconnect in that case-control and cohort studies have found positive associations between several types of cancer and food intake with high levels of saturated fats, suggesting their role in carcinogenicity (Hu *et al.* 2013).

4.8.5 Triterpenoid (ursolic acid)

a. ^{1}H and ^{13}C NMR spectroscopy

The fractionation of Fraction 8 from the DCM leaf extract of *Vallaris glabra* led to the isolation of Compound **6**, identified as ursolic acid (UA). Its ^{1}H and ^{13}C NMR spectra and spectral data are shown in **Figure 4.16**, **Figure 4.17** and **Table 4.26**. NMR spectra matched those reported by Hamzah & Lajis (1998), and Park *et al.* (2005).

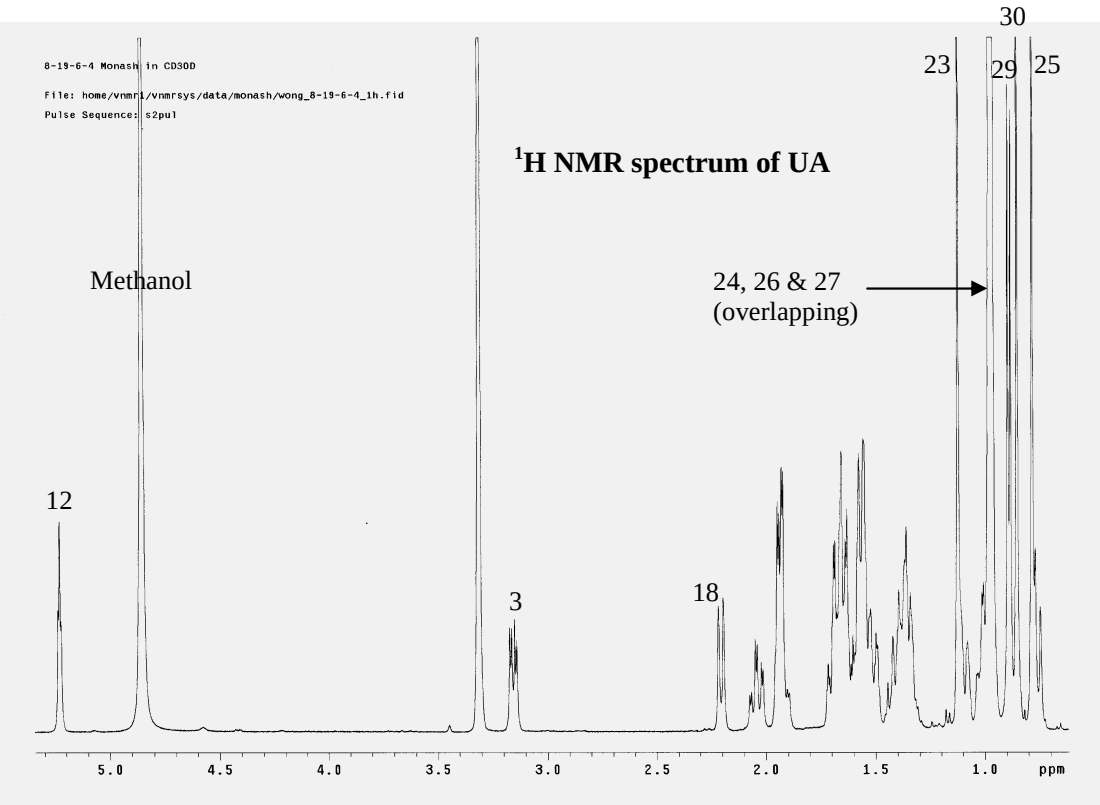
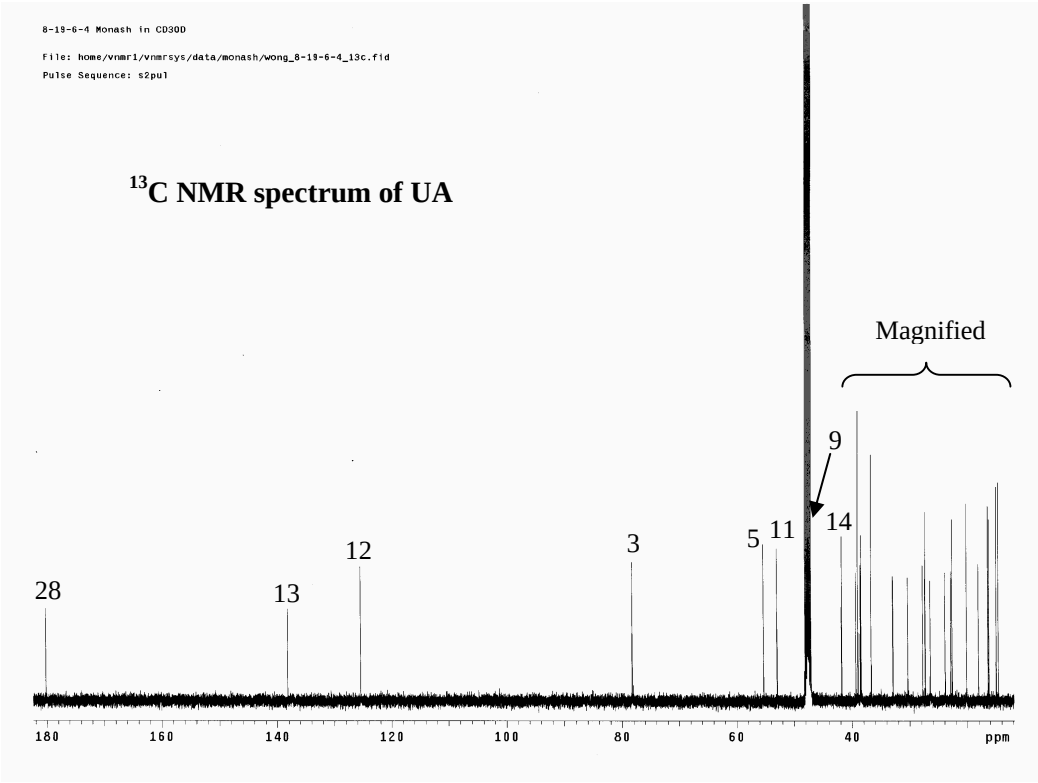


Figure 4.16 ^{1}H NMR spectrum (500 MHz) of ursolic acid (UA) in deuterated methanol



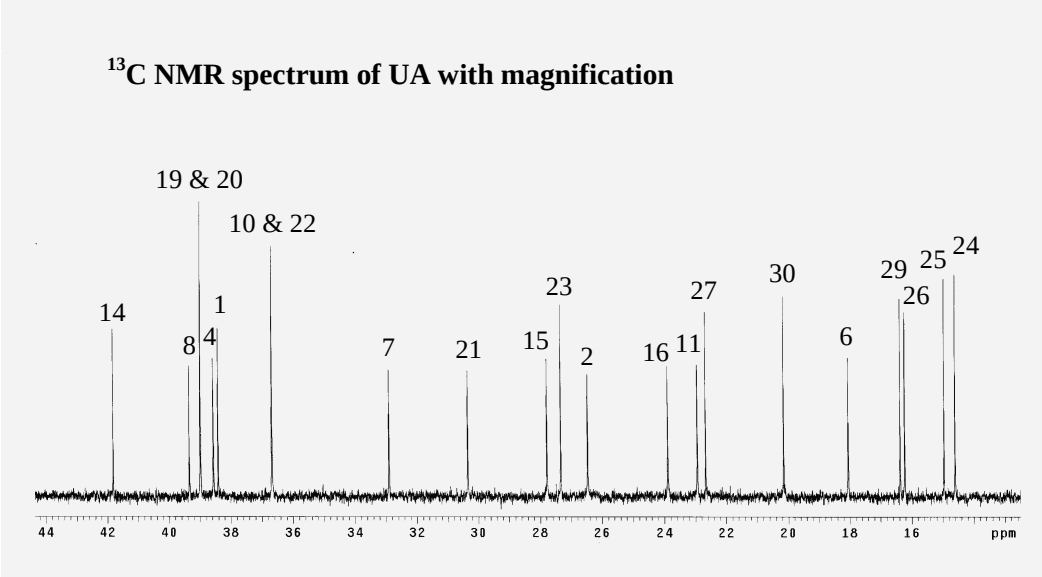


Figure 4.17 ^{13}C NMR spectrum of ursolic acid (UA) in deuterated methanol with magnification

In the ^{1}H NMR spectrum of UA, the doublet at 2.20 ppm with J = 11.3 Hz indicated that H18 and H19 are *trans* to one another (Othman *et al.* 1996; Hamzah & Lajis 1998). The coupling between single protons at C18 and C19 would result in a doublet. This doublet appears if the compound is of the α-type triterpene whereby the two groups attached to C19 are hydrogen and methyl. For a β-type triterpene whereby two protons are attached to C19, the coupling between protons at C18 and C19 would give a quartet. Note that in UA, both the methyl groups at ring E are secondary whereas in oleanolic acid, the two methyl groups are tertiary (Hamzah & Lajis 1998). Multiplet at 5.23 ppm which integrated for one proton is assigned to the olefinic proton of C12 which is coupled to protons at C11.

It was noted that the ^{13}C NMR spectrum of UA revealed a total of 30 carbon resonances, which could be identified as seven methyl, nine methylene and seven methine groups, along with seven quaternary carbons, one of which represents a carboxylic acid (Jaki *et al.* 2008).

Table 4.26 ^{1}H and ^{13}C NMR spectral data of ursolic acid from leaves of *Vallaris glabra*

Atom	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
	Thesis data (2013)		Hamzah & Lajis (1998)		Park *et al.* (2005)	
1	39.9		39.8		39.8	
2	27.9		27.8		27.8	
3	79.7	3.15 (1H, *dd* 11.5, 4.5)	79.6	3.14 (1H, *m*)	79.6	3.14 (1H, *dd* 11.2, 4.8)
4	40.0		39.9		39.9	
5	56.8		56.7		56.7	
6	19.5		19.4		19.4	
7	34.4		34.3		34.3	
8	40.8		40.7		40.7	
9	48.5		47.6		47.6	
10	38.1		38.1		38.1	
11	24.4		24.3		24.3	
12	127	5.23 (1H, *t* 3.5)	127	5.21 (1H, *m*)	127	5.22 (1H, *t* 3.6)
13	140		140		140	
14	43.3		42.8		42.8	
15	29.2		29.2		29.2	
16	25.3		25.3		25.3	
17	48.5		47.6		47.6	
18	54.4	2.21 (1H, *d* 11.0)	54.3	2.20 (1H, *d* 11.3)	54.3	2.19 (1H, *d* 11.2)
19	40.4		40.4		40.4	
20	40.4		40.4		40.4	
21	31.8		31.7		31.7	
22	38.1		38.1		38.1	
23	28.8	1.08 (3H, *s*)	28.7	1.10 (3H, *s* Me)	28.7	0.97 (3H, *s*)
24	16.1	0.96 (3H, *s*)	16.0	0.93 (3H, *s* Me)	16.0	0.95 (3H, *s*)
25	16.4	0.78 (3H, *s*)	16.3	0.76 (3H, *s*)	16.3	0.77 (3H, *s*)
26	17.7	0.97 (3H, *s*)	17.6	0.95 (3H, *s*)	17.6	0.84 (3H, *s*)
27	24.1	0.98 (3H, *s*)	24.0	0.96 (3H, *s* Me)	24.0	1.11 (3H, *s*)
28	182		182		182	
29	17.8	0.89 (3H, *d* 6.5)	17.8	0.87 (3H, *d*)	17.8	0.88 (3H, *d* 6.8)
30	21.6	0.85 (3H, *d* 6.5)	21.5	0.83 (3H, *d*)	21.5	0.96 (3H, *d* 6.8)

Values of ^{1}H (500 MHz) and ^{13}C (125.7 MHz) are in ppm (δ), and of *J* (bracketed) are in Hz. Values of ^{13}C and ^{1}H matched those of Hamzah & Lajis (1998) and Park *et al.* (2005).

As rightly pointed out by Jaki *et al.* (2008), the complete analysis of the NMR spectra of triterpenoids such as UA remains a challenge particularly due to the lack of ^{1}H NMR dispersion of the signals of the pentacyclic CH skeleton. The presence of higher order spin systems, severe signal overlap, and complex long-range coupling impede the structure elucidation. They added that the structure of UA has often been derived from reports that are typically limited to carbon shifts and rather incomplete proton assignments (e.g. Weis & Seebacher 2002).

b. APCI-MS spectrometry

Following NMR analysis, the identity of UA was reaffirmed using APCI-MS. The mass spectrum showed a $[M-H]^-$ molecular ion peak at 455.7 m/z, which corresponds to the molecular formula of $C_{30}H_{48}O_3$. Other peaks were observed at 472 and 911 m/z [**Figure 4.18**].

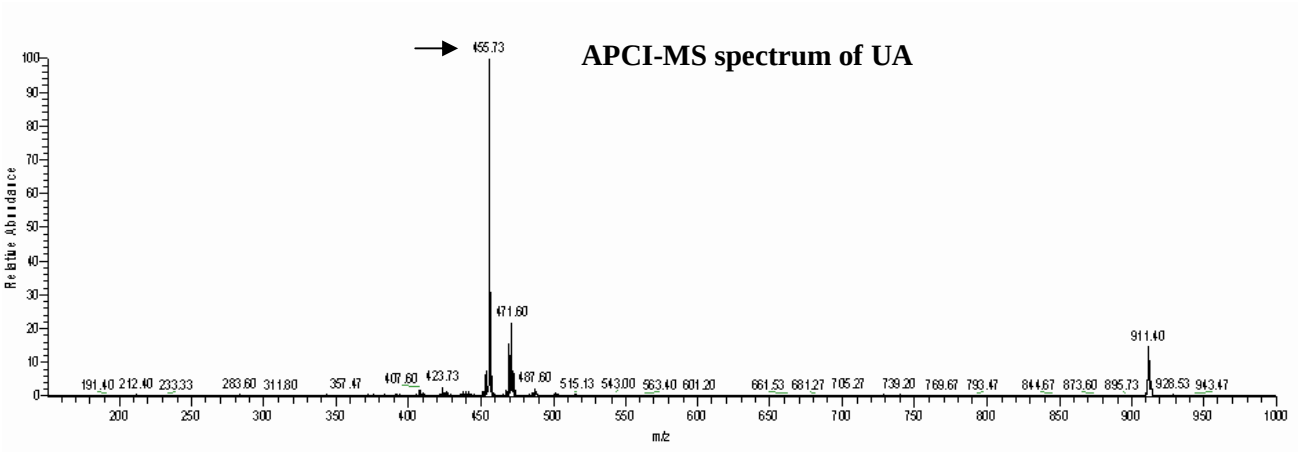


Figure 4.18 APCI-MS spectrum of ursolic acid (UA) in negative mode $[M-H]^-$

c. Ursolic acid

Ursolic acid (UA) or 3β-hydroxy-12-urs-12-en-28-oic acid has a molecular formula of $C_{30}H_{48}O_3$ [**Figure 4.19**], molecular weight of 456.7 and melting point of 283–285°C.

UA is a natural pentacyclic triterpenoid that is widely found in plants as free acids or aglycones of saponins (Liu 1995). UA is dominant in fruits such as cranberries,

apples, figs and olives, and in medicinal herbs such as lavender, oregano, rosemary and thyme (Ovesná *et al.* 2006; Bishayee *et al.* 2011).

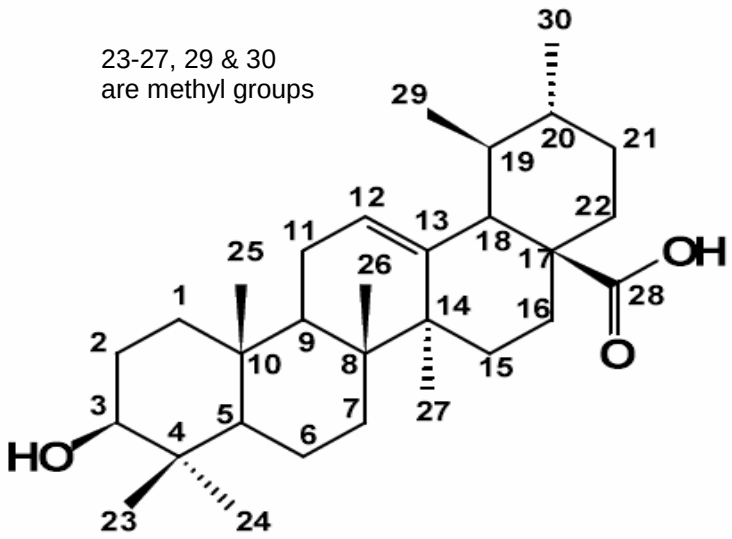


Figure 4.19 Molecular structure of ursolic acid with numbering system (Silva *et al.* 2008)

UA has been long considered to be biologically inactive, however, in recent years, it has attracted considerable interest because of its pharmacological effects combined with low toxicity (Liu 1995; Novotny *et al.* 2001; Ikeda *et al.* 2008). *In vitro* and *in vivo* studies have demonstrated that UA has many important biological functions that include antioxidant, anti-inflammatory, hepatoprotective, gastroprotective, anticancer, anti-tumour, antimicrobial, antiviral, antiallergic, antipruritic, antiangiogenic and spasmolytic (Liu 1995 & 2005; Ovesná *et al.* 2006; Bishayee *et al.* 2011). An increasing number of studies have reported that UA exhibited cytotoxicity against a variety of cancer cells [**section 4.10**].

During the last decade, over 700 research articles have been published on UA and oleanolic acid, reflecting the great interest and progress in understanding these triterpenoids (Liu 2005). The articles included isolation and purification from various plants and herbs, chemical modifications to make more effective and water-soluble derivatives, pharmacological benefits, toxicity effects, and clinical use in various diseases including anticancer chemotherapies. Muto *et al.* (1990) reported that UA is

among the eight compounds actively investigated for cancer chemo-preventive properties by scientists in Japan, funded by the Ministry of Health and Welfare.

UA and oleanolic acid share similar molecular structures but have different sites of the methyl group on ring E (Ovesná *et al.* 2006). If the methyl group at C19 of UA is moved to C20, it changes to oleanolic acid [**Figure 4.20**].

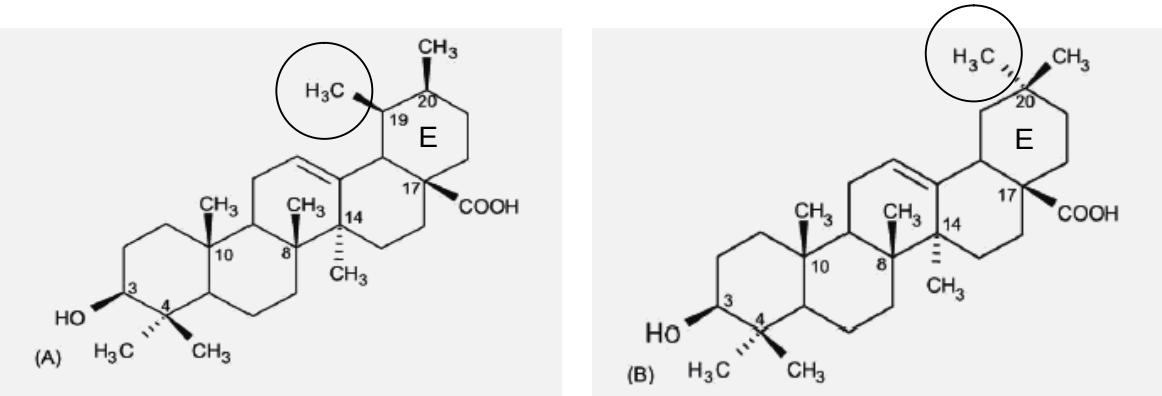


Figure 4.20 Molecular structures of ursolic acid (A) and oleanolic acid (B) (Ovesná *et al.* 2006)

4.9 CAFFEOYLQUINIC ACID CONTENT

The caffeoylquinic acid content (CQAC) of fresh leaf extracts of *Vallaris glabra* and five other Apocynaceae species was analysed using reversed-phase HPLC and results are shown in **Table 4.27**. The HPLC chromatograms of leaf extracts of the six species of Apocynaceae and flower extract of *Lonicera japonica* are shown in **Appendix VI**. CQAC was expressed as mg CGA/100 g.

Commercially purchased HPLC standard 5-CQA (chlorogenic acid) was used to construct the calibration curve [**Figure 4.21**]. CQAC was determined using peak areas. The calibration equation of peak area (mAU*s) against concentration of CGA (mg/L) was y = 24.262x (R^2= 1.000). 5-CQA was eluted at 7.1 min on the HPLC. As 3-CQA and 4-CQA had similar retention times (RT) of 5.9 min when eluted, they were estimated as a single aggregate.

Table 4.27 Caffeoylquinic acid content of leaf extracts of six Apocynaceae species using reversed-phase HPLC (fresh weight)

Species	CQA content	
	3-CQA & 4-CQA	5-CQA
Vallaris glabra	370 ± 15 [a]	353 ± 25 [b]
Alstonia angustiloba	19.2 ± 2.8 [cd]	155 ± 24 [d]
Dyera costulata	ND	253 ± 32 [c]
Kopsia fruticosa	39.7 ± 9.9 [b]	270 ± 63 [bc]
Nerium oleander	46.9 ± 4.4 [b]	537 ± 103 [a]
Plumeria obtusa	14.3 ± 4.7 [d]	245 ± 60 [cd]
Lonicera japonica	23.2 ± 2.2 [c]	173 ± 13 [d]

Caffeoylquinic acid (CQA) content is in mg CGA/100 g of samples (fresh weight). Values are means ± SD (n = 3). For each column, values followed by the same letter (a–d) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test. Flowers of *Lonicera japonica* were included as positive control. ND = not detected.

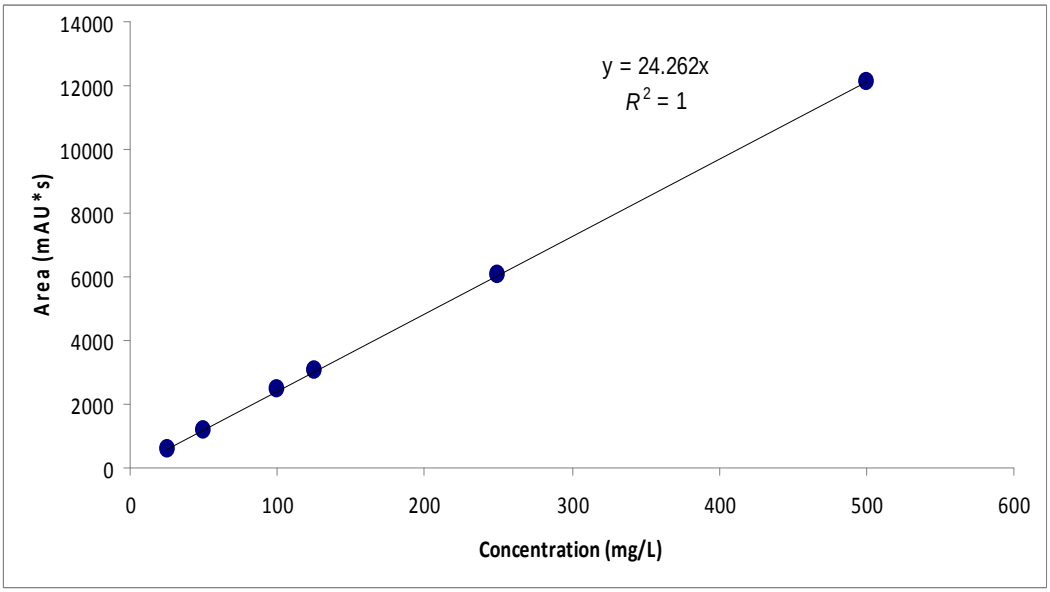


Figure 4.21 Standard curve of commercial chlorogenic acid (area vs. concentration)

It is interesting to note that leaves of all the six Apocynaceae species contained significant amounts of chlorogenic acid. *Nerium oleander* had the highest content of 5-CQA (537 ± 103 mg CGA/100 g) followed by *Vallaris glabra* (353 ± 25 mg CGA/100 g), which were respectively three and two times higher than the amount of 5-CQA in the flowers of *Lonicera japonica* (173 ± 13 mg CGA/100 g), the commercial source of 5-CQA. The 5-CQA content of *Nerium oleander* and *Vallaris glabra* leaves also surpasses *Etlingera elatior* (294 ± 53 mg CGA/100 g) and *Ipomoea batatas* (115 ± 16 mg CGA/100 g) reported by Chan *et al.* (2009). Although *Dyera costulata* had the highest CQAC [**Table 4.12**], the 5-CQA content of *Dyera costulata* (253 ± 32 mg CGA/100 g) was comparable to that of *Vallaris glabra* and *Kopsia fruticosa* (270 ± 63 mg CGA/100 g). 5-CQA content of *Dyera costulata*, *Kopsia fruticosa* and *Plumeria obtusa* (245 ± 60 mg CGA/100 g) were significantly higher than that of *Lonicera japonica* flowers. *Alstonia angustiloba* (155 ± 24 mg CGA/100 g) had comparable amounts of 5-CQA.

3-CQA and 4-CQA content of *Vallaris glabra* (370 ± 15 mg CGA/100 g) was the highest among the species screened and about 16 times higher than that of the flowers of *Lonicera japonica* (23.2 ± 2.2 mg CGA/100 g). *Nerium oleander* (46.9 ± 4.4 mg CGA/100 g), *Kopsia fruticosa* (39.7 ± 9.9 mg CGA/100 g) and *Alstonia angustiloba* (19.2 ± 2.8 mg CGA/100 g) had significantly higher 3-CQA and 4-CQA content than that of *Lonicera japonica* flowers. 3-CQA and 4-CQA were not detected in the leaves of *Dyera costulata*. The presence of other isomeric forms of CQA could account for the high amounts of CQAC in the methanol leaf extract of *Dyera costulata*.

4.10 ANTIPROLIFERATIVE ACTIVITY OF COMPOUNDS

The antiproliferative activity of the five compounds (3-CQA, 4-CQA, 5-CQA, QG and SA) isolated from leaf extracts of *Vallaris glabra* against HT-29, MCF-7, MDA-MB-231 and SKOV-3 cancer cells is shown in **Table 4.28**.

Table 4.28 Antiproliferative activity of compounds isolated from leaf extracts of *Vallaris glabra* against HT-29, MCF-7, MDA-MB-231 and SKOV-3 cancer cells

Compound	IC_{50} (µg/mL)			
	HT-29	MCF-7	MDA-MB-231	SKOV-3
3-*O*-Caffeoylquinic acid (3-CQA)	> 200	> 200	> 200	> 200
4-*O*-Caffeoylquinic acid (4-CQA)	> 200	> 200	> 200	> 200
5-*O*-Caffeoylquinic acid (5-CQA)	> 200	> 200	> 200	> 200
Quercetin-3-*O*-glucoside (QG)	> 200	> 200	> 200	> 200
Stearic acid (SA)	59 ± 6.0 (40 ± 9.8)	102 ± 16 (49 ± 5.7)	73 ± 8.8 (31 ± 4.3)	78 ± 4.8 (26 ± 2.7)

IC_{50} (µg/mL) is the concentration of compounds resulting in 50% inhibition of cell density. Bracketed values are GI_{50} (µg/mL), the concentration of compounds resulting in 50% reduction in cell growth. Values are means ± SD.

Results showed that 3-CQA, 4-CQA, 5-CQA and QG isolated from methanol leaf extract of *Vallaris glabra* did not have any inhibitory activity (based on IC_{50}) against the cancer cells tested. SA isolated from DCM leaf extract of *Vallaris glabra* was the only exception. It displayed weak inhibitory activity against HT-29 (59 ± 6.0 µg/mL), MCF-7 (102 ± 16 µg/mL), MDA-MB-231 (73 ± 8.8 µg/mL) and SKOV-3 (78 ± 4.8 µg/mL) cancer cells.

SA displayed weaker inhibitory activity on MCF-7 and MDA-MB-231 cancer cells when compared to standards such as curcumin and tamoxifen. Against these two cancer cell lines, GI_{50} of curcumin was 4.1 and 8.7 µg/mL, and GI_{50} of tamoxifen was 8.3 and 4.6 µg/mL, respectively (Cheah *et al.* 2008).

Previous studies on the antiproliferative properties of SA have reported variable results. At 120 µM, SA showed no toxicity towards 14 human leukemic cell lines (Finstad *et al*. 1998).

SA was reported to be toxic against five human cancer cell lines comprising HT-29, SuSa, 833K, RT112 and RT4 (Fermor *et al.* 1992). IC_{50} values of SA were 26.9, 9.7, 17.6, 15.3 and 26.8 μM, respectively. Growth inhibition of SA against HOG-1 cervical cancer cells was dose-related (Gleeson *et al.* 1990). At 75 μM, SA inhibited the growth of HOG-1 cells after 48 h in culture and at 125 μM, cell death occurred. SA isolated from aerial parts of *Oldenlandia diffusa* inhibited the growth of human hepatoma cells of HepG2 and Hep3B, but not human normal cells of WRL-68 (Tang *et al.* 2007). Against HepG2 (IC_{50} of 90 μM) and Hep3B (IC_{50} of 130 μM), SA was more effective than other fatty acids.

SA was found to have protective effect against breast cancer. Women with higher SA composition in their phospholipid layer had reduced risk of breast cancer (Chajès *et al*. 1999). SA inhibits the growth of carcinogen-induced mammary tumours in mice (Habib *et al.* 1987). There is accumulating evidence suggesting that desaturation of C18 of stearic to oleic acid disturbs cell membrane fluidity, a property that could promote cancer (Habib *et al*. 1987; Li *et al*. 1994).

It was unfortunate that the yield of ursolic acid (UA) isolated from the DCM leaf extract of *Vallaris glabra* was very low. The TLC examination showed that UA was present in substantial amounts. However, there were multiple co-eluting compounds that mandated many isolation steps. UA being the major component in this mixture was finally isolated *via* chloroform precipitation. The many isolation steps resulted in a low yield of only 0.01 g of pure UA. This amount was only sufficient for structural elucidation. Nevertheless, a review of existing literature was made on the cytotoxicity of UA with particular reference to the tested cancer cells.

In general, there is a wealth of references on the anticancer properties of UA including those of the cancer cells used in this study. Most of these publications concluded that UA is a potent chemo-preventive agent for cancer. IC_{50} values of 18 and 50 μM (8 and 23 μg/mL) have been reported for HT-29 colon cancer cells and SKOV-3 ovary cancer cells, respectively (Shan *et al.* 2009; Song *et al.* 2012). The inhibitory effect of UA against MCF-7 and MDA-MB-231 breast cancer cells has also been reported and is referenced in the following paragraphs.

Against HT-29 colon cancer cells, UA inhibited cell growth in a dose- and time-dependent manner (Shan *et al.* 2009). The median inhibition concentration IC_{50} values for 24-, 48- and 72-hour treatment were 26, 20 and 18 µM, respectively. Results showed that UA induced apoptosis in HT-29 cells by suppressing the epidermal growth factor receptor (EGFR) or mitogen-activated protein kinase (MAPK) pathway. Earlier, UA has been reported to have strong antiproliferative and apoptotic effects on HT-29 cells and the effects may be mediated by activation of alkaline sphingomyelinase (Andersson *et al.* 2003).

Against SKOV-3 ovary cancer cells, UA exerted cytotoxicity with IC_{50} of 50 µM (Song *et al.* 2012). Apoptotic bodies were observed in UA-treated SKOV-3 cells. Findings suggested that UA induced apoptosis *via* activation of caspase and phosphorylation of glycogen synthase kinase 3 beta (GSK 3*β*) in SKOV-3 cancer cells. The cytotoxic activity of some UA derivatives was stronger than UA against SKOV-3 cancer cells (Meng *et al.* 2010).

4.11 APOPTOTIC ACTIVITY OF EXTRACT

4.11.1 Nuclear Staining

In apoptosis, DNA fragmentation is the hallmark of apoptotic cells (Kerr *et al.* 1972). To examine the nuclear morphological changes in response to DCM leaf extract of *Vallaris glabra*, both control and treated MDA-MB-231 breast cancer cells were stained with fluorescent dye Hoechst 33342 and visualised under a fluorescent microscope. The Hoechst nuclear stain binds to the AT-rich regions of the DNA and exhibits enhanced fluorescence (Cheah *et al.* 2008; Zakaria *et al.* 2009).

MDA-MB-231 cells were chosen as a candidate for apoptotic studies due to its fast doubling rate and have been demonstrated to undergo apoptosis. MDA-MB-231 cells have high invasive ability and tend to establish resistance against programmed cell death stimuli (Cheah *et al.* 2008). They are known as triple negative because they do not express receptors for steroid hormones of oestrogen and progesterone or tyrosine kinase Her-2 (Liu *et al.* 2009). Thus, the ability of plant extracts to arrest their proliferation is beneficial for the prevention and treatment of invasive cancer.

Results of Hoechst staining of control MDA-MB-231 cells and cells treated with DCM leaf extract of *Vallaris glabra* showed that the dye was able to diffuse through intact membranes of the cells and stain their DNA. At 1.56, 3.13 and 6.25 µg/mL of leaf extract, the cells remained uniformly stained and showed no fluorescence in the nucleus [**Figure 4.22**]. The cells were neither apoptotic nor exhibit fragmentation of DNA.

MDA-MB-231 cancer cells treated with higher concentrations (12.5, 25.0 and 50.0 µg/mL) of leaf extract began to display apoptotic morphology [**Figure 4.23**]. As the concentration of extract was increased, there was stronger and more intense fluorescence. Fluorescence was seen in the nuclear region of the cells, indicating the presence of DNA fragmentation. Cell shrinkage, chromatin condensation, nuclear fragmentation and apoptotic bodies (small highly fluorescent spherical masses) were visualised. These observations provided evidence that the DCM leaf extract of *Vallaris glabra* had an apoptotic effect on MDA-MB-231 cells.

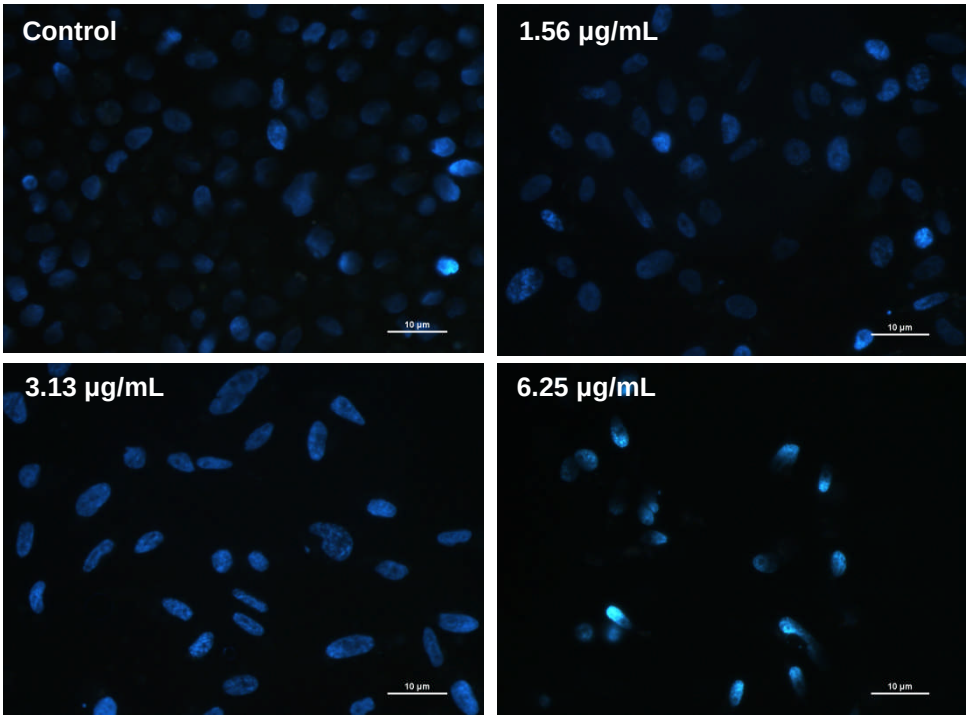


Figure 4.22 The control and DCM leaf extracts of *Vallaris glabra* at 1.56, 3.13 and 6.25 µg/mL showed no apoptotic effects on treated MDA-MB-231 breast cancer cells

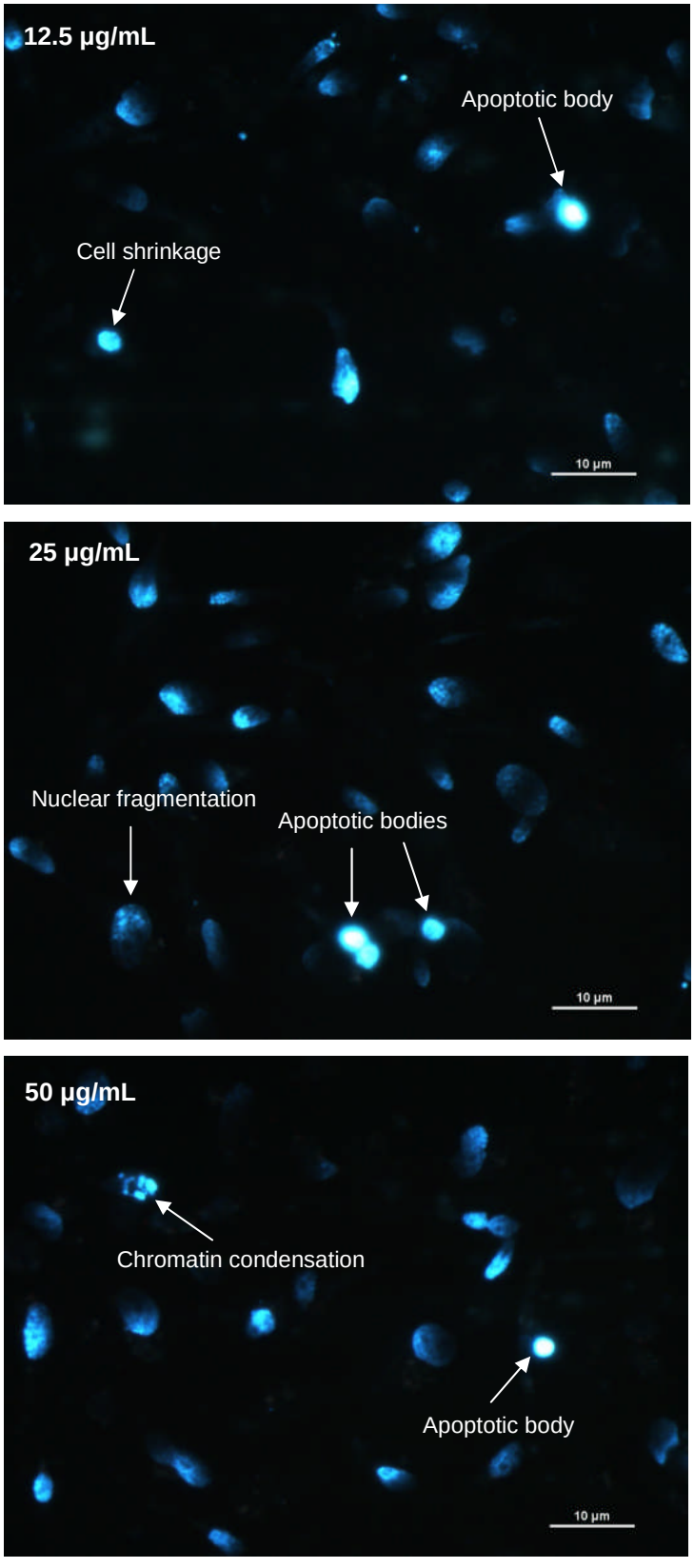


Figure 4.23 DCM leaf extracts of *Vallaris glabra* at 12.5, 25.0 and 50.0 µg/mL showed apoptotic effects on treated MDA-MB-231 breast cancer cells. Arrows indicate DNA fragmentation and chromatin condensation in cells.

Similar apoptotic morphology of cancer cells stained with Hoechst 33258 was reported for MCF-7 cells treated with *Eurycoma longifolia* root extract at 30 µg/mL (Tee & Azimahtol 2005), for MCF-7 cells treated with xanthorrhizol at 5 and 10 µg/mL (Cheah *et al.* 2006), and for MDA-MB-231 cells treated with xanthorrhizol at 5−20 µg/mL (Cheah *et al.* 2008).

The entire apoptotic process can be divided into several steps (Häcker 2000; Martelli *et al.* 2001; Ray & Corcoran 2009). They are cell shrinkage; chromatin or nuclear condensation; formation of apoptotic bodies; and phagocytosis of apoptotic bodies. Cell shrinkage is the earliest change where the cytoplasm condenses and the entire cell shrinks. Chromatin or nuclear condensation is one of the most widely observed and distinguishing features of apoptosis. At this stage, condensing fragments of chromatin coalesce and the nucleus breaks down into several fragments. Subsequently, the cell breaks up into several smooth surfaced apoptotic bodies that contain tightly compacted organelles and nuclear fragments. Finally, these apoptotic bodies are engulfed by phagocytes.

4.11.2 Caspase Colorimetry

MDA-MB-231 cells were treated with varying concentrations (1.56–25.0 µg/mL) of DCM leaf extract of *Vallaris glabra*. Fold-increase in activity of caspase-3, -6, -8 and -9 was determined by comparing the results of treated samples with those of the control. A graph plotting concentration of DCM leaf extract against fold-increase in caspase activity is shown in **Figure 4.24**.

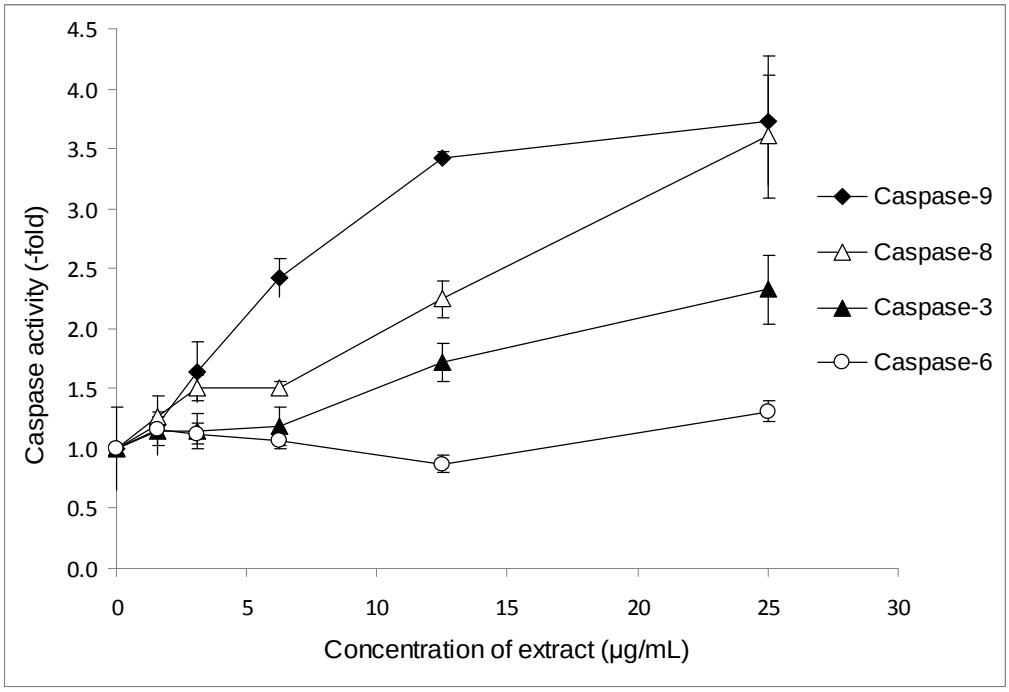


Figure 4.24 Graph of fold-increase in the activity of caspase-3, -6, -8 and -9 vs. concentration of DCM leaf extract (µg/mL) of *Vallaris glabra*

Two caspases analysed showed fairly strong fold-increase activity with increasing concentration of DCM leaf extract of *Vallaris glabra*. They are caspase-8 and -9. At 3.13 and 6.25 µg/mL of extract, caspase-8 remained at 1.50 fold. At 12.5 and 25.0 µg/mL of extract, activity increased 2.25 and 3.61 fold, respectively. Caspase-9 showed a slightly different trend of increase. At 3.13, 6.25 and 12.5 µg/mL of extract, activity strongly increased 1.64, 2.42 and 3.42 fold, respectively. Activity culminated to 3.73 fold at 25.0 µg/mL of extract. Fold-increase activity of caspase-3 was weak with caspase-6 hardly showing any activity. Caspase-3 showed no discernable activity at 3.13 and 6.25 µg/mL of extract. Activity was 1.72 and 2.33 fold at 12.5 and 25.0 µg/mL of extract. Caspase-6 showed slight decline in activity from 3.13–12.5 µg/mL of extract. Activity was only 1.31 fold at 25.0 µg/mL of extract.

Results of the caspase colorimetric assay showed that the DCM leaf extract of *Vallaris glabra* modulates caspase-8, -9 and -3 but not caspase-6 which indicated that the extract induced apoptosis through both the extrinsic (death-receptor) and intrinsic (mitochondrial) pathways. The DCM leaf extract could have activated both the intrinsic and extrinsic pathways of apoptosis owing to its complex mixtures of

compounds. Apoptosis induced by the DCM leaf extract was concentration dependent with fold-increase in caspase activities becoming more apparent at 12.5 and 25.0 µg/mL.

Similarly, an increase in caspase-8, -9 and -3 activities has been reported after MDA-MB-231 cancer cells were treated with 250 µM of quercetin (Chien *et al.* 2009), and with 40 mM of metformin (Liu *et al.* 2009). Likewise, the induction of apoptosis of MDA-MB-231 cells involving caspase-8, -9 and -3 was also reported for sanguinarine (Choi *et al.* 2008) and 2-methoxyestradiol (LaVallee *et al.* 2003). On the contrary, MDA-MB-231 cancer cells treated with xanthorrhizol (0−20 µg/mL) was reported to modulate caspase-3 and -9 activity, but not caspase-6 and -8 (Cheah *et al.* 2008).

For other cancer cells, Liu *et al.* (2006) reported an increase in caspase-8, -9 and -3 activities after promyelocytic leukemia HL-60 cells were treated with extracts from *Narcissus tazetta.* Apoptosis of HepG2 cancer cells when treated with cardiotonic steroids (bufalin and cinobufagin) from the skin and venom glands of the Asiatic toad, also involved the activation of caspase-8, -9 and -3 (Qi *et al.* 2011).

Caspases are the executioners of apoptosis, and in mammals, caspase-2, -8, -9 and -10 are the initiator caspases, while caspase-3, -6 and -7 are the effector caspases (Riedl & Shi 2004). AIthough initiator and effector caspases share general structural features, their activation, inhibition and release of inhibition are differentially regulated. An initiator caspase is characterised by an extended N-terminal region which comprises one or more adaptor domains that are important for its functions, whereas an effector caspase usually contains 20–30 residues in its prodomain sequence.

The activation of caspases has been well-reviewed (Hengartner 2000; Riedl & Shi 2004; Shi 2005; Fulda 2009). Activation of effector caspases is mediated by specific initiator caspases through proteolytic cleavage. As a consequence of the intra-chain cleavage, the catalytic activity of effector caspases is enhanced by several orders of magnitude. Effector caspases, once activated, are responsible for the proteolytic degradation of a broad spectrum of cellular targets that ultimately leads to cell death.

In contrast, the activation of initiator caspases and their regulation are quite complex, involving adaptor protein complexes. In mammalian cells, caspase-8 operates *via* the extrinsic (death-receptor) pathway and caspase-9 operates *via* the intrinsic (mitochondrial) pathway. The extrinsic pathway is initiated by the binding of an extracellular death ligand to its cell-surface death receptor. The intrinsic pathway is triggered in response to a wide array of death stimuli that are generated from within the cell. Activation of caspase-8 is mediated by the death-inducing signalling complex (DISC) while caspase-9 is activated by the apoptosome. DISC is assembled following binding of death ligand to its receptor and contains Fas-associated death domain (FADD) and caspase-8.

Chapter V

CONCLUSION

Leaf extracts of ten Apocynaceae species assessed for antiproliferative (APF) activity showed that six species (*Alstonia angustiloba*, *Calotropis gigantea*, *Catharanthus roseus*, *Nerium oleander*, *Plumeria obtusa* and *Vallaris glabra*) yielded positive results. DCM and DCM:MeOH extracts of *Vallaris glabra* showed strong APF activities against all six human cancer cell lines of MCF-7, MDA-MB-231, HeLa, HT-29, SKOV-3 and HepG2.

Leaf extracts of all five Apocynaceae species (*Alstonia angustiloba*, *Calotropis gigantea*, *Dyera costulata*, *Kopsia fruticosa* and *Vallaris glabra*) assessed for antiplasmodial (APM) activity were effective against chloroquine-resistant K1 strain of *Plasmodium falciparum.* All four extracts of *Vallaris glabra* displayed effective APM activity.

Vallaris glabra was chosen as the plant for further investigation. Among the four different leaf extracts of *Vallaris glabra*, the DCM extract showed strongest APF and APM activities. Using column chromatography, stearic acid (SA) and ursolic acid (UA) were isolated from this extract. From the MeOH extract, 3-O-caffeoylquinic acid (3-CQA), 4-O-caffeoylquinic acid (4-CQA), 5-O-caffeoylquinic acid (5-CQA) and quercetin 3-O-glucoside (QG) were isolated. These isolated compounds were identified by ^{1}H-NMR, ^{13}C-NMR and MS analyses

APF activity of the five compounds (3-CQA, 4-CQA, 5-CQA, QG and SA) isolated from leaf extracts of *Vallaris glabra* were tested against four cancer cell lines. Only SA displayed weak inhibitory activity. UA was not tested due to low yield but a review of existing literature showed that it has strong anticancer properties including those of the cancer cells used in this study.

MDA-MB-231 breast cancer cells treated with DCM leaf extract of *Vallaris glabra* and stained with fluorescent dye Hoechst 33342, showed evidence that the extract had an apoptotic effect on the MDA-MB-231 cells, based on nuclear morphological changes. Caspase colorimetry showed that the apoptotic effect involved activation of caspase-8, -9 and -3, but not caspase-6, indicating that the extract induced apoptosis through both the extrinsic (death-receptor) and intrinsic (mitochondrial) pathways.

Vallaris glabra is a unique plant because it displayed both strong and broad-spectrum APF and APM activities. The species would be of interest to the pharmaceutical industry as a potential candidate for anticancer and antimalarial drug discovery. With much higher CQA content in the leaves of *Vallaris glabra* than flowers of *Lonicera japonica* (the commercial source), it can serve as a promising alternative source of CQA.

During the course of this study, two papers have been published in international refereed journals. ‘Assessment of antiproliferative and antiplasmodial activities of five selected Apocynaceae species’ was published in *BMC Complementary and Alternative Medicine* and ‘Antiproliferative and phytochemical analyses of leaf extracts of ten Apocynaceae species’ was published in *Pharmacognosy Research*. The papers were jointly authored with my supervisor and scientists from the Institute of Medical Research (IMR), Malaysia.

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ADDENDUM

During the course of this study, two papers have been published in international refereed journals based on the Ph.D. work. They were jointly authored with my supervisor and scientists from the Institute of Medical Research (IMR), Malaysia. The publications are summarised below with copies of their reprints included in **Appendix VII**.

Publication No. 1

Wong, S.K., Lim, Y.Y., Abdullah, N.R. & Nordin F.J., 2011. Assessment of anti-proliferative and antiplasmodial activities of five selected Apocynaceae species. *BMC Complementary and Alternative Medicine*, 11, 3. http://www.biomedcentral.com/1472-6882/11/3 (Indexed by BioMed Central)

Background: Studies have shown that the barks and roots of some Apocynaceae species have anticancer and antimalarial properties. In this study, leaf extracts of five selected species of Apocynaceae used in traditional medicine (*Alstonia angustiloba*, *Calotropis gigantea*, *Dyera costulata*, *Kopsia fruticosa* and *Vallaris glabra*) were assessed for antiproliferative (APF) and antiplasmodial (APM) activities, and analysed for total alkaloid content (TAC), total phenolic content (TPC) and radical-scavenging activity (RSA). As *Vallaris glabra* leaf extracts showed wide spectrum APF and APM activities, they were further screened for saponins, tannins, cardenolides and terpenoids.

Methods: APF and APM activities were assessed using the sulphorhodamine B and lactate dehydrogenase assays, respectively. TAC, TPC and RSA were analysed using Dragendorff precipitation, Folin-Ciocalteu and DPPH assays, respectively. Screening for saponins, tannins, cardenolides and terpenoids were conducted using the frothing, ferric chloride, Kedde and vanillin-H_2SO_4 tests, respectively.

Results: Leaf extracts of *Alstonia angustiloba*, *Calotropis gigantea* and *Vallaris glabra* displayed positive APF activity. Dichloromethane (DCM) extract of *Calotropis gigantea*, and DCM and DCM:MeOH extracts of *Vallaris glabra* showed strong APF activity against all six human cancer cell lines tested. DCM extract of *Alstonia angustiloba* was effective against three cancer cell lines. Against MCF-7 and MDA-MB-231 cell lines, DCM extract of *Calotropis gigantea* was stronger than standard drugs of xanthorrhizol, curcumin and tamoxifen. All five species were effective against K1 strain of *Plasmodium falciparum* and three species (*Calotropis gigantea*, *Dyera costulata* and *Kopsia fruticosa*) were effective against 3D7 strain. Against K1 strain, all four extracts of *Vallaris glabra* displayed effective APM activity. Extracts of *Dyera costulata* were effective against 3D7 strain. Selectivity index values of extracts of *Alstonia angustiloba*, *Calotropis gigantea* and *Vallaris glabra* suggested that they are potentially safe for use to treat malaria. Extracts of *Kopsia fruticosa* had the highest TAC while *Dyera costulata* had the highest TPC and RSA. Phytochemical screening of extracts of *Vallaris glabra* also showed the presence of terpenoids, tannins and saponins.

Conclusions: Leaf extracts of *Calotropis gigantea* and *Vallaris glabra* showed great promise as potential candidates for anticancer drugs as they inhibited the growth of all six cancer cell lines. Against K1 strain of *Plasmodium falciparum*, all four extracts of *Vallaris glabra* displayed effective APM activity. The wide spectrum APF and APM activities of *Vallaris glabra* are reported for the first time and this warrants further investigation into its bioactive compounds.

Publication No. 2

Wong, S.K., Lim, Y.Y., Abdullah, N.R. & Nordin F.J., 2011. Antiproliferative and phyto-chemical analyses of leaf extracts of ten Apocynaceae species. *Pharmacognosy Research*, 3(2), 100−6. (Indexed by PubMed)

Background: The anticancer properties of Apocynaceae species are well known in barks and roots but less so in leaves.

Materials and Methods: In this study, leaf extracts of 10 Apocynaceae species were assessed for antiproliferative (APF) activities using the sulphorhodamine B assay. Their extracts were also analysed for total alkaloid content (TAC), total phenolic content (TPC), and radical scavenging activity (RSA) using the Dragendorff precipitation, Folin-Ciocalteu, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) assays, respectively.

Results: Leaf extracts of *Alstonia angustiloba*, *Calotropis gigantea*, *Catharanthus roseus*, *Nerium oleander*, *Plumeria obtusa*, and *Vallaris glabra* displayed positive APF activities. Extracts of *Allamanda cathartica*, *Cerbera odollam*, *Dyera costulata*, and *Kopsia fruticosa* did not show any APF activity. Dichloromethane (DCM) extract of *Calotropis gigantea*, and DCM and DCM:MeOH extracts of *Vallaris glabra* showed strong APF activities against all six human cancer cell lines. Against breast cancer cells of MCF-7 and MDA-MB-231, DCM extracts of *Calotropis gigantea* and *Nerium oleander* were stronger than or comparable to standard drugs of xanthorrhizol, curcumin, and tamoxifen. All four extracts of *Nerium oleander* were effective against MCF-7 cells. Extracts of *Kopsia fruticosa* had the highest TAC while those of *Dyera costulata* had the highest TPC and RSA. Extracts of *Calotropis gigantea* and *Vallaris glabra* inhibited the growth of all six cancer cell lines while all extracts of *Nerium oleander* were effective against MCF-7 cells.

Conclusion: Extracts of *Calotropis gigantea*, *Vallaris glabra*, and *Nerium oleander* therefore showed great promise as potential candidates for anticancer drugs. The wide-spectrum APF activities of these three species are reported for the first time and their bioactive compounds warrant further investigation.

Manuscripts

After this thesis was sent for external examination, three manuscripts were prepared for submission to international peer-reviewed journals. ‘Antiproliferative activity of *Vallaris glabra* (Apocynaceae)’ was submitted to *Pharmacognosy Magazine*.

'Caffeoylquinic acids in leaves of selected Apocynaceae species: their isolation and content' was submitted and accepted for publication in Oct-Dec 2013 issue of *Pharmacognosy Research.* 'Botany, uses, phytochemistry and pharmacology of selected Apocynaceae species: A review' was submitted and accepted for publication in Jul-Sep 2013 issue of *Pharmacognosy Communications.*

Abstracts of the manuscripts are shown below:

Submitted to *Pharmacognosy Magazine*

Antiproliferative activity of *Vallaris glabra* (Apocynaceae)

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Abstract

Background: Our earlier study on the antiproliferative (APF) activity of leaves of ten Apocynaceae species showed that leaves of *Vallaris glabra* possessed strong and broad-spectrum properties. In this study, extracts, fractions and compounds from *V. glabra* leaves, and extracts from stems and flowers were assessed for APF activity. **Methods:** Plant parts of *V. glabra* were sequentially extracted with hexane (Hex), dichloromethane (DCM) and methanol (MeOH). APF activity was assessed using the sulforhodamine B assay. Four human cancer cell lines (HT-29, MCF-7, MDA-MB-231, and SKOV-3) were tested. Compounds were isolated using column chromatography and identified by ^{1}H-NMR, ^{13}C-NMR and ESI-MS analyzes. Caffeoylquinic acid content (CQAC) of leaves of *V. glabra* was quantified using reversed-phase HPLC, with comparison with five other Apocynaceae species. Possible mechanisms of apoptosis of DCM leaf extract were elucidated using Hoechst nuclear staining and caspase colorimetric assays.

Results: Stem and flower extracts of *V. glabra* displayed effective inhibition against MCF-7 and SKOV-3 cancer cells. Both leaves and flowers possessed broad-spectrum APF activity. From the MeOH leaf extract of *V. glabra*, three caffeoylquinic acids (3-*O*-caffeoylquinic acid, 4-*O*-caffeoylquinic acid and 5-*O*-caffeoylquinic acid) and a flavonol (quercetin 3-*O*-glucoside), and from the DCM leaf extract, a fatty acid (stearic acid) and a triterpenoid (ursolic acid) were isolated. The 5-CQA content of *V. glabra* was twice that of flowers of *Lonicera japonica* (the commercial source) while its 3-CQA and 4-CQA content was 16 times higher. APF activity of the compounds isolated from leaf extracts of *V. glabra* showed that stearic acid displayed weak inhibitory activity. Although the yield of ursolic acid was too low for analysis, a review of existing literature showed that there is a wealth of references on the anticancer properties of ursolic acid including those of the cancer cells used in this study. MDA-MB-231 cancer cells treated with DCM leaf extract of *V. glabra* and stained with fluorescent Hoechst 33342 dye, provided evidence that the extract had an apoptotic effect on the cells, based on nuclear morphological changes. Caspase colorimetry showed that the apoptotic effect involved activation of caspase-8, -9 and -3, but not caspase-6. **Conclusion:** Leaves and flowers of *V. glabra* displayed strong and broad-spectrum APF activity. The CQA content of leaves was much higher than flowers of *L. japonica.* DCM leaf extract of *V. glabra* had an apoptotic effect on MDA-MB-231 cancer cells, which involved activation of caspase-8, -9 and -3. The potential of *V. glabra* as a candidate species for anticancer drug discovery and as an alternative source of CQA warrants further investigation. Such studies should include other *Vallaris* species.

Accepted by *Pharmacognosy Research*

Caffeoylquinic acids in leaves of selected Apocynaceae species: their isolation and content

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Abstract

Background: Three compounds isolated from the methanol (MeOH) leaf extract of *Vallaris glabra* (Apocynaceae) were those of caffeoylquinic acids (CQAs). This prompted a quantitative analysis of their contents in leaves of *V. glabra* in comparison with those of five other Apocynaceae species (*Alstonia angustiloba*, *Dyera costulata*, *Kopsia fruticosa*, *Nerium oleander*, and *Plumeria obtusa*), including flowers of *Lonicera japonica* (Japanese honeysuckle), the commercial source of chlorogenic acid (CGA). **Materials and Methods:** Compound were isolated by column chromatography, and identified by NMR and MS analyses. CQA content of leaf extracts was determined using reversed-phase HPLC. **Results:** From the MeOH leaf extract of *V. glabra*, 3-CQA, 4-CQA, and 5-CQA or CGA were isolated. Content of 5-CQA of *V. glabra* was two times higher than flowers of *L. japonica*, while 3-CQA and 4-CQA content was 16 times higher. **Conclusion:** With much higher CQA content than the commercial source, leaves of *V. glabra* can serve as a promising alternative source.

Accepted by *Pharmacognosy Communications*

Botany, uses, phytochemistry and pharmacology of selected Apocynaceae species: A review

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ABSTRACT

The family Apocynaceae consists of tropical trees, shrubs and vines. Characteristic features of the family are that almost all species produce milky sap. Leaves are simple, opposite or whorled; flowers are large, colourful and slightly fragrant with five contorted lobes; and fruits are in pairs. With the inclusion of species of Asclepiadaceae, the family has now enlarged from two to five sub-families. The expanded family now comprises more than 250 genera and 2000 species. In traditional medicine, Apocynaceae species are used to treat gastrointestinal ailments, fever, malaria, pain and diabetes, including skin and ecto-parasitic diseases. Some are important timber species while many are planted as ornamentals. Non-medicinal uses include food, poisons, fodder, wood, ornamentals, dye and perfume. Species of Apocynaceae have been reported to possess anticancer and antimalarial properties. Species having cytotoxic activity include *Allamanda*, *Alstonia*, *Calotropis*, *Catharanthus*, *Cerbera*, *Nerium*, *Plumeria*, *Tabernaemontana* and *Vallaris*. Species of *Alstonia*, *Calotropis*, *Dyera*, *Kopsia* and *Vallaris* are also known to have antimalarial properties. Prompted by their anticancer and antimalarial properties; the botany, uses, phytochemistry and pharmacology of ten selected Apocynaceae species (*Allamanda cathartica*, *Alstonia angustiloba*, *Calotropis gigantea*, *Catharanthus roseus*, *Cerbera odollam*, *Dyera costulata*, *Kopsia fruticosa*, *Nerium oleander*, *Plumeria obtusa* and *Vallaris glabra*) belonging to ten genera are reviewed.

Other Publications

During the course of this study, other papers have been published in international journals based on research work done previously. Titles of publications are listed below with abstracts shown in **Appendix VIII**.

Wong, S.K., Wong, L.F., Lim, Y.Y. & Chan, E.W.C., 2010. Effects of drying treatments on the antioxidant properties of leaves and teas of *Alpinia* species (Zingiberaceae). *Journal of Tropical Medicinal Plants*, 11(1), 97−105.

Wong, S.K., Lim, Y.Y. & Chan, E.W.C., 2010. Evaluation of antioxidant, antityrosinase and antibacterial activities of selected *Hibiscus* species. *Ethnobotancial Leaflets*, 14, 781−96.

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Appendix I

CHEMICALS AND CONSUMABLES

BIOASSAYS

Extraction

Liquid nitrogen (Malaysia Oxygen Sdn. Bhd., Malaysia)

Methanol, analytical grade (Merck KGaA, Germany)

Hexane, analytical grade (Fisher Scientific, UK)

Dichloromethane, analytical grade (Merck KGaA, Germany)

Total alkaloid content

Bismuth nitrate, ≥ 98% (Sigma-Aldrich, USA)

Glacial acetic acid, ≥ 99.9% (Sigma-Aldrich, USA)

Potassium iodide (VWR International Ltd., UK)

Sodium sulphide flake (Ajax Finechem, Australia)

Thiourea (R&M Marketing, UK)

Boldine (Sigma-Aldrich, USA)

Total phenolic content

Folin-Ciocalteu's reagent (Fluka, USA)

Gallic acid, 98% (Fluka, USA)

Sodium carbonate anhydrous, 99% (Fluka, USA)

Caffeoylquinic acid content

Sodium molybdate dehydrate, > 99.0% (Acros Organics, USA)

Potassium dihydrogen orthophosphate, 99.7% (Fisher Scientific, UK)

Dipotassium hydrogen phosphate, > 99.0% (Merck KGaA, Germany)

Chlorogenic acid, ≥ 95% (Sigma-Aldrich, USA)

Free radical scavenging activity

L-ascorbic acid, 99.7% (Merck KGaA, Germany)

2,2-diphenyl-1-picrylhydrazyl (DPPH), 90% (Sigma, USA)

Tyrosinase inhibition

3,4-Dihydroxy-L-phenylalanine (Sigma-Aldrich, USA)

Dimethyl sulphoxide (Fisher Scientific, USA)

Tyrosinase (Sigma-Aldrich, USA)

Qualitative phytochemical screening

Glacial acetic acid (R&M Marketing, UK)

Iron (III) chloride hexahydrate (R&M Chemicals, UK)

Sulphuric acid (H_2SO_4), 95–97% (HmbG Chemicals, Germany)

Cell culture

Foetal bovine serium (Sigma-Aldrich, USA)

Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, USA)

Roswell Park Memorial Institute 1640 Medium (RPMI-1640; Sigma-Aldrich, USA)

L-Glutamine ≥ 99% (Sigma-Aldrich, USA)

Sodium hydrogen carbonate (HmbG Chemicals, Germany)

Penicillin-streptomycin 1 x liquid, [+] 10,000 units/mL

Penicillin [+] 10,000 µg/mL

Streptomycin (Invitrogen Co., Carlsbad, California, USA)

Phosphate buffer saline tablets (Merck KGaA, Germany)

Trypsin-EDTA 0.5% (Invitrogen Co., Carlsbad, California, USA)

Cell culture plastic wares

96 tissue culture test plates (TPP, Switzerland)

25 cm^2 flask with canted neck (Corning Incorporated, USA)

75 cm^2 flask with filter cap; nunclon^{TM} (Nunc A/S, Germany)

100 x 20 mm cell culture dish (Corning Incorporated, USA)

150 x 25 mm cell culture dish (SPL Lifesciences Co., Ltd., Korea)

15 mL polypropylene conical tube; BD Falcon^{TM} (Becton Dickinson Labware, USA)

50 mL polypropylene conical tube; BD Falcon^{TM} (Becton Dickinson Labware, USA)

500 mL filter-top, 0.22 µm PES (TPP, Switzerland)

5 mL serological pipette (Greiner Bio-One, Switzerland)

10 mL serological pipette; BD Falcon^{TM} (Becton Dickinson Labware, USA)

25 mL serological pipette (Corning Incorporated, USA)

Antiproliferative activity

Sulphorhodamine B, 75% (Sigma-Aldrich, USA)

Trichloroacetic acid, ≥ 99% (Sigma-Aldrich, USA)

Dimethyl sulphoxide, ≥ 99.9% (Sigma-Aldrich, USA)

Trizma base, ≥ 99.9% (Sigma-Aldrich, USA)

Glacial acetic acid (R&M Marketing, UK)

Antiplasmodial activity

Dimethyl sulphoxide, ≥ 99.9% (Sigma-Aldrich, USA)

Artemisinin, 98% (Sigma-Aldrich, USA)

Mefloquine hydrochloric, ≥ 98% (Sigma-Aldrich, USA)

Nitroblue tetrazolium (Sigma-Aldrich, USA)

Phenazine ethosulphate (Sigma-Aldrich, USA)

Hoechst nuclear staining assay

Hoechst 33342 powder (Sigma-Aldrich,USA)

Paraformaldehyde crystalline, reagent grade (Sigma-Aldrich, USA)

Gel Mount^{TM} Aqueous Mounting Medium (Sigma-Aldrich, USA)

35 x 10 mm dish (Corning Incorporated, USA)

Microscope slides 25.4 x 76.2 mm , 1–1.2 mm thick (Sail Brand, China)

Cover glass 22 x 22 mm

Caspase colorimetric assay

Quick-Start Bradford 1 x Dye Reagent (Bio-Rad Laboratories Inc., USA)

Caspase-3 /CPP32 colorimetric assay kit (BioVision Research, USA)

Caspase-6 colorimetric assay kit (BioVision Research, USA)

Caspase-8/FLICE colorimetric assay kit (BioVision Research, USA)

Caspase-9 colorimetric assay kit (BioVision Research, USA)

NATURAL PRODUCT RESEARCH

Solvents used in extraction for isolation

Hexane, industrial grade (HmbG Chemicals, Germany)

Dichloromethane, industrial grade (HmbG Chemicals, Germany)

Methanol, industrial grade (HmbG Chemicals, Germany)

Solvents used in isolation

Acetone, analytical grade (Merck KGaA, Germany)

Chloroform, analytical grade (Merck KGaA, Germany)

Ethanol, analytical grade (HmbG Chemicals, Germany)

Ethyl acetate, analytical grade (Merck KGaA, Germany)

Hexane, analytical grade (Fisher Scientific, UK)

Methanol, analytical grade (Merck KGaA, Germany)

Isolation

Chromatorex ODS (Fuji Silysia)

Diaion HP-20 (Supelco, USA)

LiChroprep® RP-18 (Merck KGaA, Germany)

MCI gel CHP 20P (Supelco, USA)

Silica gel 60 (Merck KGaA, Germany)

Sephadex LH-20 (Sigma, USA)

TLC silica gel 60 F254, aluminium sheets 20 x 20 cm (Merck KGaA, Germany)

90 mm qualitative filter paper; Advantec (Toyo Roshi Kaisha, Ltd., Japan)

150 mm qualitative filter paper; Whatman™ (GE Healthcare UK Ltd., UK)

185 mm qualitative filter paper; Whatman™ (GE Healthcare UK Ltd., UK)

HPLC

Cromolith SemiPrep RP18e 100-10 mm (Merck KGaA, Germany)

Acetonitrile HPLC grade (Fischer Scientific, USA)

Hexane C_6H_{14} (Fisher Scientific, USA)

Hydrochloric acid, >37% (Riedel-deHaen, USA)

Sodium diethyldithiocarbamate trihydrate (Fluka, USA)

Sodium hydroxide, 99% (BDH Analar, UK)

Methanol HPLC grade (Fischer Scientific, USA)

Trifluoroacetic acid (Sigma-Aldrich, USA)

Nylon membrane filter 0.45 µm, 47 mm diameter, Whatman™ (GE Healthcare UK Limited, UK)

Structure elucidation

Methanol-D4 deuteration degree ≥ 99.8% (Merck KGaA, Germany)

Chloroform-D1 deuteration degree ≥ 99.8% (Merck KGaA, Germany)

Dimethyl sulphoxide-D6 deuteration degree ≥ 99.8% (Merck KGaA, Germany)

Appendix II

EQUIPMENT AND APPARATUS

BIOASSAYS

BIO-Tek Power Wave XS Microplate Scanning Spectrophotometer

Digital Dry Bath AccuBlock™ (Labnet International Inc., USA)

Freeze-dryer (Christ Alpha 1-4, Salm en Kipp, the Netherlands)

Heating mantle (MTOPS, Model: MSE102)

Hot plate (Favorit H50707V2)

Incubator (TS606-G/2 WTW GmbH, Germany)

Microcomputer pH meter Model HI 8424 (Hana Instruments, Portugal)

Refrigerated microcentrifuge machine Model 5415R (Eppendorf , USA)

Vortex mixer (Labnet International Inc., USA)

Water bath (Memmert, Germany)

Weighing balance (A&D Measurement HR-200)

Weighing balance (Scaltec SPO62)

-86°C Ultra-Low Temperature Freezer (Sanyo Electric Co. Ltd., Japan)

-20°C Freezer (GS 5203 Liebherr Comfort, Liebherr-Hausgeräte GmbH, Germany)

NATURAL PRODUCT RESEARCH

Extraction

Heated ultrasonic bath (Model LC 130/H, Elam, Germany)

Orbital Shaker (Protech Model 719)

Rotary vacuum evaporator N-N series (Eyela Tolyo Rikakikai Co. Ltd., Japan)

Vacuum pump Aspirator A-35 (Eyela Tolyo Rikakikai Co. Ltd., Japan)

Vacuum pump DryFast® Model 2044 (Welch-llmac, USA)

Isolation

HPLC (Agilent Technologies 1200 Series, Germany)

C18 column, 4.6 x 150 mm, 5 µm (Agilent Eclipse XDB)

Phenyl column 100 x 4.6 mm, 5 µm (Thermo Scientific BDS Hypersil)

HPLC (Agilent Technologies 1200 series, Germany) with computer operating

ChemStation software, quartenary pump and UV-visible diode-array detector

HPLC (Shimadzu, Japan) with computer operating LC Solution software, quaternary pump, UV-visible diode-array detector and fraction collector

Vacuum manifold LiChrolut® (Merck KGaA, Germany)

TLC visualiser, UV 254 nm (CAMAG, Switzerland)

Money reader, UV 365 nm

Glassware used in isolation

10 x 370 mm glass column, socket 14/23 (Favorit, Malaysia)

20 x 450 mm glass column (Favorit, Malaysia)

450 x 530 mm glass column, socket 45/40 (Favorit, Malaysia)

16 x 125 mm glass column (Favorit, Malaysia)

25 x 150 mm test tubes (Duran GmbH, Germany)

Structure elucidation

Bruker DRX 300 MHz spectrometer (NMR)

Varian Unity Inova 500 MHz spectrometer (NMR)

Perkin Elmer Flexar SQ 300 mass spectrometer (ESI-MS)

Thermo Finnigan LCQ Deca spectrometer (APCI-MS)

Thermo Finnigan Trace GC-PolarisQ system (EI-MS)

Cell culture

CO_2 incubator , Thermo Scientific Heraeus® BB15 (Thermo Electron Corp., Germany)

Thermo Scientific Heraeus Megafuge 40 Centrifuge Series (Thermo Fisher Scientific Inc.)

Integra Pipetboy CH-7205 (INTEGRA Biosciences AG, Switzerland)

Antiproliferative activity

Multi-channel electronic pipette, 20–300 µL, 12 channel, Eppendorf Research® Pro 300 (Eppendorf AG, Germany)

Microplate spectrophotometer, Power Wave XS (BioTek Instruments Inc., USA)

Monitoring nuclear stained apoptotic cells

Fluorescent microscope (Olympus BX41, Japan)

Nikon DS-Fi1c camera (Nikon Instruments Inc., Japan)

Caspase colorimetric assay

Refrigerated microcentrifuge machine Model 5415R (Eppendorf, USA)

Multimode microplate reader, Tecan Infinite® 200 Pro (Tecan Trading AG, Switzerland)

Appendix III

HUMAN CANCER CELL LINES

Cell line: **HeLa**
Source: Cervix
Disease: Cervical carcinoma
Morphology: Epithelial
Growth properties: Adherent

Cell line: **MCF-7**
Source: Breast
Disease: Breast adenocarcinoma
Morphology: Epithelial
Growth properties: Adherent

Cell line: **HepG2**
Source: Liver
Disease: Hepatocellular carcinoma
Morphology: Epithelial
Growth properties: Adherent

Cell line: **MDA-MB-231**
Source: Breast
Disease: Breast adenocarcinoma
Morphology: Epithelial
Growth properties: Adherent

Cell line: **HT-29**
Source: Colon
Disease: Colorectal adenocarcinoma
Morphology: Epithelial
Growth properties: Adherent

Cell line: **SKOV-3**
Source: Ovary
Disease: Ovarian adenocarcinoma
Morphology: Epithelial
Growth properties: Adherent

Adapted from American Type Culture Collection (ATCC)

Appendix IV

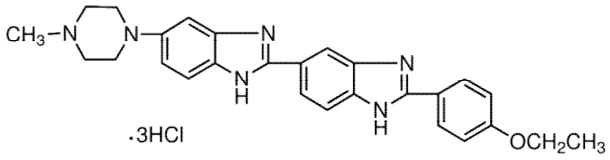
SIGMA HOECHST 33342 DYE

Product name: Bisbenzimide H 33342

Synonym: Hoechst 33342

Molecular formula: $C_{27}H_{28}N_6O \cdot 3HCl \cdot 3H_2O$

Molecular weight: 616.0



Product description

Bisbenzimide Hoechst 33342 is a specific stain for AT-rich regions of double-stranded DNA and has been shown to displace several known DNA intercalators. This fluorescent dye has been used in sorting living cells based on DNA content, used in flow cytometry for the determination of DNA content, and in visualising chromatin distribution in living cells. It has also been used in studying the initial stages apoptosis and cell cycle distribution. Chromosomes that are dividing or replicating will not stain with this dye.

Appendix V

BIOVISION COLORIMETRIC ASSAY KITS

Detection absorbance: 400 or 405 nm

Sample type: Cell and tissue lysates

Species reactivity: Mammalian

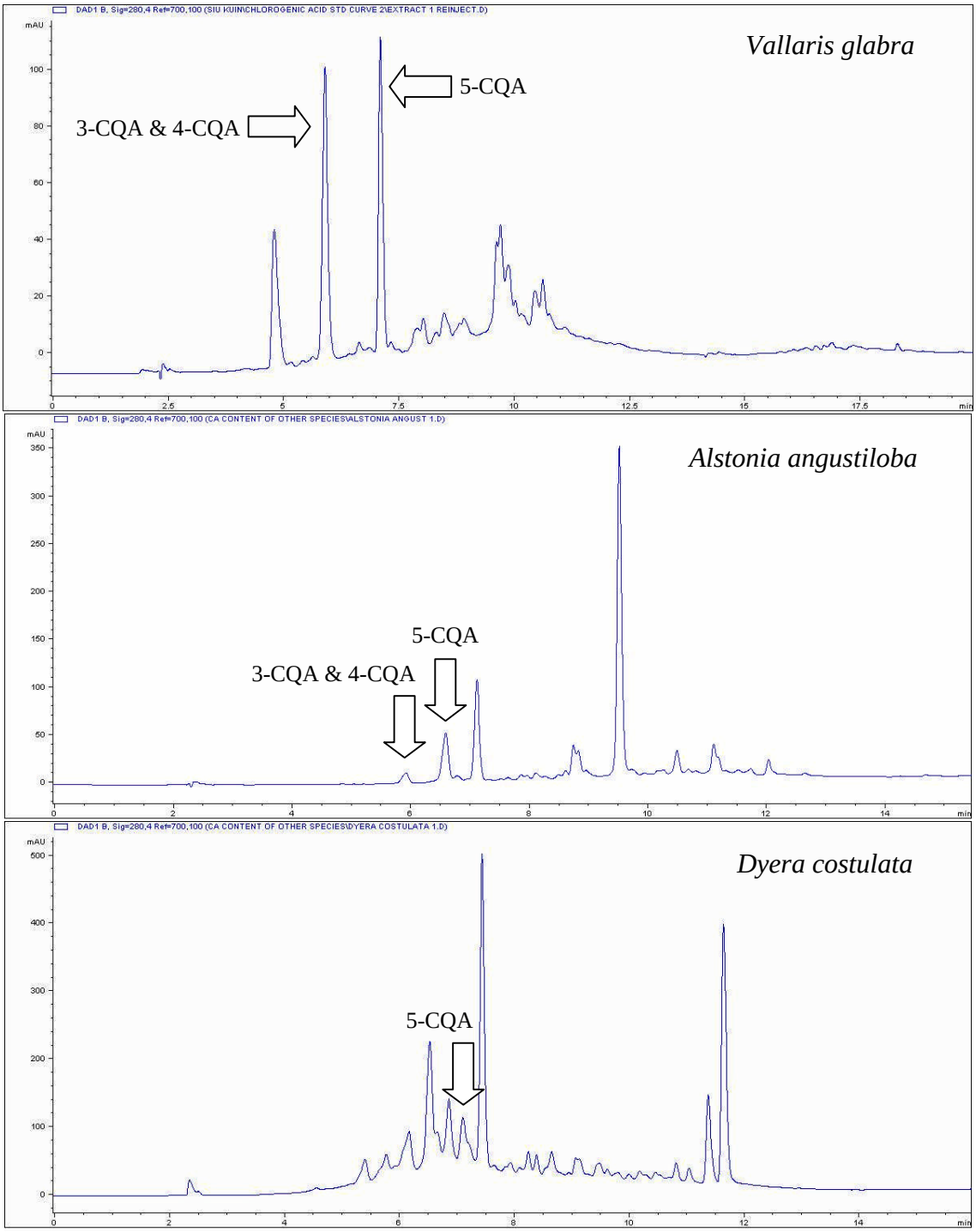
Applications

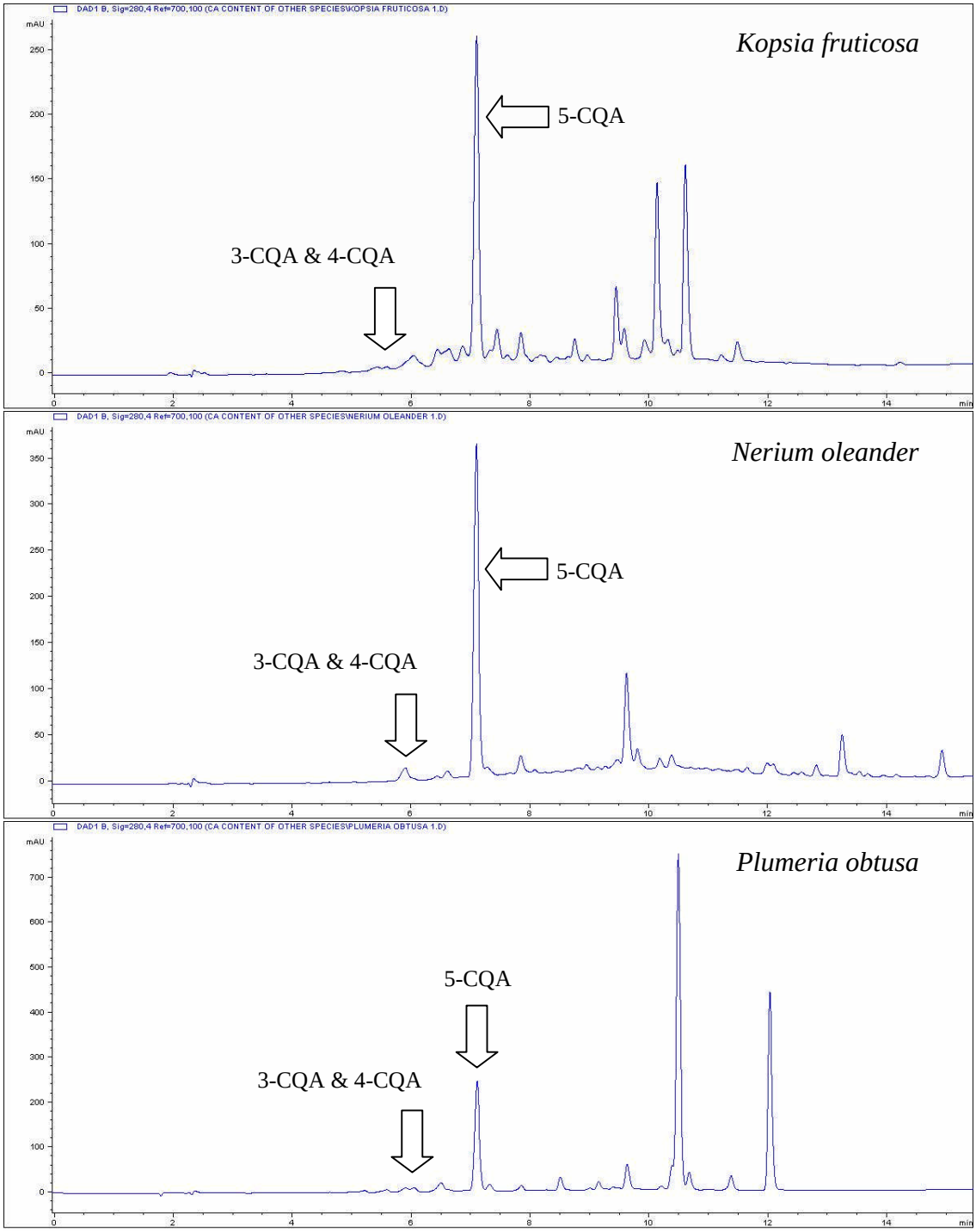
The kits detect early or middle stages of apoptosis. Comparison of the absorbance of p-nitroanilide (pNA) from an apoptotic sample with an untreated control allows determination of the fold-increase in caspase activity. They are based on spectrophotometric detection of pNA after cleavage from the labelled substrate.

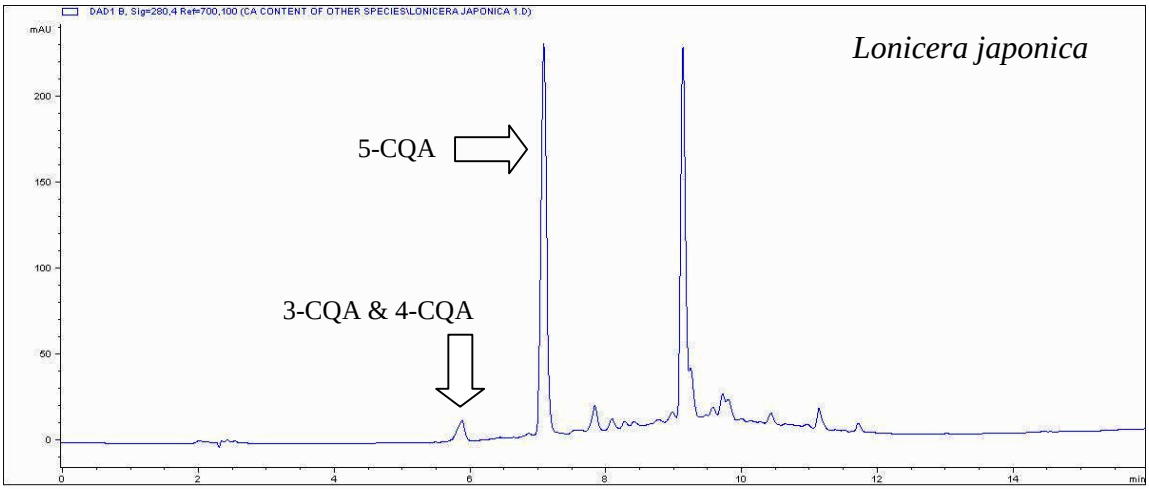
Caspase	Sequence recognition	Labelled substrate
3	DEVD (Asp-Glu-Val-Asp)	DEVD-pNA
6	VEID (Val-Glu-Ile-Asp)	VEID-pNA
8	IETD (Ile-Glu-Thr-Asp)	IETD-pNA
9	LEHD (Leu-Glu-His-Asp)	LEHD-pNA

Appendix VI

HPLC CHROMATOGRAMS OF CQA




DAD1 B, Sig=280,4 Ref=700,100 (CA CONTENT OF OTHER SPECIES\KOPSIA FRUTICOSA 1.D)
mAU
Kopsia fruticosa
5-CQA
3-CQA & 4-CQA
min
DAD1 B, Sig=280,4 Ref=700,100 (CA CONTENT OF OTHER SPECIES\NERIUM OLEANDER 1.D)
mAU
Nerium oleander
5-CQA
3-CQA & 4-CQA
min
DAD1 B, Sig=280,4 Ref=700,100 (CA CONTENT OF OTHER SPECIES\PLUMERIA OBTUSA 1.D)
mAU
Plumeria obtusa
5-CQA
3-CQA & 4-CQA
min



Monitored at 280 nm, 3-CQA and 4-CQA were eluted at 5.9 min
while 5-CQA was eluted at 7.1 min

RESEARCH ARTICLE Open Access

Assessment of antiproliferative and antiplasmodial activities of five selected Apocynaceae species

Siu Kuin Wong[1], Yau Yan Lim[1*], Noor Rain Abdullah[2], Fariza Juliana Nordin[2]

Indexed by PubMed

Abstract

Background: Studies have shown that the barks and roots of some Apocynaceae species have anticancer and antimalarial properties. In this study, leaf extracts of five selected species of Apocynaceae used in traditional medicine (*Alstonia angustiloba*, *Calotropis gigantea*, *Dyera costulata*, *Kopsia fruticosa* and *Vallaris glabra*) were assessed for antiproliferative (APF) and antiplasmodial (APM) activities, and analysed for total alkaloid content (TAC), total phenolic content (TPC) and radical-scavenging activity (RSA). As *V. glabra* leaf extracts showed wide spectrum APF and APM activities, they were further screened for saponins, tannins, cardenolides and terpenoids.

Methods: APF and APM activities were assessed using the sulphorhodamine B and lactate dehydrogenase assays, respectively. TAC, TPC and RSA were analysed using Dragendorff precipitation, Folin-Ciocalteu and DPPH assays, respectively. Screening for saponins, tannins, cardenolides and terpenoids were conducted using the frothing, ferric chloride, Kedde and vanillin-H_2SO_4 tests, respectively.

Results: Leaf extracts of *A. angustiloba*, *C. gigantea* and *V. glabra* displayed positive APF activity. Dichloromethane (DCM) extract of *C. gigantea*, and DCM and DCM:MeOH extracts of *V. glabra* showed strong APF activity against all six human cancer cell lines tested. DCM extract of *A. angustiloba* was effective against three cancer cell lines. Against MCF-7 and MDA-MB-231 cell lines, DCM extract of *C. gigantea* was stronger than standard drugs of xanthorrhizol, curcumin and tamoxifen. All five species were effective against K1 strain of *Plasmodium falciparum* and three species (*C. gigantea, D. costulata and K. fruticosa*) were effective against 3D7 strain. Against K1 strain, all four extracts of *V. glabra* displayed effective APM activity. Extracts of *D. costulata* were effective against 3D7 strain. Selectivity index values of extracts of *A. angustiloba*, *C. gigantea* and *V. glabra* suggested that they are potentially safe for use to treat malaria. Extracts of *K. fruticosa* had the highest TAC while *D. costulata* had the highest TPC and RSA. Phytochemical screening of extracts of *V. glabra* also showed the presence of terpenoids, tannins and saponins.

Conclusions: Leaf extracts of *C. gigantea* and *V. glabra* showed great promise as potential candidates for anticancer drugs as they inhibited the growth of all six cancer cell lines. Against K1 strain of *P. falciparum*, all four extracts of *V. glabra* displayed effective APM activity. The wide spectrum APF and APM activities of *V. glabra* are reported for the first time and this warrants further investigation into its bioactive compounds.

Background

The family Apocynaceae consists of about 250 genera and 2000 species of tropical trees, shrubs and vines [1,2]. A characteristic feature of the family is that almost all species produce milky sap. Leaves are simple, opposite and whorled. Flowers are large and colourful.

In traditional medicine, Apocynaceae species are used to treat gastrointestinal ailments, fever, malaria, pain and diabetes [2]. Of the five species studied, roots and leaves of *Calotropis gigantea* (L.) Aiton are used to treat skin and liver diseases, leprosy, dysentery, worms, ulcers, tumours and earaches [3]. Its latex has been reported to have wound healing properties [4]. *Kopsia fruticosa* (Ker.) A. DC. is used to treat sore and syphilis, and has cholinergic effects [5]. Leaves and barks of *Dyera costulata* Hook have been used for treating fever, inflammation and pain [6]. Stems, leaves and latex of *Alstonia*

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angustiloba Miq. have been used for gynaecological problems and skin sores in Indonesia [7]. Leaves are externally applied to treat headache in Malaysia [8]. *Vallaris glabra* Kuntze is well known in Thailand because the scent of its flowers is similar to that of pandan leaves and aromatic rice [9]. Its use in traditional medicine has not been reported, and its bioactivity and phytochemistry have yet to be studied. Species of Apocynaceae have also been reported to possess anticancer properties [2,10]. Species having cytotoxic activity include those of *Allamanda* [11], *Alstonia* [12,13], *Cerbera* [14,15], *Nerium* [16,17], *Plumeria* [18] and *Tabernaemontana* [19]. Species of Apocynaceae, notably those of *Alstonia*, are also known to have antimalarial properties [20-22].

Prompted by the anticancer and antimalarial properties of Apocynaceae, leaf extracts of five selected species used in traditional medicine were assessed for antiproliferative (APF) activity against six human cancer cell lines and for antiplasmodial (APM) activity against two strains of *Plasmodium falciparum*. Their extracts were also analysed for total alkaloid content, total phenolic content and radical-scavenging activity. Having wide spectrum APF and APM activities, leaf extracts of *V. glabra* were further screened for saponins, tannins, cardenolides and terpenoids. Information from the screening will serve as a useful guide to further work on isolating compounds with APF and APM activities.

Methods

Plant materials

Species studied were *A. angustiloba*, *C. gigantea*, *D. costulata*, *K. fruticosa* and *V. glabra.* Leaf samples of *A. angustiloba*, *C. gigantea* and *V. glabra* were collected from Puchong (3°2'42"N; 101°37'12"E), Sunway (3°4'30"N; 101°36'8" E) and Kepong (3°12'14"N; 101°37'50"E) in Selangor, Malaysia, respectively. Those of *D. costulata* and *K. fruticosa* were collected from the Forest Research Institute Malaysia (3°14'6"N; 101°37'58"E). Identification of species was based on documented descriptions and illustrations [1,2]. With brief descriptions of their morphology and location of collection, the voucher specimens of these species (WSK01, WSK02, WSK03, WSK04 and WSK05, respectively) were deposited in the herbarium of Monash University Sunway Campus in Malaysia.

Extraction of leaves

For crude extraction, fresh leaves of each species (40 g) were cut into small pieces and freeze-dried overnight. Dried samples were blended and extracted with 250 ml of methanol (MeOH) three times for 1 h each time. Samples were filtered and the solvent was removed using a rotary evaporator (Eyela). The dried crude extracts were stored at -20°C for further analysis. For sequential extraction, fresh leaves of each species (40 g) were freeze-dried, ground and extracted successively with hexane (HEX), dichloromethane (DCM), DCM: MeOH (1:1) and MeOH (HmbG Chemicals). For each solvent, the suspension of ground leaves in 250-300 ml of solvent was shaken for 1 h on the orbital shaker. After filtering, the samples were extracted two more times for each solvent. Solvents were removed with a rotary evaporator to obtain the dried extracts, which were stored at -20°C for further analysis.

Antiproliferative activity

Antiproliferative (APF) activity of extracts (25 μg/ml) was initially screened for growth inhibitory activity against three human cancer cell lines (MCF-7, MDA-MB-231 and HeLa) using the sulphorhodamine B (SRB) assay [23]. Growth inhibitory activity with less than 50% cell growth was considered positive while that with more than 50% cell growth was considered negative. Extracts with positive growth inhibition were further tested against six human cancer lines (MCF-7, MDA-MB-231, HeLa, HT-29, SKOV-3 and HepG2) using six different extract concentrations. Human cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were seeded 24 h prior to treatment in 96-well plates at densities of 10,000-20,000 cells/well. Each cell line was designated one plate. Initial cell population of each cell lines prior to addition of extracts was determined by fixing with trichloroacetic acid (TCA) (Sigma). Extracts were dissolved in dimethyl sulphoxide (DMSO) (Sigma) and serially diluted from 8-25 μg/ml. Control cultures were treated with the same volume of DMSO. The concentration of DMSO was kept within 1% to avoid any interference with cell viability. After the addition of extracts, the plates were incubated for 48 h. After incubation, the cells were fixed with 50 μl of cold 50% TCA and incubated for 1 h at 4°C. The plates were then washed with tap water and air dried. Cells were stained with 100 μl of 0.4% SRB solution (Sigma) diluted with 1% acetic acid followed by incubation for 10 min at room temperature. Unbound dye was removed by washing with 1% acetic acid. Bound stain was then solubilised with 200 μl of 10 mM trizma base (Sigma). Absorbance of each well at 505 nm was obtained using a microplate reader. Dose-response curves were constructed to obtain GI_{50} or concentration of extract that causes growth inhibition (GI) of cells by 50%. GI_{50} was calculated using the formula $[(T_z - T_i)/(T_c - T_i)] \times 100 = 50$ where T_z is the absorbance of cells treated with extracts or drugs at the end of incubation, T_i is the absorbance of cells prior to treatment with extracts or drugs and T_c is the absorbance of untreated cells at the end of incubation [24]. IC_{50} or inhibition concentration

at which there is a 50% reduction in cells was obtained using the formula $T_z/T_c \times 100 = 50$. Activity is considered to be effective when GI_{50} value ≤20 μg/ml [25].

Antiplasmodial activity

Antiplasmodial (APM) activity of extracts was assessed *in vitro* in human blood using the lactate dehydrogenase assay with slight modifications [26]. Chloroquine-resistant K1 and chloroquine-sensitive 3D7 strains of *Plasmodium falciparum* were tested. Standard drugs of artemisinin (Sigma) and mefloquine (Sigma) were used as positive controls. Extracts dissolved in DMSO (10 mg/ml) were diluted with deionised water to 320 μg/ml. The solution was serially diluted two-fold six times to give seven different concentrations. Aliquots of each concentration (10 μl) were transferred into 96-well microtiter plates. Parasitised red blood cell suspensions (1% parasitaemia, 190 μl) were added to each well. Parasitised and non-parasitised red blood cells were used as negative controls. The plates were incubated for 24 h at 37°C in a candle jar and were subsequently chilled at -20°C to lyse the red blood cells. The plates were then allowed to cool to room temperature, and 20 μl of blood suspension was dispensed into a new microtiter plate containing 100 μl MALSTAT™ reagent (Flow Inc.), and 20 μl of nitroblue tetrazolium (Sigma) and phenazine ethosulphate (Sigma) mixture. Absorbance was measured with a plate reader at 630 nm. Percentage inhibition at each concentration was determined and the mean of EC_{50} values of parasite viability was calculated using probit analysis. EC_{50} or effective concentration is the extract concentration that kills 50% of malaria parasites. Activity is effective if EC_{50} value ≤10 μg/ml [27]. The selectivity index (SI) for APM activity was calculated based on the ratio of cytotoxicity (IC_{50}) on HepG2 and MCF-7 cells to APM activity (EC_{50}) on chloroquine-resistant K1 strain.

Analysis of TAC, TPC and RSA

Total alkaloid content

Total alkaloid content (TAC) of extracts was determined using the Dragendorff precipitation assay [28]. For each species, extracts (15 mg) were dissolved in 1 ml of distilled water that was acidified to pH 2.0-2.5 with 0.01 M HCl. Analysis was conducted in triplicate. Alkaloids were then precipitated with 0.4 ml of Dragendorff reagent. After washing with 0.5 ml of distilled water to remove traces of the reagent, the precipitate was later treated with 0.4 ml of 1% sodium sulphide, resulting in a brownish-black precipitate. Precipitates formed at each stage were recovered by centrifugation at 14,000 rpm for 1 min. The resulting precipitate was dissolved in 0.2 ml of concentrated nitric acid and diluted to 1 ml with distilled water. Addition of 2.5 ml of 3% thiourea to 0.5 ml aliquots of this solution resulted in a yellow colored complex. Absorbance was measured at 435 nm and TAC was expressed as boldine equivalent in milligram per gram of extract. The calibration equation for boldine (Sigma) was $y = 1.068x$ ($R^2 = 0.9959$) where y is absorbance and × is mg/ml of boldine. Dragendorff reagent was prepared by dissolving 0.8 g of bismuth nitrate (Sigma) in 40 ml of distilled water and 10 ml of glacial acetic acid. The resulting solution was mixed with 20 ml of 40% potassium iodide.

Total phenolic content

Total phenolic content (TPC) of extracts was determined using the Folin-Ciocalteu (FC) assay [29-31]. Extracts (300 μl in triplicate) were introduced into test tubes followed by 1.5 ml of FC reagent (Fluka) at 10 times dilution and 1.2 ml of sodium carbonate (Fluka) at 7.5% w/v. The tubes were allowed to stand for 30 min in the dark before absorbance was measured at 765 nm. TPC was expressed as gallic acid (GA) equivalent in milligram per gram of extract. The calibration equation for GA (Fluka) was $y = 0.0111x - 0.0148$ ($R^2 = 0.9998$) where y is absorbance and × is mg/ml of GA.

Radical-scavenging activity

Radical-scavenging activity (RSA) of extracts was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay [29-31]. Different dilutions of extracts (1 ml in triplicate) were added to 2 ml of DPPH (Sigma). The concentration of DPPH used was 5.9 mg in 100 ml of methanol. Absorbance was measured at 517 nm after 30 min. RSA was calculated as IC_{50}, the concentration of extract to scavenge 50% of the DPPH radical. RSA was then expressed as ascorbic acid equivalent antioxidant capacity (AEAC) using the equation: AEAC (mg ascorbic acid/g) = $IC_{50(ascorbate)}/IC_{50(sample)} \times 10^5$. IC_{50} of ascorbic acid used for calculation of AEAC was 0.00387 mg/ml.

Phytochemical screening

Qualitative phytochemical screening for saponins, tannins, cardenolides and terpenoids from leaf extracts of *V. glabra* was carried out using standard phytochemical procedures [32,33]. The frothing test was used for saponins, the ferric chloride test for tannins, the Kedde test for cardenolides and the vanillin-H_2SO_4 test for terpenoids.

Results and Discussion

APF activity

Initial screening of leaf extracts of five Apocynaceae species against three human cancer cell lines (MCF-7, MDA-MB-231 and HeLa) showed that DCM, DCM:MeOH and MeOH extracts of *A. angustiloba*, *C. gigantea* and *V. glabra* had growth inhibitory activity (data not shown). DCM and DCM:MeOH extracts of

V. glabra, and DCM extract of *C. gigantea* inhibited all three cancer cell lines. HEX and MeOH extracts of all three species did not show inhibition, with the exception of MeOH extract of *V. glabra*. Extracts of *D. costulata* and *K. fruticosa* did not show any APF activity.

Extracts of the three species were further tested against six human cancer cell lines (MCF-7, MDA-MB-231, HeLa, HT-29, SKOV-3 and HepG2). Results showed that DCM extract of *A. angustiloba* inhibited only MDA-MB-231, HeLa and SKOV-3 cell lines with GI_{50} values of 20, 20 and 16 μg/ml, respectively (Table 1). DCM and DCM:MeOH extracts of *C. gigantea* inhibited all cancer cell lines except for DCM:MEOH extract against MDA-MB-231. APF activity of DCM extract of *C. gigantea* was the strongest with GI_{50} values ranging from 1.3 to 3.3 μg/ml. Against MCF-7 and MDA-MB-231, GI_{50} of DCM extract of *C. gigantea* (1.9 and 1.3 μg/ml) was stronger than that of xanthorrhizol (11 and 8.7 μg/ml), curcumin (4.1 and 8.7 μg/ml) and tamoxifen (8.3 and 4.6 μg/ml), respectively. DCM and DCM:MeOH extracts of *V. glabra* inhibited all cell lines with GI_{50} values ranging from 7.5-12 μg/ml and 5.8-13 μg/ml, respectively. In addition, MeOH extract of *V. glabra* also inhibited the growth of MCF-7 and HepG2. Against MCF-7, GI_{50} of DCM and DCM:MeOH extracts of *V. glabra* (7.7 and 7.0 μg/ml) was stronger than xanthorrhizol (11 μg/ml) and comparable to tamoxifen (8.3 μg/ml), respectively.

To the best of our knowledge, this study represents the first report of cytotoxic activity from DCM leaf extract of *A. angustiloba* and DCM and DCM:MeOH extracts of *V. glabra*. Earlier studies have reported cytotoxic activity from the root bark of *Alstonia macrophylla* Wall. ex G. Don [12] and the stem bark of *Alstonia scholaris* R. Br. [13]. A recent study on *Vallaris solanacea* (Roth) Kuntz has reported potent cell growth inhibitory activity of cardenolide glycosides isolated from the plant [34]. The finding of strong APF activity from DCM and DCM:MeOH extracts of *C. gigantea* from this study is supported by an earlier report that DCM leaf extracts of *C. gigantea* had strong inhibitory activity against KB, BC and NCI-H187 cancer cell lines [35]. Ethanol root extracts of *C. gigantea* were also reported to be cytotoxic to K562 and SGC-7901 human cell lines [36].

APM activity

Against chloroquine-resistant K1 strain of *P. falciparum*, leaves of *V. glabra* were most effective as all four extracts had APM activity with EC_{50} less than 10 μg/ml (Table 2). DCM extract was the strongest with EC_{50} of 0.85 μg/ml. Three extracts of *A. angustiloba*, and two extracts of *C. gigantea*, *D. costulata* and *K. fruticosa* showed APM activity. It should be noted that DCM:MeOH extracts of all five species displayed APM activity with *A. angustiloba* having the strongest activity (EC_{50} of 0.46 μg/ml).

Against chloroquine-sensitive 3D7 strain of *P. falciparum*, extracts of *A. angustiloba* and *V. glabra* showed

Table 1 Antiproliferative activity of leaf extracts of three Apocynaceae species against six human cancer cell lines[a]

Species	[c]Leaf extract	[b]GI_{50} (μg/ml)					
		MCF-7	MDA-MB-231	HeLa	HT-29	SKOV-3	HepG2
Alstonia angustiloba	HEX	-	-	-	-	-	-
	DCM	-	20 ± 1.7	20 ± 1.1	-	16 ± 1.4	-
	DCM:MeOH	-	-	-	-	-	-
	MeOH	-	-	-	-	-	-
Calotropis gigantea	HEX	-	-	-	-	-	-
	DCM	1.9 ± 0.2	1.3 ± 0.3	2.5 ± 0.5	3.3 ± 0.2	2.5 ± 0.2	1.8 ± 1.7
	DCM:MeOH	13 ± 0.3	-	15 ± 1.0	24 ± 0.7	20 ± 2.3	16 ± 3.5
	MeOH	-	-	-	-	-	-
Vallaris glabra	HEX	-	-	-	-	-	-
	DCM	7.7 ± 1.3	12 ± 2.0	9.8 ± 1.5	9.3 ± 2.0	7.5 ± 4.5	7.6 ± 0.2
	DCM:MeOH	7.0 ± 2.5	13 ± 6.3	8.5 ± 2.9	12 ± 1.2	7.7 ± 2.4	5.8 ± 1.2
	MeOH	16 ± 2.1	-	-	-	-	19 ± 0.9
[d]**Standard drug**							
Xanthorrhizol		11 ± 0.7	8.7 ± 0.8				
Curcumin		4.1 ± 0.9	8.7 ± 0.8				
Tamoxifen		8.3 ± 0.6	4.6 ± 0.5				

[a] Initial screening showed that extracts of *Dyera costulata* and *Kopsia fruticosa* did not show any APF activity.

[b] GI_{50} (μg/ml) is growth inhibition (GI) of cancer cell lines by 50%. Inhibition is not effective (-) with values >20 μg/ml. MCF-7 and MDA-MB-231, HeLa and SKOV-3, HT-29, and HepG2 are human breast, cervical, colon and liver cancer cell lines, respectively.

[c] HEX, hexane; DCM, dichloromethane; MeOH, methanol.

[d] Data on standard drugs of xanthorrhizol, curcumin and tamoxifen against MCF-7 and MDA-MD-231 cell lines are from an earlier publication [24].

Table 2 Antiplasmodial (APM) activity and selectivity index (SI) of leaf extracts of Apocynaceae species

Species	[c]Leaf extract	[a]EC_{50} (μg/ml) of APM activity		[b]IC_{50} (μg/ml) of APF activity		[b]SI of APM activity	
		K1	3D7	HepG2	MCF-7	HepG2	MCF-7
Alstonia angustiloba	HEX	7.81	-				
	DCM	-	-				
	DCM:MeOH	0.46	-	>25.0	>25.0	NC	NC
	MeOH	6.46	-				
Calotropis gigantea	HEX	5.82	-				
	DCM	-	-				
	DCM:MeOH	0.97	3.29	>25.0	16.8	NC	17.3
	MeOH	-	-				
Dyera costulata	HEX	-	-				
	DCM	-	8.31				
	DCM:MeOH	7.52	2.13				
	MeOH	7.74	3.56				
Kopsia fruticosa	HEX	-	-				
	DCM	-	7.14				
	DCM:MeOH	4.35	-				
	MeOH	1.01	-				
Vallaris glabra	HEX	1.00	-	>25.0	>25.0	NC	NC
	DCM	0.85	-	>25.0	12.0	NC	14.1
	DCM:MeOH	8.45	-				
	MeOH	8.42	-				
[d]**Standard drug**							
Artemisinin		0.001	0.001				
Mefloquine		0.008	0.018				

[a] EC_{50} or effective concentration (μg/ml) is the extract concentration that kills 50% of malaria parasites. Activity is not effective (-) if EC_{50} value >10 μg/ml. K1 and 3D7 are chloroquine-resistant and chloroquine-sensitive strains of *Plasmodium falciparum*, respectively.

[b] IC_{50} of antiproliferative (APF) activity and SI of APM activity against HepG2 and MCF-7 cells were calculated only for the most potent extracts against K1 strain with EC_{50} ≤1.00 μg/ml. NC, non-cytotoxic with SI values >25.0.

[c] HEX, hexane; MeOH, methanol; DCM, dichloromethane.

[d] Standard drugs of artemisinin and mefloquine are used as positive controls.

no activity. Extracts of *D. costulata* were the exception in that DCM, DCM:MeOH and MeOH extracts showed positive APM activity with EC_{50} of 8.31, 2.13 and 3.56 μg/ml, respectively. Generally, extracts were less effective against 3D7 strain.

This finding complements an earlier report that alkaloids from the extracts of *Alstonia* species were effective against chloroquine-resistant K1 strain but not against chloroquine-sensitive T9-96 strain [22]. Previous studies on the APM activity of Apocynaceae were focused on *Alstonia* species [21,22]. This study is the first to report the APM activity of leaf extracts of *D. costulata*, *C. gigantea*, *K. fruticosa* and *V. glabra*. A notable finding is the APM activity of *V. glabra* against K1 strain in all solvent fractions.

Selectivity index

The selectivity index (SI) for APM activity indicates the safety of an extract to be used for antimalarial therapy [37]. The index was calculated based on the ratio of cytotoxicity (IC_{50}) on HepG2 and MCF-7 cells to APM activity (EC_{50}). The SI was calculated for the most potent extracts against K1 strain of *P. falciparum* with EC_{50} ≤1.00 μg/ml. They were DCM:MeOH extracts of *A. angustiloba* (0.46 μg/ml) and *C. gigantea* (0.97 μg/ml), and HEX and DCM extracts of *V. glabra* (1.00 and 0.85 μg/ml), respectively (Table 2). Against HepG2 cells, IC_{50} values were all >25.0 μg/ml, and against MCF-7 cells, IC_{50} values were >25.0, 16.8, >25.0 and 12.0 μg/ml, respectively.

Against HepG2 cells, HEX and DCM extracts of *V. glabra*, and DCM:MeOH extracts of *A. angustiloba* and *C. gigantea* were non-cytotoxic with SI values >25.0 (Table 2). Against MCF-7 cells, the DCM extract of *V. glabra* and the DCM:MeOH extract of *C. gigantea* had SI values of 14.1 and 17.3, respectively. The HEX extract of *V. glabra* and the DCM:MeOH extract of *A. angustiloba* were non-cytotoxic with SI values >25.0.

Recently, a study on the antimalarial and cytotoxic activity of plants in the Democratic Republic of Congo considered SI values of extracts >10 as high [38]. With SI values relatively higher than 10, the extracts of *A. angustiloba*,

C. gigantea and *V. glabra* are potentially safe for use to treat malaria.

Analysis of TAC, TPC and RSA

Of the five species analysed, MeOH crude and DCM extracts of *K. fruticosa* had the highest TAC (100 and 129 mg BE/g of extract), respectively (Table 3). Other species with high TAC were *D. costulata* and *A. angustiloba* with MeOH crude and DCM:MeOH extracts having values in the range 58-68 and 23-58 mg BE/g of extract, respectively. Based on TAC, the ranking of species was: *K. fruticosa* >*D. costulata* >*A. angustiloba* >*C. gigantea* ≈ *V. glabra*. Extracts of *D. costulata* had the highest TPC and strongest RSA. MeOH crude, DCM:MeOH and MeOH extracts yielded TPC values of 319, 354 and 279 mg GAE/g of extract, and RSA values of 377, 349 and 278 mg AA/g of extract, respectively. Based on TPC and RSA, the ranking of species was: *D. costulata* >*V. glabra* >*K. fruticosa* >*A. angustiloba* >*C. gigantea*. There is a strong correlation between TPC and RSA of extracts (R^2 = 0.955) but not with TAC (R^2 = 0.112). Correlation of results of phytochemical analysis with APF and APM activities remains unclear. Extracts of *C. gigantea* and *V. glabra* showed strong APF activity. The former had low TAC and TPC, while the latter had low TAC but high TPC. Extracts of *V. glabra* and *D. costulata* were effective against K1 and 3D7 strains of *P. falciparum*, respectively. The former had low TAC but high TPC, while the latter had high TAC and TPC.

Phytochemical screening

Phytochemical screening showed the presence of terpenoids in all leaf extracts of *V. glabra* except MeOH extract while saponins and tannins were present in DCM and DCM:MeOH extracts, and in DCM:MeOH and MeOH extracts, respectively (Table 4). Cardenolides were not detected.

Terpenoids are used for the treatment of human diseases such as cancer and malaria, and infectious diseases caused by virus and bacterial [39]. Taxol and artimesinin are renowned terpenoid-based anticancer and antimalarial drugs, respectively. Terpenoids inhibit the growth or induce apoptosis of breast cancer cells such as MCF-7, MDA-MB-231 and T47D [40]. They are among the most important natural antimalarial drugs, which also include quinones and alkaloids [41].

Tannins are water-soluble polyphenols that are present in many plant foods [42]. Literature on the effects of tannins on human health is vast and sometimes conflicting. Incidences of esophageal cancer have been attributed to consumption of tannin-rich foods such as herbal teas, suggesting that tannins might be carcinogenic. However, reports have indicated a negative association between consumption of tea and the incidence of cancer. Teas with high tannin content have been suggested to be anticarcinogenic

Table 3 Phytochemical analysis of total alkaloid content, total phenolic content and radical-scavenging activity of leaf extracts of Apocynaceae species

Species	MeOH crude extract	[a]Sequential extract			
		HEX	DCM	DCM:MeOH	MeOH
[b]**Total alkaloid content (mg BE/g)**					
Kopsia fruticosa	100 ± 4.2	63 ± 1.1	129 ± 4.0	99 ± 2.5	46 ± 1.6
Dyera costulata	58 ± 2.5	2.4 ± 0.6	11 ± 1.8	68 ± 2.7	41 ± 2.4
Alstonia angustiloba	23 ± 0.3	13 ± 2.3	27 ± 3.1	58 ± 1.2	27 ± 1.0
Calotropis gigantea	2.7 ± 0.3	3.7 ± 1.0	8.6 ± 1.2	9.6 ± 1.9	9.2 ± 2.7
Vallaris glabra	2.7 ± 0.4	4.4 ± 0.8	8.9 ± 0.6	9.2 ± 0.2	8.7 ± 2.2
[b]**Total phenolic content (mg GAE/g)**					
Dyera costulata	319 ± 5.5	21 ± 0.2	23 ± 0.1	354 ± 6.0	279 ± 3.0
Vallaris glabra	99 ± 3.8	15 ± 0.9	24 ± 0.5	134 ± 1.0	164 ± 13
Kopsia fruticosa	83 ± 1.1	39 ± 0.5	20 ± 0.5	129 ± 1.0	84 ± 1.0
Alstonia angustiloba	68 ± 2.0	17 ± 0.6	24 ± 0.6	96 ± 1.1	94 ± 1.1
Calotropis gigantea	28 ± 0.8	14 ± 0.6	44 ± 1.7	42 ± 0.8	33 ± 0.5
[b]**Radical-scavenging activity (mg AA/g)**					
Dyera costulata	377 ± 25	15 ± 0.3	9.3 ± 0.5	349 ± 21	278 ± 5.4
Vallaris glabra	84 ± 0.5	6.0 ± 0.3	8.4 ± 0.4	77 ± 2.2	119 ± 6.8
Kopsia fruticosa	63 ± 3.2	12 ± 1.4	7.5 ± 0.3	70 ± 2.0	48 ± 1.5
Alstonia angustiloba	29 ± 0.9	10 ± 0.2	5.7 ± 0.7	50 ± 1.2	46 ± 2.2
Calotropis gigantea	7.6 ± 0.4	5.6 ± 0.3	6.1 ± 0.4	8.0 ± 0.6	14 ± 1.3

[a] HEX, hexane; DCM, dichloromethane; MeOH, methanol.

[b] BE, boldine equivalent; GAE, gallic acid equivalent; AA, ascorbic acid. Values are in milligram per gram of extract.

Table 4 Phytochemical screening of leaf extracts of *Vallaris glabra*

[a]Leaf extract	[b]Qualitative test			
	[c]Saponin	[c]Tannin	[c]Cardenolide	[c]Terpenoid
HEX	-	-	-	+++
DCM	+	-	-	++
DCM:MeOH	++	++	-	++
MeOH	-	++	-	-

[a] HEX, hexane; DCM, dichloromethane; MeOH, methanol.

[b] Strong (+++), moderate (++) and weak (+) presence, and absent (-). Classification was based on observation of colour intensity and amount of precipitate.

[c] Saponins, tannins, cardenolides and terpenoids were screened using the frothing, ferric chloride, Kedde and vanillin-H_2SO_4 tests.

and antimutagenic which may be related to their antioxidative property in protecting cellular oxidative damage against lipid peroxidaton and superoxide radicals. The antimicrobial activities of tannins are well documented.

Saponins are naturally occurring glycosides with a distinctive foaming characteristic and bitter taste [43,44]. They have a wide range of properties, which include both beneficial and detrimental effects on human health. Saponins affect the immune system in ways that help to protect the human body against cancers, and also lower cholesterol levels. They decrease blood lipids, lower cancer risks and lower blood glucose response.

Conclusions

The DCM extract of *C. gigantea*, and DCM and DCM: MeOH extracts of *V. glabra* inhibited the growth of all six human cancer cell lines. Against MCF-7 and MDA-MB-231 human cell lines, DCM leaf extract of *C. gigantea* had stronger APF activity than standard drugs of xanthorrhizol, curcumin and tamoxifen. With wide spectrum APF activity, leaves of these two species are therefore promising candidates as alternative resources for anticancer drugs. Against K1 strain of *P. falciparum*, all four extracts of *V. glabra* displayed effective APM activity. Selectivity index values suggested that extracts of *V. glabra* are potentially safe for use to treat malaria. The wide spectrum APF and APM activities of *V. glabra* are reported for the first time. This warrants further investigation into its bioactive compounds.

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Authors' contributions

The study was conducted by SKW as part of her PhD program in Monash University Sunway Campus in Malaysia which is supervised by YYL. Part of the experiments was done in collaboration with Noor Rain and Fariza Juliana from the Institute of Medical Research. SKW analysed the data and drafted the manuscript which was edited and revised by YYL, with comments from counterparts. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Antiproliferative and phytochemical analyses of leaf extracts of ten Apocynaceae species

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ABSTRACT

Background: The anticancer properties of Apocynaceae species are well known in barks and roots but less so in leaves. **Materials and Methods:** In this study, leaf extracts of 10 Apocynaceae species were assessed for antiproliferative (APF) activities using the sulforhodamine B assay. Their extracts were also analyzed for total alkaloid content (TAC), total phenolic content (TPC), and radical scavenging activity (RSA) using the Dragendorff precipitation, Folin–Ciocalteu, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) assays, respectively. **Results:** Leaf extracts of *Alstonia angustiloba*, *Calotropis gigantea*, *Catharanthus roseus*, *Nerium oleander*, *Plumeria obtusa*, and *Vallaris glabra* displayed positive APF activities. Extracts of *Allamanda cathartica*, *Cerbera odollam*, *Dyera costulata*, and *Kopsia fruticosa* did not show any APF activity. Dichloromethane (DCM) extract of *C. gigantea*, and DCM and DCM:MeOH extracts of *V. glabra* showed strong APF activities against all six human cancer cell lines. Against breast cancer cells of MCF-7 and MDA-MB-231, DCM extracts of *C. gigantea* and *N. oleander* were stronger than or comparable to standard drugs of xanthorrhizol, curcumin, and tamoxifen. All four extracts of *N. oleander* were effective against MCF-7 cells. Extracts of *Kopsia fruticosa* had the highest TAC while those of *Dyera costulata* had the highest TPC and RSA. Extracts of *C. gigantea* and *V. glabra* inhibited the growth of all six cancer cell lines while all extracts of *N. oleander* were effective against MCF-7 cells. **Conclusion:** Extracts of *C. gigantea*, *V. glabra*, and *N. oleander* therefore showed great promise as potential candidates for anticancer drugs. The wide-spectrum APF activities of these three species are reported for the first time and their bioactive compounds warrant further investigation.

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INTRODUCTION

The family Apocynaceae consists of about 250 genera and 2000 species of tropical trees, shrubs, and vines.[1] With the inclusion of species of Asclepiadaceae, the family has now been enlarged from two to five subfamilies.[2] Characteristic features of the family are that almost all species produce milky sap; leaves are simple, opposite, or whorled; flowers are large, colorful, and slightly fragrant with five contorted lobes; and fruits are in pairs.[1,3]

In traditional medicine, Apocynaceae species are used to treat gastrointestinal ailments, fever, malaria, pain, and diabetes.[1] Of the 10 species studied, leaves of *Allamanda cathartica* are used as a purgative or emetic in Southeast Asia.[4] Leaves are also used as an antidote, and for relieving coughs and headaches. Stems, leaves, and latex of *Alstonia angustiloba* are used for gynecological problems and skin sores in Indonesia.[5] Leaves are externally applied to treat headache in Malaysia.[6] Roots and leaves of *Calotropis gigantea* are used to treat skin and liver diseases, leprosy, dysentery, worms, ulcers, tumours, and earache.[7] Its latex has wound-healing properties.[8] A decoction of all parts of *Catharanthus roseus* is used to treat malaria, diarrhea, diabetes, cancer, and skin diseases.[9] The species is also well known as an oral hypoglycemic agent. Extracts prepared from leaves have been used as an antiseptic agent for healing wounds and as a mouthwash to treat toothache. In Southeast Asia, leaves of *Cerbera odollam* are used in aromatic bath by women after childbirth.[10] Leaves, bark and latex are emetic and purgative, and seeds are toxic and

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strongly purgative. Leaves and bark of *Dyera costulata* have been used for treating fever, inflammation, and pain.[11] *Kopsia fruticosa* has cholinergic effects and is used to treat sore and syphilis.[12] *Nerium oleander* is highly poisonous with no reported benefits in traditional medicine. In Asia, a decoction of leaves of *Plumeria obtusa* is used for treating wounds and skin diseases.[13] Its latex and bark are known to have purgative and diuretic properties. *Vallaris glabra* is well known in Thailand because the scent of its flowers is similar to that of pandan leaves and aromatic rice.[14] Its use in traditional medicine is not known.

Apocynaceae species have been reported to possess anticancer properties. They include those of *Allamanda*,[15] *Alstonia*,[16,17] *Calotropis*,[18-20] *Catharanthus*,[21] *Cerbera*,[22,23] *Nerium*,[24,25] *Plumeria*,[26] and *Tabernaemontana*.[27]

Prompted by the anticancer properties found in many species of Apocynaceae, leaf extracts of 10 species were assessed for antiproliferative activity against six human cancer cell lines. Their extracts were also analyzed for total alkaloid content, total phenolic content, and radical scavenging activity.

MATERIALS AND METHODS

Plant materials

The 10 Apocynaceae species studied were *A. cathartica*, *Alstonia angustiloba*, *Calotropis gigantea*, *Catharanthus roseus*, *Cerbera odollam*, *Dyera costulata*, *Kopsia fruticosa*, *Nerium oleander*, *Plumeria obtusa*, and *Vallaris glabra* [Figure 1]. Their common or vernacular names and brief descriptions are given in [Table 1]. Leaves of the species were collected from Sunway, Puchong, or Kepong, all in the state of Selangor, Malaysia. Identification of species was based on documented descriptions and illustrations.[1,3] The voucher specimens of these species were deposited in the herbarium of Monash University Sunway Campus.

Extraction of leaves

For crude extraction, fresh leaves of each species (40 g) were cut into small pieces and freeze-dried overnight. Dried samples were blended and extracted with 250 ml of methanol (MeOH) three times for 1 h each time. Samples were filtered and the solvent was removed using a rotary evaporator (Eyela). The dried crude extracts were stored at –20°C for further analysis. For sequential extraction, fresh leaves of each species (40 g) were freeze-dried, ground, and extracted successively with hexane, dichloromethane (DCM), DCM:MeOH (1:1), and MeOH (HmbG Chemicals). For each solvent, the suspension of ground leaves in 250–300 ml of solvent was shaken for 1 h on the orbital shaker. After filtering, the samples were extracted two more times for each solvent. Solvents were removed with a rotary evaporator to obtain the dried extracts, which were stored at –20°C for further analysis.

Antiproliferative activity

Antiproliferative (APF) activity of extracts (25 μg/ml) was initially screened for growth inhibitory activity against three human cancer cell lines (MCF-7, MDA-MB-231, and HeLa) using the sulforhodamine B (SRB) assay.[28-30] Growth inhibitory activity with less than 50% cell growth was considered positive while that with more than 50% cell growth was considered negative. Extracts with positive growth inhibition were further tested against six human cancer lines (MCF-7, MDA-MB-231, HeLa, HT-29, SKOV-3, and HepG2) using six different extract concentrations. Human cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The

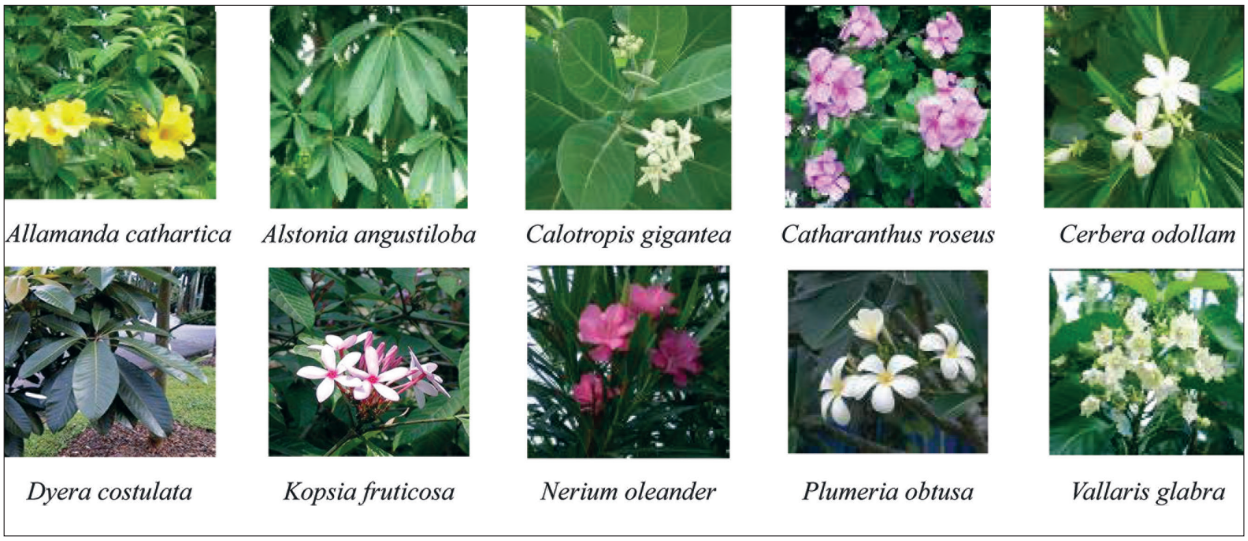

Figure 1: The ten Apocynaceae species studied

Table 1: Common or vernacular names and brief description of Apocynaceae species studied

Species (common/vernacular name)	Brief description
Allamanda cathartica L. (Common allamanda)	A shrub with trumpet-shaped yellow flowers similar in size as leaves which are in whorls
Alstonia angustiloba Miq. (Pulai)	A medium-sized tree with leaves in whorls and having fine secondary veins
Calotropis gigantea (L.) Aiton (Giant milkweed)	A shrub with pale green leaves and white or lilac flowers with a crown rising from the center
Catharanthus roseus (L.) G. Don (Madagascar periwinkle)	A common ornamental shrub with oval to oblong leaves and white, pink or purple flowers with a dark colored center
Cerbera odollam Gaertner (Pong-pong)	A tree bearing white flowers in clusters and rounded fruits that are green when young and red when mature
Dyera costulata Hook (Jelutong)	A tall timber tree with straight columnar bole, leaves in whorls and latex which was an important source of chewing gum
Kopsia fruticosa (Ker.) A. DC. (Pink kopsia)	A shrub with large glossy leaves and clusters of light pink flowers resembling those of Ixora
Nerium oleander L. (Oleander)	An ornamental shrub with thick narrow leaves in pairs or whorls and bearing clusters of pink, red, or purple flowers
Plumeria obtusa L. (Frangipanni)	A tree producing dark green, glossy and oval leaves and white fragrant flowers with a yellow center
Vallaris glabra Kuntze (Kesidang)	A woody climber producing clusters of white flowers with a scent characteristic of pandan leaves or fragrant rice

cells were seeded 24 h prior to treatment in 96-well plates at densities of 10,000–20,000 cells/well. Each cell line was designated one plate. Initial cell population of each cell lines prior to addition of extracts was determined by fixing with trichloroacetic acid (TCA) (Sigma). Extracts were dissolved in dimethyl sulfoxide (DMSO) (Sigma) and serially diluted from 8–25 μg/ml. Control cultures were treated with the same volume of DMSO. The concentration of DMSO was kept within 1% to avoid any interference with cell viability. After the addition of extracts, the plates were incubated for 48 h. After incubation, the cells were fixed with 50 μl of cold 50% TCA and incubated for 1 h at 4°C. The plates were then washed with tap water and air dried. Cells were stained with 100 μl of 0.4% SRB solution (Sigma) diluted with 1% acetic acid followed by incubation for 10 min at room temperature. Unbound dye was removed by washing with 1% acetic acid. Bound stain was then solubilized with 200 μl of 10 mM trizma base (Sigma). Absorbance of each well at 505 nm was obtained using a microplate reader. Dose–response curves were constructed to obtain GI_{50} or growth inhibition of cell lines by 50%. Activity is considered to be effective when GI_{50} value ≤20 μg/ml.[31]

Analysis of TAC, TPC, and RSA

Total alkaloid content (TAC) of extracts was determined using the Dragendorff precipitation assay.[32] For each species, extracts (15 mg) were dissolved in 1 ml of distilled water that was acidified to pH 2.0–2.5 with 0.01 M HCl. Analysis was conducted in triplicate. Alkaloids were then precipitated with 0.4 ml of Dragendorff reagent. Washed with 0.5 ml of distilled water to remove traces of the reagent, the precipitate was later treated with 0.4 ml of 1% sodium sulfide, resulting in a brownish-black precipitate. Precipitates formed at each stage were recovered by centrifugation at 14,000 rpm for 1 min. The resulting precipitate was dissolved in 0.2 ml of concentrated nitric acid and diluted to 1 ml with distilled water. Addition of 2.5 ml of 3% thiourea to 0.5 ml aliquots of this solution resulted in a yellow-colored complex. Absorbance was measured at 435 nm and TAC was expressed as boldine equivalent in milligram per gram of extract. The calibration equation for boldine (Sigma) was $y = 1.068x$ ($R^2 = 0.9959$) where y is absorbance and x is mg/ml of boldine. Dragendorff reagent was prepared by dissolving 0.8 g of bismuth nitrate (Sigma) in 40 ml of distilled water and 10 ml of glacial acetic acid. The resulting solution was mixed with 20 ml of 40% potassium iodide.

Total phenolic content (TPC) of extracts was determined using the Folin–Ciocalteu (FC) assay.[33] Extracts (300 μl in triplicate) were introduced into test tubes followed by 1.5 ml of FC reagent (Fluka) at 10 times dilution and 1.2 ml of sodium carbonate (Fluka) at 7.5% w/v. The tubes were allowed to stand for 30 min in the dark before absorbance was measured at 765 nm. TPC was expressed as gallic acid (GA) equivalent in milligram per gram of extract. The calibration equation for GA (Fluka) was $y = 0.0111x - 0.0148$ ($R^2 = 0.9998$) where y is absorbance and x is mg/ml of GA.

Radical scavenging activity (RSA) of extracts was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay.[33] Different dilutions of extracts (1 ml in triplicate) were added to 2 ml of DPPH (Sigma). The concentration of DPPH used was 5.9 mg in 100 ml of methanol. Absorbance was measured at 517 nm after 30 min. RSA was calculated as IC_{50}, the concentration of extract to scavenge 50% of the DPPH radical. RSA was then expressed as ascorbic acid equivalent antioxidant capacity (AEAC) using the equation of AEAC (mg ascorbic

acid/g of extract) = $IC_{50(ascorbate)}/IC_{50(sample)} \times 10^5$. IC_{50} of ascorbic acid used for calculation of AEAC was 0.00387 mg/ml.

RESULTS AND DISCUSSION

Initial screening of leaf extracts of 10 Apocynaceae species against three human cancer cell lines (MCF-7, MDA-MB-231, and HeLa) showed that extracts of *A. angustiloba*, *C. gigantea*, *C. roseus*, *N. oleander*, *P. obtusa*, and *V. glabra* displayed positive growth inhibitory activity, that is, inhibition with ≤50% cell growth [Table 2]. DCM extract of *C. gigantea*, and DCM and DCM:MeOH extracts of *N. oleander* and *V. glabra* inhibited all three cancer cell lines. All four extracts of *N. oleander* were effective against MCF-7 cells. Only DCM:MeOH extract of *C. roseus* was active against MCF-7 and HeLa cells. Hexane extract of *N. oleander* inhibited MCF-7 cells while hexane extract of *P. obtusa* inhibited MCF-7 and HeLa cells. MeOH extracts of *N. oleander* and *V. glabra* inhibited MCF-7 cells. In general, DCM and DCM:MeOH extracts of these species were the most effective. All extracts of *A. cathartica*, *C. odollam*, *D. costulata*, and *K. fruticosa* did not show any APF activity.

Contrary to findings of this study, cytotoxic activities have been reported in species of *Cerbera*, *Allamanda*, and *Kopsia*. Methanol extract of leaves of *C. odollam* strongly inhibited MCF-7 and T47D cells.[23] Cardenolides from seeds of *C. odollam* had cytotoxic activity against KB, BC, and NCI-H187 cells.[34] Potent cytotoxic activity was reported in ethanol extracts of fruits and leaves of *Cerbera manghas*.[35] Cardenolides from roots of *C. manghas* also showed APF activity.[22] Ethanol root extracts of *A. schottii* and *A. blanchetti* displayed stronger cytotoxicity against K-562 cells than leaf and stem extracts.[15] Valparicine from the stem bark of *Kopsia arborea* showed cytotoxic effects against KB and Jurkat cells[36] while kopsimaline from leaves and stem bark of *Kopsia singapurensis* was found to inhibit KB cells.[37]

Table 2: Leaf extracts of Apocynaceae species with positive growth inhibitory activity against three human cancer cell lines

Species	Leaf extract[3]	Growth inhibitory activity[1]		
		MCF-7[2]	MDA-MB-231[2]	HeLa[2]
Alstonia angustiloba	DCM	-	+	+
Calotropis gigantea	DCM	+	+	+
	DCM:MeOH	+	-	+
Catharanthus roseus	DCM:MeOH	+	-	+
Nerium oleander	HEX	+	-	-
	DCM	+	+	+
	DCM:MeOH	+	+	+
	MeOH	+	-	-
Plumeria obtusa	HEX	+	-	+
	DCM	-	-	+
Vallaris glabra	DCM	+	+	+
	DCM:MeOH	+	+	+
	MeOH	+	-	-

[1]Growth inhibitory activity with <50% cell growth is considered positive (+) while that with >50% cell growth is considered negative (-); [2]MCF-7 and MDA-MB-231 are human breast cancer cells, and HeLa are human cervical cancer cells; [3]HEX = Hexane, DCM = Dichloromethane, and MeOH = Methanol.

Extracts of the six species were further tested against six human cancer cell lines (MCF-7, MDA-MB-231, HeLa, HT-29, SKOV-3, and HepG2). Results showed that DCM extract of *A. angustiloba* inhibited only MDA-MB-231, HeLa, and SKOV-3 cells with GI_{50} values of 20, 20, and 16 μg/ml, respectively [Table 3]. DCM extract of *C. gigantea* strongly inhibited all cancer cell lines with GI_{50} values ranging from 1.3–3.3 μg/ml. Against MCF-7 and MDA-MB-231 cells, GI_{50} of DCM extract of *C. gigantea* (1.9 and 1.3 μg/ml) was much stronger than that of xanthorrhizol (11 and 8.7 μg/ml), curcumin (4.1 and 8.7 μg/ml), and tamoxifen (8.3 and 4.6 μg/ml), respectively.[30] DCM:MeOH extract of *C. roseus* strongly inhibited MCF-7 and HeLa cells with GI_{50} of 3.5 and 4.7 μg/ml, respectively. All four extracts of *N. oleander* were effective against MCF-7 cells with GI_{50} ranging from 3.7–12 μg/ml. DCM and DCM:MeOH extracts inhibited all cell lines except HepG2 cells. Hexane extract of *P. obtusa* was effective against MCF-7 and HeLa cells, while its DCM extract was effective against HeLa cells. DCM and DCM:MeOH extracts of *V. glabra* inhibited all cell lines with GI_{50} values ranging from 7.5–12 μg/ml and 5.8–13 μg/ml, respectively. In addition, MeOH extract of *V. glabra* also inhibited the growth of MCF-7 and HepG2 cells. Against MCF-7 cells, GI_{50} of DCM and DCM:MeOH extracts of *V. glabra* (7.7 and 7.0 μg/ml) was stronger than xanthorrhizol (11 μg/ml) and comparable to tamoxifen (8.3 μg/ml), respectively.[30]

To the best of our knowledge, this study represents the first report of APF activities from leaf extracts of *A. angustiloba*, *P. obtusa*, and *V. glabra*. Earlier studies have reported cytotoxic activity from the root bark of *Alstonia macrophylla*[16] and from the stem bark of *Alstonia scholaris*.[17] Iridoids isolated from the bark of *Plumeria rubra* were cytotoxic.[26] A recent study reported potent cell growth inhibition of cardenolide glycosides isolated from *Vallaris solanacea*.[38] The finding of strong APF activities from DCM and DCM:MeOH extracts of *C. gigantea* from this study is supported by earlier reports that leaf and root extracts of *C. gigantea* had strong inhibitory activity against cancer cells.[18-20] Isolated from leaves of *N. oleander*, pentacyclic triterpenoids were cytotoxic to KB cells.[24] Against HL60 and K562 cells, the stem extract of *N. oleander* displayed stronger cytotoxic activity than leaf and root extracts.[39]

Table 3: Antiproliferative activity of leaf extracts of Apocynaceae species with positive growth inhibitory activity against six human cancer cell lines

Species	Leaf extract[3]	Growth inhibition (GI_{50})[1]					
		MCF-7[2]	MDA-MB-231[2]	HeLa[2]	SKOV-3[2]	HT-29[2]	HepG2[2]
Alstonia angustiloba	DCM	-	20 ± 1.7	20 ± 1.1	16 ± 1.4	-	-
Calotropis gigantea	DCM	1.9 ± 0.2	1.3 ± 0.3	2.5 ± 0.5	2.5 ± 0.2	3.3 ± 0.2	1.8 ± 1.7
	DCM:MeOH	13 ± 0.3	-	15 ± 1.0	20 ± 2.3	24 ± 0.7	16 ± 3.5
Catharanthus roseus	DCM:MeOH	3.5 ± 0.1	-	4.7 ± 0.6	-	-	-
Nerium oleander	HEX	11 ± 0.9	-	-	12 ± 2.8	-	-
	DCM	3.7 ± 0.1	5.2 ± 0.7	5.1 ± 0.7	4.5 ± 1.0	6.1 ± 0.9	-
	DCM:MeOH	4.3 ± 0.2	18 ± 2.3	6.8 ± 0.9	7.6 ± 2.1	9.2 ± 2.2	-
	MeOH	12 ± 1.1	-	-	-	-	-
Plumeria obtusa	HEX	5.7 ± 0.8	-	10 ± 1.4	-	-	-
	DCM	-	-	19 ± 4.0	-	-	-
Vallaris glabra	DCM	7.7 ± 1.3	12 ± 2.0	9.8 ± 1.5	7.5 ± 4.5	9.3 ± 2.0	7.6 ± 0.2
	DCM:MeOH	7.0 ± 2.5	13 ± 6.3	8.5 ± 2.9	7.7 ± 2.4	12 ± 1.2	5.8 ± 1.2
	MeOH	16 ± 2.1	-	-	-	-	19 ± 0.9

[1]GI_{50} (μg/ml) is growth inhibition of cancer cell lines by 50% and inhibition is not effective (-) if values >20 μg/ml; [2]MCF-7 and MDA-MB-231, HeLa and SKOV-3, HT-29, and HepG2 are human breast, cervical, colon, and liver cancer cells, respectively; [3]HEX = Hexane, DCM = Dichloromethane, and MeOH = Methanol

It is interesting to note that out of four leaf extracts of *C. roseus* tested against six cell lines, only DCM:MeOH extract inhibited MCF-7 and HeLa cells. The species is well known for its indole alkaloids notably vinblastine and vincristine, which are used to treat Hodgkin's disease and acute leukemia in children, respectively.[1] It can be inferred that the APF activities of *C. roseus* may be cell line specific unlike those of *C. gigantea*, *N. oleander*, and *V. glabra* which are wide spectrum, inhibiting most or all cell lines tested.

Of the 10 species analyzed, MeOH crude and DCM extracts of *K. fruticosa* had the highest TAC (100 and 129 mg BE/g of extract), respectively [Table 4]. Other species with moderately high TAC were *D. costulata*, *C. roseus*, and *A. angustiloba* with MeOH crude and DCM:MeOH extracts having values ranging from 23–58 and 58–68 mg BE/g of extract, respectively. Based on TAC, the species can be ranked as high (*K. fruticosa*), moderate (*D. costulata*, *C. roseus*, and *A. angustiloba*), and low (*C. odollam*, *C. gigantea*, *V. glabra*, *P. obtusa*, *A. cathartica*, and *N. oleander*). Extracts of *D. costulata* had the highest TPC and strongest RSA. MeOH crude, DCM:MeOH, and MeOH extracts of *D. costulata* yielded TPC values of 319, 354, and 279 mg GAE/g of extract, and RSA values of 377, 349, and 278 mg AA/g of extract, respectively. Compared to *D. costulata*, extracts of other species can be categorized as moderate to low.

The high TAC of leaf extracts of *K. fruticosa* may be attributed to the presence of alkaloids identified as fruticosamine, fruticosine, and kopsine.[40] The presence of flavonols identified as 3'7-dimethoxyquercetin and quercetin-3-*O*-α-L-rhamnopyranoside[11] may contribute to the high TPC and RSA of leaf extracts of *D. costulata*.

Overall, there is a strong correlation between TPC and RSA of extracts (R^2 = 0.992) but not with TAC (R^2 = 0.135). The strong correlation between TPC and RSA of extracts affirms that Apocynaceae species with higher concentration of phenolic compounds in the leaves also have stronger radical scavenging capacity. This would mean that the phenolic compounds are the main contributors to the antioxidant potential of leaves. Similar findings have been reported in medicinal plants and herbs,[41-43] plants of industrial interest,[44] wild edible fruits,[45] and mushrooms.[46] The correlation of results of phytochemical analysis with APF activities remains unclear. Extracts of *C. gigantea*, *N. oleander*, and *V. glabra* which showed strong APF activities had low TAC, and moderate to low TPC and RSA.

CONCLUSION

Out of 10 species of Apocynaceae, leaf extracts of *A. angustiloba*, *C. gigantea*, *C. roseus*, *N. oleander*, *P. obtusa*, and *V. glabra* displayed positive APF activities. DCM extract of *C. gigantea*, and DCM and DCM:MeOH extracts of *V. glabra* inhibited the growth of all six human cancer cell lines. Against MCF-7 and MDA-MB-231 breast cancer cells, DCM extracts of *C. gigantea* and *N. oleander* were stronger than or comparable to standard drugs of xanthorrhizol, curcumin, and tamoxifen. All four extracts of *N. oleander* were effective against MCF-7 cells. With wide-spectrum APF activities, leaves of these three species are therefore promising candidates as alternative resources for anticancer drugs. Their wide-spectrum APF activities are reported for the first time and this warrants further investigation into their bioactive compounds.

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Table 4: Total alkaloid content, total phenolic content, and radical scavenging activity of leaf extracts of Apocynaceae species

Species	MeOH crude extract[1]	Sequential extract[1]			
		HEX	DCM	DCM:MeOH	MeOH
TAC (BE mg/g extract)[2]					
Kopsia fruticosa	100 ± 4.2	63 ± 1.1	129 ± 4.0	99 ± 2.5	46 ± 1.6
Dyera costulata	58 ± 2.5	2.4 ± 0.6	11 ± 1.8	68 ± 2.7	41 ± 2.4
Catharanthus roseus	37 ± 2.8	18 ± 0.9	9.1 ± 0.8	76 ± 5.5	35 ± 3.3
Alstonia angustiloba	23 ± 0.3	13 ± 2.3	27 ± 3.1	58 ± 1.2	27 ± 1.0
Cerbera odollam	2.8 ± 0.9	3.6 ± 0.5	3.0 ± 0.5	5.0 ± 1.5	3.2 ± 0.4
Calotropis gigantea	2.7 ± 0.3	3.7 ± 1.0	8.6 ± 1.2	9.6 ± 1.9	9.2 ± 2.7
Vallaris glabra	2.7 ± 0.4	4.4 ± 0.8	8.9 ± 0.6	9.2 ± 0.2	8.7 ± 2.2
Plumeria obtusa	2.3 ± 0.3	2.3 ± 0.4	2.5 ± 0.1	4.3 ± 1.3	5.8 ± 1.7
Allamanda cathartica	2.0 ± 0.1	4.4 ± 1.3	8.7 ± 0.9	12 ± 1.0	8.7 ± 2.1
Nerium oleander	1.4 ± 0.7	1.6 ± 0.0	2.8 ± 0.8	3.9 ± 0.7	8.3 ± 0.5
TPC (GAE mg/g extract)[2]					
Dyera costulata	319 ± 5.5	21 ± 0.2	23 ± 0.1	354 ± 6.0	279 ± 3.0
Vallaris glabra	99 ± 3.8	15 ± 0.9	24 ± 0.5	134 ± 1.0	164 ± 13
Plumeria obtusa	85 ± 0.9	21 ± 1.7	52 ± 1.3	104 ± 4.0	134 ± 3.0
Kopsia fruticosa	83 ± 1.1	39 ± 0.5	20 ± 0.5	129 ± 1.0	84 ± 1.0
Alstonia angustiloba	68 ± 2.0	17 ± 0.6	24 ± 0.6	96 ± 1.1	94 ± 1.1
Nerium oleander	56 ± 3.8	18 ± 0.3	23 ± 0.1	57 ± 0.3	29 ± 0.3
Catharanthus roseus	53 ± 2.5	24 ± 0.5	46 ± 1.4	75 ± 0.7	61 ± 1.1
Allamanda cathartica	37 ± 0.8	11 ± 0.4	27 ± 0.2	39 ± 0.9	42 ± 0.5
Cerbera odollam	30 ± 4.1	19 ± 0.7	26 ± 0.5	30 ± 0.6	40 ± 0.9
Calotropis gigantea	28 ± 0.8	14 ± 0.6	44 ± 1.7	42 ± 0.8	33 ± 0.5
RSA (AA mg/g extract)[2]					
Dyera costulata	377 ± 25	15 ± 0.3	9.3 ± 0.5	349 ± 21	278 ± 5.4
Vallaris glabra	84 ± 0.5	6.0 ± 0.3	8.4 ± 0.4	77 ± 2.2	119 ± 6.8
Plumeria obtusa	66 ± 3.5	4.9 ± 0.2	10 ± 0.9	58 ± 0.1	115 ± 5.3
Kopsia fruticosa	63 ± 3.2	12 ± 1.4	7.5 ± 0.3	70 ± 2.0	48 ± 1.5
Nerium oleander	42 ± 1.3	6.4 ± 0.1	8.5 ± 0.6	48 ± 1.2	33 ± 0.2
Alstonia angustiloba	29 ± 0.9	10 ± 0.2	5.7 ± 0.7	50 ± 1.2	46 ± 2.2
Catharanthus roseus	24 ± 0.8	13 ± 0.5	21 ± 0.5	33 ± 1.1	31 ± 0.3
Allamanda cathartica	17 ± 1.1	11 ± 0.1	7.9 ± 0.8	18 ± 1.4	24 ± 2.0
Cerbera odollam	15 ± 1.0	16 ± 0.4	5.5 ± 0.1	14 ± 1.3	25 ± 0.7
Calotropis gigantea	7.6 ± 0.4	5.6 ± 0.3	6.1 ± 0.4	8.0 ± 0.6	14 ± 1.3

[1]HEX = Hexane, DCM = Dichloromethane, and MeOH = Methanol; [2]BE = Boldine equivalent, GAE = Gallic acid equivalent, AA = Ascorbic acid, TAC = Total alkaloid content, TPC = Total phenolic content, and RSA = Radical scavenging activity

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Appendix VIII

OTHER PUBLICATIONS

Wong, S.K., Wong, L.F., Lim, Y.Y. & Chan, E.W.C., 2010. Effects of drying treatments on the antioxidant properties of leaves and teas of *Alpinia* species (Zingiberaceae). *Journal of Tropical Medicinal Plants*, 11(1), 97−105.

Fresh leaves of five *Alpinia* species were screened for antioxidant properties (AOP) with comparison to three commercial ginger species. AOP assessed included total phenolic content (TPC), ascorbic acid equivalent antioxidant capacity (AEAC) and ferric reducing power (FRP). Effects of thermal and non-thermal drying treatments on AOP of leaves of three *Alpinia* species were analysed. Based on TPC, AEAC and FRP, leaves of *Alpinia* species screened can be categorized into high, moderate and weak categories. Thermal drying of leaves resulted in significant loss of TPC and AEAC values. Air-dried leaves of *Alpinia zerumbet* showed drastic losses in AOP while freeze-dried leaves had significant gains. The freeze-dried tea of *A. zerumbet* was superior to the commercial tea for all AOP studied.

Wong, S.K., Lim, Y.Y. & Chan, E.W.C., 2010. Evaluation of antioxidant, anti-tyrosinase and antibacterial activities of selected *Hibiscus* species. *Ethnobotancial Leaflets*, 14, 781−96.

Leaves and flowers of six *Hibiscus* species used in traditional medicine were evaluated for antioxidant properties (AOP), and antityrosinase and antibacterial activities. Information on the selected species is meagre and this study would contribute new and additional knowledge on the bioactivities of Malvaceae. AOP were assessed for total phenolic content (TPC), total anthocyanin content (TAC), ascorbic acid equivalent antioxidant capacity (AEAC), ferric reducing power (FRP), ferrous ion chelating (FIC) ability and lipid peroxidation inhibition (LPI) activity. Antityrosinase and antibacterial activities were assessed using the modified dopachrome and disc diffusion methods, respectively. Leaves and flowers of *Hibiscus tiliaceus* showed outstanding AOP. Leaves of species with high TPC and

AEAC had low FIC ability and *vice versa.* Red flowers of species which yielded the highest TAC also displayed high FIC ability and LPI activity. Leaves of *H. tiliaceus* had the strongest tyrosinase inhibition activity. At 1 mg extract/disc, leaves of *Hibiscus sabdariffa* and *H. tiliaceus* were found to inhibit Gram-positive bacteria. Leaves of all four *Hibiscus* species showed no antibacterial activity on Gram-negative bacteria. At 2 mg extract per disc, leaves of *H. sabdariffa* displayed inhibition on Gram-positive and Gram-negative bacteria. This is first report of leaf extracts of *H. sabdariffa* inhibiting Gram-negative bacteria. Adding 1 mM of ethylene-diamine tetraacetic acid (EDTA) to the agar slightly enhanced the antibacterial activity of leaves of *H. sabdariffa* on Gram-negative bacteria. EDTA rendered streptomycin ineffective against *Salmonella choleraesuis* but enhanced the efficiency of the antibiotic against *P. aeruginosa*.

Chan, E.W.C., Lim, Y.Y., Chong, K.L., Tan, J.B.L. & **Wong, S.K.**, 2010. Antioxidant properties of tropical and temperate herbal teas. *Journal of Food Composition and Analysis*, 23(2), 185−9.

Antioxidant properties (AOP) of thirteen tropical and five temperate herbal teas were screened. Comparisons were made with green, oolong and black teas of *Camellia sinensis*. The AOP studied were total phenolic content, radical-scavenging activity, ferric-reducing power and ferrous ion-chelating (FIC) ability. Tropical herbal teas were more diverse in types and more variable in AOP values than temperate herbal teas. Herbal teas generally had lower antioxidant values than teas of *C. sinensis*. Exceptions were lemon myrtle, guava and oregano teas with AOP comparable to black teas. FIC ability of mint and peppermint teas was significantly stronger than all *C. sinensis* teas.

Chan, E.W.C., Lim, Y.Y & **Wong, S.K.**, 2011. Antioxidant properties of ginger leaves: An overview. *Free Radicals and Antioxidants*, 1(1), 6−16.

Past studies on the antioxidant properties (AOP) of ginger species were confined to rhizomes. Although leaves of ginger species have been used for food flavouring and in traditional medicine, little research has been done on their AOP until recent years. This overview is on recent work done on the AOP of ginger leaves. Emphasis is on

variation between species, comparison with rhizomes and flowers, altitudinal variation, effects of thermal and non-thermal drying methods, herbal teas, and commercial potentials. Of 26 ginger species, belonging to nine genera and three tribes, AOP of leaves were strongest in *Etlingera* followed by *Alpinia* and *Hedychium*. Eleven out of 14 species (78%) had significantly higher values in leaves than in rhizomes. Similar trends were also observed in other species of *Zingiber*, *Boesenbergia* and *Elettariopsis*. Leaves of highland populations of *Etlingera* had higher values than their lowland counterparts. Thermal drying of leaves of four species led to drastic declines in AOP but freeze drying led to significantly increase for leaves of *Etlingera elatior* and *Alpinia zerumbet*. AOP of hot-water extracts of the freeze-dried tea of *A. zerumbet* were found to be significantly higher than the commercial tea. A protocol to produce a standardised herbal extract of chlorogenic acid (CGA) from *E. elatior* leaves (40% purity) has been developed. With high CGA content of 1.6 times that of commercial extracts from honeysuckle flowers (25% purity), the standardised extract has great potential to be developed into functional foods and other health products.

Chan, E.W.C., Lim, Y.Y. & **Wong, S.K.**, 2011. Phytochemistry and pharmacological activities of *Etlingera elatior*: A review. *Pharmacognosy Journal*, 3(22), 6−10.

Etlingera elatior are large ginger plants growing in clumps. Rhizomes are stout, strongly aromatic and found just below ground level. Crushed leaves emit a pleasant sour fragrance which is distinctive of the species. Leaves are entirely green with young leaves sometimes flushed pink. Inflorescences, borne on erect stalks protruding from the ground, are large and attractive with showy bracts. Native to Malaysia and Indonesia, *E. elatior* is widely cultivated in Southeast Asia. The species is used as food, condiment, medicine, and ornament. The current knowledge on the phytochemistry of leaves, inflorescences and rhizomes of *E. elatior* is reviewed. Some insights on the pharmacological properties of the species are discussed. They include antioxidant, antibacterial, antifungal, tyrosinase inhibition, cytotoxic and hepatoprotective activities.