MELODINUS EUGENIIFOLIUS.

(APOCYNACEAE JUSS.):

A PHYTOCHEMICAL AND

PHARMACOLOGICAL STUDY

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THESIS SUBMITTED TO THE

UNIVERSITY OF NOTTINGHAM FOR

THE DEGREE OF DOCTOR OF

PHILOSOPHY

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DECLARATION

I, Lu Yao, declare that this thesis is my own work. It is being submitted for the Degree of Doctor of Philosophy, at the School of Pharmacy, Faculty of Sciences, University of Nottingham. It has not been submitted before for any degree or examination at this or any other University.

.....

Signature

.....

Date

DEDICATION

To my beloved parents, J. X. Lu and J. H. Pi, and my beloved sister Y. Lu.

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I offer my greatest thanks to my beloved parents and dear sister for their ongoing support and motivation throughout my life.

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ABSTRACT

Melodinus eugeniifolius. a very rare medicinal plant belongs to the family of Apocynaceae. This family consists of 424 genera and 1,500 species of trees. In the Asia-Pacific region, about 20 species of Apocynaceae are used to treat various diseases like cancer, cardiovascular diseases and infectious diseases. Furthermore, almost no pharmacological and phytochemical studies in genera *Melodinus,* no pharmacological and phytochemical studies in this plant as well. This study was undertaken to screen the phytochemical and phytochemical aspects on the plant *Melodinus eugeniifolius.*

Qualitative phytochemical analysis of the crude extract was determined for the presence of tannins, flavonoids, alkaloids, saponins and sterols. Phytochemical analysis of *Melodinus eugeniifolius.* revealed presence of alkaloids, cardiac glycosides, sterols, steroids and flavonoids in crude extracts of leaves and barks. The crude extracts of the plant were subjected to several biological assays and were then focused on anti-cancer aspect.

Bacterial infections have been known to generate extensive formation of free radicals which is becoming increasingly recognized in the

pathogenesis of the many human diseases. *Melodinus eugeniifolius* leaves and barks were screened for the antibacterial activities, among the different extracts tested, all the extracts displayed remarkable antibacterial activity against *Staphylococcus aureus*, *Bacillus cereus* and *Escherichia coli*.

The role of free radicals and active oxygen is becoming increasingly recognized in the pathogenesis of the many human diseases, including cancer, neurodegenerative diseases, ageing, and atherosclerosis. The FRAP assay (Ferric reducing antioxidant power), DPPH radical scavenging assay (1,1-diphenyl-2-picryl hydrazyl radical reducing power methods), and β-carotene bleaching assays were employed to determine the radical scavenging activity of the plant extracts using 96-well microtiter plate. Percentage decolourisation was plotted against the concentration of the sample and the EC_{50} values were determined. Vitamin C (l-ascorbic acid), Gallic Acid and Quercetin were used as positive control. Ethanol extracts showed significant antioxidant activity. In this test, the ethanol extract of leaves and barks exhibited profound antioxidant activities. The EC50 (μg/mL) for the ethanol extract of barks was as good as Gallic acid.

Plant substances continue to serve as a wellspring of drugs for the world population and several plant-based drugs are in extensive clinical use. Preliminary antiparasitic screening of six extracts of *Melodinus eugeniifolius* leaves and barks were screened for their *in vitro* antileishmanial and antitumor activities, among the different extracts tested, the ethanol and hexane extract of barks showed significant antileishmanial activities.

Cancers remain the leading cause of death worldwide and the search for novel anti-cancer agents from medicinal plants has become crucial. Infections and free radical generation are recognized in the pathogenesis of cancer. Six different extractions of *Melodinus eugeniifolius* leaves and barks were screened for their *in vitro* antitumor activities, among the different extracts tested, the chloroform extracts of barks and leaves showed significant antitumor activities and would not damage the normal cells. According to the current result, susceptible cell lines were found to be the HT-29 human colon cancer cell line, HCT-116 human colon cancer cell line, MCF-7 human breast cancer cell line, Caco-2 human colon adenocarcinoma cell line, HK-1 human nasopharyngeal cancer cell line, and MRC-5 human lung fibroblast cell line.

Results from our phytochemical analysis revealed that the chloroform extract of leaves and barks of *Melodinus eugeniifolius* accumulate substantial amounts of alkaloids and cardiac glycosides which could be well correlated with the activities measured.

Bioassay guided isolation and nine chemical compounds were isolated by column chromatography fractions and were identified by NMR techniques. Five indole alkaloids were isolated and were identified as yohimbine hydrochloride, tabersonine, yohimbine, β-yohimbine, and leuconolam respectively. The new alkaloid, having a pentacyclic diazaspiro system, was named Melodinoid. Other constituents identified were epigallocatechin and loganic acid. Some of the compounds isolated from the plant were subjected to biological assays and were then focused on anti-cancer aspect.

Alkaloids always present significant anticancer capacity. Three alkaloids isolated from *Melodinus eugeniifolius* were screened by MTT assays against two cancer cells and a normal cell. All the compounds revealed significant anticancer capacity on the two human cancer cell lines, and not damage the normal cells.

Hence, further research on the mechanism of some of the isolated compounds with target pharmacological activities, relative *in vivo* anticancer tests, and even clinical trials are recommended to ascertain the efficacy, safety and other mechanisms of action to application in the pharmaceutical industry as natural therapeutic agents

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Post presentation at the 2013 Diabetic Foot Global Conference on the 21th-23th of March 2013 at Los Angeles, USA. – **"A Note on the Treatment of Diabetic Foot by the 'Heat - Clearing Method' (Qing Fa) of Traditional Chinese Medicine"**

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LIST OF ABBREVIATIONS AND SYMBOLS

A

AST	Antimicrobial susceptibility testing
ATCC	American Type Culture Collection

B

BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene

C

°C	Degree Celsius
CEA	Carcinoembryonic antigen
$CDCl_3$	Deuterated chloroform
CDOD	Deuteromethanol
cm	Centimetre
CO_2	Carbon dioxide
COX	Cyclooxygenase isozymes

D

δ	Chemical shifts
DMSO	Dimethyl sulfoxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl

E

EDTA	Ethylene diamine tetra-acetic acid
ELSD	Evaporative Light Scattering Detector

F

FCS	Foetal calf serum
$FeCl_3$	Ferric chloride
$FeSO_4$	Ferric sulphate
FRAP	Ferric reducing antioxidant potential
FRIM	Forest Research Institute Malaysia
5-FU	5-Fluorouracil

G

g	Gram
GI_{50}	Half maximal growth inhibition

H

h	Hour
^1H	Proton
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
HPLC	High Performance Liquid Chromatograph
hrs	Hours
H_2SO_4	Sulphuric acid

I

IAPT	International Association for Plant Taxonomy
IC_{50}	Half maximal inhibitory concentration
IgA	Immunoglobulin A
IR	Infrared spectroscopy

L

L	Litre

M

m	Metre

M	Molar
mg	Milligram
mm	Millimetre
mL	Millilitre
mM	Millimolar
min	Minute
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MS	Mass Spectroscopy

N

NA	Not available
NaCl	Sodium chloride
NADOC	Sodium deoxycholate
NCCLS	National Committee for Clinical Laboratory Standards
NCE	New Chemical Entities
nm	Nanometre
NMR	Nuclear magnetic resonance
NPC	Nasopharyngeal carcinoma

P

%	Percentage
PBS	Phosphate buffered saline
pH	Potentiometric hydrogen ion concentration
ppm	Parts per million

R

ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute

S

SCI	Science Citation Index

T

TBHQ	Tert-butylhydroquinone
TCM	Traditional Chinese Medicine
TLC	Thin layer chromatography
TPTZ	2,4,6- Tris (2-pyridyl) -1,3,5 -triazine

U

µg	Microgram
µL	Microlitre
µM	Micromolar
µmol	Micromole
UV	Ultraviolet
UNMC	University of Nottingham Malaysia Campus

V

v/v	Volume per volume

W

WCSP	World Checklist of Seed Plants
WHO	World Health Organisation
w/v	Weight per volume

CHAPTER Ⅰ

GENERAL INTRODUCTION

1.1 BACKGROUND

The scope of pharmacognosy is defined as the study of physical, chemical, biochemical and biological properties of drugs, drug substances, or potential drugs or drug substances of natural origin as well as the search for new drugs from natural origin as well as the search for new drugs from natural sources [1]. Since time immemorial, plants have been used extensively as a source of medicines for the treatment of various human ailments [2]. According to the World Health Organisation (WHO), approximately 80% of the people in developing countries still rely on traditional medicines for their primary health care needs [3], and a major part of the traditional therapy involves the use of plant extracts or their active constituents [4]. Moreover, about 25% to 50% of current pharmaceuticals are plant-derived natural products, indicating the significance and efficacy of plants as an indispensable pharmacological tool [5].

Natural products offer unmatched chemical diversity with structural complexity and biological potency [5]. It is estimated about 100-fold

higher hit rate for natural products over synthetic compounds. Natural products occupy different chemical space, the natural product databases contain many mainstay chemical products [5]. Natural products compounds not only serve as drugs or templates for drugs but also lead to a better understanding of targets and pathways involved in disease process [6]. Besides, natural products also create opportunities for additional drug targets to be identified and exploited in these pathways. For instance, the elucidation of the anti-inflammatory mechanism of action of aspirin led to the discovery of the cyclooxygenase isozymes COX-1 and -2, which were used in the development of novel anti-inflammatory drugs [6].

There has been a tremendous resurgence of interest in medicinal plants in recent years [7]. This revival might be attributed to several driving factors such as rise in population, insufficient supply of drugs in certain parts of the world, prohibitive cost of treatments for common, ailments, side effects of several allopathic drugs in current usage as well as development of resistance to currently used drugs for diseases [8, 9]. Consequently, exploitation of medicinal plants for bioactive compounds is of great potential and could be an alternative source of providing new vistas for novel drug discovery and development [10, 11].

Over half of the world's top 25 best-selling pharmaceuticals drugs in 1991 owed their origin to natural products [11]. Higher plant-derived products represent around 25% of the total number of clinically used drugs and include the classical drugs atropine, codeine, digoxin, morphine and quinine [1]. The number of drug molecules obtained and developed industrially from plants and used in modern medicines increased drastically from 121 in 1995, 130 in 1997, 143 in 2000 and 166 in 2006. From 1981 and 2000, 61% of the small molecule - new chemical entities (NCE) that were introduced as drugs worldwide can be traced to or were inspired by natural products. Between 2001 and 2005, 23 new drugs derived from natural products were introduced for the treatment of bacterial and fungal infection, cancer, diabetes, dyslipidemia, atopic dermatitis, Alzheimer's disease and genetic disease such as tyrosinaemia and Gaucher disease [12]. Table 1.1 lists the drugs derived from plants in the global market, their clinical uses and sources [12, 13].

Table 1.1: Clinical uses and sources of drugs derived from plants in global market.

Drug	Clinical Use/Action	Plant source
Atropine	Anticholinergic	*Atropa belladonna*
Colchicine	Antitumor, antigout agent	*Colchicum autumonale*
Digitoxin	Cardiotonic	*Digitalis purpurea*
Emetine	Emetic, amoebicide	*Cephaelis ipecacuanha*
Morphine	Analgesic	*Papaver somniferum*
Pilocarpine	Parasympathomimetic	*Pilocarpus jaborandi*
Quinine	Antimalarial	*Cinchona ledgeriana*
Apomorphine HCl	Potent dopamine receptor agonist (Parkinson's disease)	*Papaver somniferum*
Tiotropium bromide	Longer-acting antibronchospasm	*Atropa belladonna*
Nitisinone	For hereditary tyrosinaemia type-1	*Callistemone citrinus*
Galanthamine HBr	Selective acetylcholinesterase Inhibitor (Alzheimer's disease)	*Galanthus nivalis*
Arteether	Antimalarial	*Artemisia annua*

1.2 OBJECTIVES

This study is aimed at a relatively overall research on a medicinal plant, which includes to extract from target plant, to assay for pharmacological activities, to isolate and purify active constituents from plants by phytochemical techniques, to characterize, elucidate and identify the structure of the pure compound. To the best of my knowledge, no detailed phytochemical and pharmacological studies have been reported on *Melodinus eugeniifolius* (Apocynaceae Juss.). Therefore, the objective in this research are:

i. An introduction of the family Apocynaceae Juss. and genus *Melodinus*. The introduction includes botanical descriptions, geographic distributions, pharmacological activities and phytochemistry.

ii. Extraction of leaves and barks of *Melodinus eugeniifolius*.

iii. Investigation of the possible secondary metabolite types present in the crude extracts of *Melodinus eugeniifolius* leaves and barks with qualitative phytochemical analysis.

iv. Investigation of the antioxidant activity of the crude extracts of *Melodinus eugeniifolius.*

v. Investigation of the in vitro antimicrobial activity of the crude extracts of *Melodinus eugeniifolius*.

vi. Investigation of the antiparasitic activity of the crude extracts of *Melodinus eugeniifolius.*

vii. Investigation of the anticancer activity of the crude extracts of *Melodinus eugeniifolius.*

viii. Isolation and identification of the chemical compounds responsible for the anticancer activity in the most active extracts.

ix. Investigation of the anticancer activity of the chemical compounds isolated from the crude extract of *Melodinus eugeniifolius* with the best anticancer potential.

CHAPTER Ⅱ

LITERATURE REVIEW

2.1 Ethnopharmacological research

The study of plants used in traditional medicine requires the effective integration of information on chemical composition of extracts, pharmacological activities of isolated compounds, as well as indigenous knowledge of traditional healers. The acquisition of ethnobotanical information remains an empirical aspect in any such study [14].

There is a great need and ethical obligation to accurately document investigative findings on plants used for health purposes. This will also aid in the efficient preservation and conservation of traditional knowledge which may potentially benefit society in general.

2.2 An introduction of the family Apocynaceae Juss. and genus *Melodinus*.

2.2.1 The family Apocynaceae Juss.

Apocynaceae is a family of flowering plants that includes trees, shrubs, herbs, stem succulents, and vines, commonly called the dogbane family [15]. The family comprises some 1,500 species divided over about 424 genera. The former family Asclepiadaceae is included in Apocynaceae according to the APG III system [16]. Members of the family are native to European, Asian, African, Australian and American tropics or subtropics, with some temperate members [15]. Many species are tall trees found in tropical rainforests, but some grow in tropical dry (xeric) environments. There are also perennial herbs from temperate zones. Some are sources of important drugs, such as cardiac glycosides, which affect heart function. These include the *Acokanthera*, *Apocynum*, *Cerbera*, *Nerium*, *Thevetia* and *Strophantus*. *Rauvolfia serpentina*, or Indian Snakeroot, yields the alkaloids reserpine and rescinnamine, which are useful tools in the treatment of high blood pressure and even some forms of psychosis. *Catharanthus roseus* yields alkaloids used in treating cancer [17, 18].

2.2.2 The genus *Melodinus*.

The genus *Melodinus* belongs to the family Apocynaceae [19]. *Melodinus* clade embraces 109 species which are mainly found in tropical Asia and Oceania [19].

2.3 Botanical descriptions of genus *Melodinus*.

The members of the genus *Melodinus* consist of stout climber or lianas. Branches dark gray, glabrous; young branchlets and leaves scaly. Petiole 5-10 mm; leaf blade oblong or narrowly elliptic, 7-18 × 2.5-5.2 cm, papery, base rounded, apex acuminate; lateral veins 10-15 pairs, nearly flat on both surfaces. Cymes umbellate, terminal and axillary, 5-6.5 cm; peduncle 1.5-2 cm, glabrous; bracts and bracteoles 3-7 mm. Pedicel 5-7 mm, pubescent. Flower buds cylindric, ca. 2 cm, glabrous outside. Sepals broadly ovate, ca. 7 5 mm, ciliate, apex acute. Corolla white, tube ca. 1.2 cm, pubescent inside; lobes oblong, ca. 1.1 cm; corona scales linear, decurrent to lower part of corolla tube, included. Ovary glabrous. Style very short. Berries globose, ca. 10.5 cm in diam [20, 21]. Distribution and detailed botanical descriptions of species belongs to genus *Melodinus* regarding their morphological characters including habit, roots, stems, branches, leaves, inflorescences, flowers, fruits, seeds,

origin and distribution, as well as herbarium samples are presented in Table 2.1, Table 2.2 and Figure 2.1 (adapted from JSTOR Plant Sciences 2014, China plant species catalogue, and Flora of China) respectively.

Table 2.1 indicates the geographic distributions of species belong to genus *Melodinus,* Table 2.2 indicates the botanical descriptions of species belong to genus *Melodinus*, and Figure 2.1 shows the herbarium samples of species belong to genus *Melodinus.* These tables and figure will help for the cognition of the genus *Melodinus* and the identification of the plant *Melodinus eugeniifolius*.

Table 2.1 Geographic distributions of species belong to genus *Melodinus*

Plant Species	Geographic distributions	Reference
M. acutiflorus F.Muell.	Papua New Guinea, Queensland, New South Wales	[22-26]
M. aeneus Baill.	New Caledonia	[27-29]
M. angustifolius Hayata	Taiwan, N Vietnam	[29, 30]
M. australis (F.Muell.) Pierre	New Guinea, Queensland, New South Wales, Northern Territory, Solomon Islands, Bismarck Archipelago, Vanuatu	[25, 26]
M. axillaris W.T.Wang	Yunnan China	[25, 31-35]
M. balansae Baill.	New Caledonia	[25, 26]
M. baueri Endl.	E Kalimantan, Papua New Guinea, Norfolk Island	[25, 26]
M. cochinchinensis (Lour.) Merr.	Indochina, W Malaysia, E Himalayas, (Assam, Bhutan, Bangladesh, etc.), Yunnan, Myanmar, Thailand, Vietnam	[28-30]
M. cumingii A.DC.	Philippines	[25, 26]
M. densistriatus Markgr.	New Guinea	[25, 26]
M. forbesii Fawc.	Bali, Lombok, Timor, Flores, Maluku, Sulawesi, New Guinea	[25, 26]
M. fusiformis Champ. ex Benth.	China (Guangdong, Guangxi, Guizhou), Indochina, Luzon	[25, 36-39]
M. glaber Turrill	Vanuatu, Fiji	[25, 26]
M. hemsleyanus Diels	Guizhou, Sichuan (Emei mountain), Yunnan.	[25, 26, 28-30]
M. honbaensis A.Chev. ex Pit.	Vietnam	[25, 26]
M. insularis (Markgr.) Fosberg	Palau	[25, 26]

Table 2.1 Geographic distributions of species belong to genus *Melodinus* (continued)

Plant Species	Geographic distributions	Reference
M. khasianus Hook.f	Guizhou, Yunnan, India.	[25, 26, 28-30]
M. magnificus Tsiang	Sunyatsenia, Guangxi	[25, 26, 28-30]
M. morsei Tsiang	Guangdong, Guangxi	[28-30]
M. orientalis Blume	Thailand, W Malaysia, Borneo, Java, Lesser Sunda Islands, Sumatra, Sulawesi, Philippines	[25, 26]
M. orientalis Blume	Thailand, W Malaysia, Borneo, Java, Lesser Sunda Islands, Sumatra, Sulawesi, Philippines	[25, 26]
M. orientalis Blume	Thailand, W Malaysia, Borneo, Java, Lesser Sunda Islands, Sumatra, Sulawesi, Philippines	[25, 26]
M. orientalis Blume	Thailand, W Malaysia, Borneo, Java, Lesser Sunda Islands, Sumatra, Sulawesi, Philippines	[25, 26]
M. philippensis A.DC.	Mindoro	[25, 26]
M. phillirаeoides Labill.	New Caledonia	[25, 26]
M. reticulatus Boiteau	New Caledonia	[25, 26]
M. scandens J.R.Forst. & G.Forst.	New Caledonia	[25, 26]
M. suaveolens (Hance) Champion ex Bentham	Guangdong, Guangxi, Hainan , Vietnam.	[25, 26, 28-30]
M. tenuicaudatus Tsiang & P.T.Li	Yunnan, Guizhou, Guangxi	[28-30, 40]
M. vitiensis Rolfe	Loyalty Islands, Fiji, Tonga, Samoa, Vanuatu	[25-27]
M. yunnanensis Tsiang & P. T. Li.	Guangxi, Yunnan	[28-30]

Table 2.2 Botanical descriptions of species belong to genus *Melodinus*

Plant Species	Botanical descriptions	Reference
M. acutiflorus F.Muell.	Tall climber, branchlets pubescent. Leaves lanceolate to ± oblong-elliptic or elliptic, mostly 4–10 cm long, 15–30 mm wide, apex shortly acute or acuminate, lower surface finely and softly pubescent; petiole c. 5 mm long. Flowers scented, mostly 3 or 5 on each axillary peduncle. Calyx lobes narrow, acute, c. 3 mm long, pubescent. Corolla white or creamy; lobes linear, 6–7 mm long, acute; scales absent at throat. Berry globose, 3–4 cm diam., red to purplish.	[22-26]
M. aeneus Baill.	Lianas to 4-5 m. Petiole 3-5 mm; leaf blade narrowly elliptic, somewhat leathery, base rounded to cuneate, apex acuminate; lateral veins subparallel, prominent on both surfaces. Cymes terminal and axillary, 1-2.5 cm, 2-12-flowered; bracts and bracteoles ovate, ca. 2 mm. Pedicel 3-5 mm. Sepals triangular, 1-3 mm, ciliate, pubescent outside. Corolla white, 5-10 mm in diam., tube 5-8 mm; lobes orbicular, ca. 65-66 mm; corona scales 5, apex 2-cleft,. Berries fusiform, 5-9 X 2-3 cm. Seeds black, triangular, and smooth. Florescence. summer-autumn.	[27-29]

Table 2.2 Botanical descriptions of species belong to genus *Melodinus* (continued)

Plant Species	Botanical descriptions	Reference
M. angustifolius Hayata	Thickets, coral rocks; 100-1000 m. Taiwan. Lianas to 5 m. Petiole 2-5 mm; leaf blade narrowly elliptic, 5-10 X 1-2.1 cm, somewhat leathery, base rounded to cuneate, apex acuminate; lateral veins subparallel, prominent on both surfaces. Cymes terminal and axillary, 1.5-2.5 cm, 3-12-flowered; bracts and bracteoles ovate, ca. 2 mm. Pedicel 2-4 mm. Sepals triangular, 1-2 mm, ciliate, pubescent outside. Corolla white, 6-10 mm in diam., tube 5-8 mm; lobes orbicular, ca. 66 mm; corona scales 5, apex 2-cleft, slightly exserted from throat. Berries fusiform, 5-9 X 2-3 cm. Seeds black, triangular, smooth. Florescence. summer-autumn.	[29, 30]
M. australis (F.Muell.) Pierre	Woody climber with twining stems, sometimes tall, branchlets glabrous. Leaves oblong-lanceolate to narrow-elliptic, 4–10 cm long, 10–35 mm wide, apex bluntly acute or acuminate, margins sometimes undulate, glabrous; petiole 4–8 mm long. Flowers mostly 5–13 on each axillary peduncle. Calyx lobes ovate, ciliate, 1–1.5 mm long. Corolla orange-yellow or white; lobes broad, 2–3 mm long, obtuse; scales sometimes present at throat. Berry ovoid to ellipsoid, mostly 4–5 cm long, 1.5–3 cm diam., orange to red.	[25, 26]

Table 2.2 Botanical descriptions of species belong to genus *Melodinus* (continued)

Plant Species	Botanical descriptions	Reference
M. axillaris W.T.Wang	Humid forests; ca. 1000 m. S Yunnan Lianas to 3 m. Branchlets angular, slightly pubescent. Petiole 5-9 mm; Leaf blade oblong, 10-18 X 3.5-6 cm, papery, glabrous, base obtuse, apex acute; lateral veins 17-20 pairs, slightly prominent on both surfaces. Inflorescences axillary, 2-5-branched, 3.5-8 cm, pubescent; peduncle 2-3 cm; bracts and bracteoles narrowly elliptic, 2-3 mm, puberulent outside. Pedicel 2-3 cm. Flower buds narrowly oblong, short pubescent outside, acuminate. Sepals ovate, 3-3.5 ca. 2 mm, glabrous outside, ciliate, apex obtuse. Corolla white, ca. 1.2 cm, tube pubescent inside; lobes oblong; corona scales villous. Filaments pubescent. Ovary glabrous. Style filiform; pistil head conical. Florescence. May.	[25, 31-35]

Table 2.2 Botanical descriptions of species belong to genus *Melodinus* (continued)

Plant Species	Botanical descriptions	Reference
M. cochinchinensis (Lour.) Merr.	Montane forests; 800-2800 m. S Yunnan [Myanmar, Thailand, Vietnam]. Lianas stout, to 10 m, glabrous except for inflorescences. Branches dark brown. Petiole 6-10 mm. Leaf blade elliptic or narrowly so, 6-19 X 2.2-6.5 cm, papery, base cuneate, apex acute or acuminate; lateral veins numerous, convergent, conspicuous. Cymes paniculate, terminal, 3-branched, 4-5.5 cm, minutely pilose; bracts and bracteoles minute. Pedicel short. Sepals orbicular or broadly elliptic, ca. 2 mm, ciliate, apex subacute to rounded. Corolla white; tube ca. 6 mm, pilose except at base; lobes ovate, ca. 3.5 mm; corona large, lobes 2-cleft, villous. Ovary glabrous. Style ca. 3 mm. Berries narrowly ellipsoid, ca. 9 X 5 cm. Seeds oblong or ovate, ca. 1.3 cm. Florescence. Apr-May, fruit. Sep-Nov.	[28-30]

Table 2.2 Botanical descriptions of species belong to genus *Melodinus* (continued)

Plant Species	Botanical descriptions	Reference
M. fusiformis Champ. ex Benth.	Sparse montane woods, valleys; 300-1500 m. Guangdong, Guangxi, Guizhou. Lianas stout, to 10 m, juvenile parts pubescent, later glabrescent. Bark gray-brown. Petiole 4-6 mm; Leaf blade elliptic or oblong, rarely narrowly elliptic, 4.5-12 X 1-5.3 cm, somewhat leathery, base cuneate or rounded, apex acuminate; lateral veins ca. 15 pairs, obliquely spreading and reticulate toward margin. Cymes terminal, 3-5 cm, 6-12-flowered. Pedicel 5-10 mm. Sepals ovate, 4-5 mm, acute. Corolla white, tube 1.2-2 cm; lobes obliquely narrow ovate or obovate, (0.8-)1.1-2 cm X 3.5-9 mm; corona scales 5, indistinct, exserted, villous, apex 2- or 3-cleft. Stamens inserted near base of corolla tube. Berries fusiform, 3.5-5.3 X 2.2-4 cm. Florescence. Apr-Sep, fruit. Jun-Dec.	[25, 36-39]

Table 2.2 Botanical descriptions of species belong to genus *Melodinus* (continued)

Plant Species	Botanical descriptions	Reference
M. hemsleyanus Diels	Sparse montane woods; 500-1500 m. Guizhou, Sichuan, Yunnan. Lianas stout, to 8 m, juvenile parts densely minutely tomentose. Petiole ca. 5 mm; leaf blade elliptic, oblong, or narrowly so, 7-15 X 4-5 cm, somewhat leathery, lustrous and glabrous adaxially, pubescent near veins abaxially, base cuneate or obtuse, apex acuminate; lateral veins ca. 10 pairs, conspicuous on both surfaces. Cymes terminal. Sepals ovate-oblong, ca. 7 mm, densely pubescent outside, apex acu-minate. Corolla white, tube ca. 1 cm, puberulent on both surfaces; lobes narrowly elliptic, ca. 8 mm; corona scales minute, unequal. Berries ellipsoid, to 7.5 X 3 cm. Seeds narrowly elliptic, ca. 9 mm. Florescence. May-Aug, fruit. Jul-Dec.	[25, 26, 28-30]
M. khasianus Hook.f	Humid forests, valleys; 1600-2900 m. Guizhou, Yunnan, India. Lianas to 10 m, glabrous except for flowers. Petiole 6-7 mm; leaf blade narrowly elliptic, 6-12 X 0.5-4 cm, base cuneate, apex short acuminate; lateral veins subparallel. Cymes or fascicles axillary near branch apex, 2.5-6.5 cm, few flowered; bracts ca. 3 mm. Sepals orbicular, ca. 3 mm, ciliate. Corolla white, tube ca. 6 mm; lobes orbicular, ca. 5.5 mm in diam., obliquely 2-lobed or 2-cleft at apex; corona scales 5, oblong. Berries ovoid, ca. 5.5 X 4 cm. Florescence. October.	[25, 26, 28-30]

Table 2.2 Botanical descriptions of species belong to genus *Melodinus* (continued)

Plant Species	Botanical descriptions	Reference
M. magnificus Tsiang	Sparse woods; 500-800 m. Guangxi. Lianas to 6 m. Branchlets dark brown, rust-colored pubescent. Petiole 6-8 mm, pubescent; leaf blade narrowly oblong, 12-21 X 4-6.5 cm, somewhat leathery, glabrous, base cuneate, apex acuminate or rarely obtuse; lateral veins 15-20 pairs, subparallel, pubescent. Cymes terminal, shorter than leaves; bracteoles sublinear, 3-5 mm, short pubescent. Sepals oblong, pubescent outside. Corolla white, puberulent outside; tube 1.5-1.7 cm, pubescent inside; lobes obliquely obovate, 1.5-1.8 cm; Corona scales thick, indistinct, minutely pilose, adnate to corolla throat, apex shortly 2-cleft. Berries ellipsoid, 6-8 X 3-4 cm. Florescence. Jun-Aug, fruit. Oct-Dec.	[25, 26, 28-30]

Table 2.2 Botanical descriptions of species belong to genus *Melodinus* (continued)

Plant Species	Botanical descriptions	Reference
M. morsei Tsiang	Montane forests. Guangdong, Guangxi. Lianas to 3 m. Branchlets tomentose. Petiole ca. 5 mm; Leaf blade broadly ovate or orbicular, 4.5-9 X 2-7 cm, leathery, glabrescent and shiny adaxially, tomentose abaxially, base rounded or truncate, apex short acuminate; lateral veins 8-10 pairs, flat adaxially, slightly prominent abaxially. Cymes terminal, 6-8-flowered; peduncle 1-2 cm, tomentose. Pedicel ca. 3 mm. Sepals ovate, 3-4 X 2-2.5 mm, ciliate, villous outside, acute. Corolla white, tube ca. 1.5 cm, minutely tomentose; lobes oblong, falcate, ca. 13 4 mm; corona scales 5, oblong, pilose, apex 2-cleft. Filaments pilose. Ovary glabrous. Berries fusiform, ca. 8.5 X 2 cm, acuminate at both ends. Seeds orbicular, ca. 6 mm in diam. Florescence. August-October, fruit. September-December.	[28-30]

Table 2.2 Botanical descriptions of species belong to genus *Melodinus* (continued)

Plant Species	Botanical descriptions	Reference
M. suaveolens (Hance) Champion ex Bentham	Open forests, humid brushwood; 100-800 m. Guangdong, Guangxi, Hainan , Vietnam. Lycimnia suaveolens Hance in Walpers, Ann. Bot. Syst. 3: 31. 1852; Melodinus laetus Champion ex Bentham. Lianas to 10 m, glabrous except for inflorescences. Petiole to 1.2 cm; Leaf blade elliptic or ovate, 5-10 X 1.8-5 cm, leathery, base attenuate to rounded, apex short acuminate. Cymes terminal and axillary. Flower buds rounded or obtuse at apex, minutely pubescent outside. Flowers fragrant. Sepals ovate, ca. 3 mm, minutely pubescent outside, apex rounded or obtuse. Corolla white, tube 1-1.4 cm; lobes 0.5-1 as long as tube, suborbicular, falcate, with a distinct notch near apex; Corona campanulate or tubular, apex 5-cleft, exserted from throat. Berries globose, 5-8 cm in diam. Florescence. May-November, Fruit. August-December.	[25, 26, 28-30]

Table 2.2 Botanical descriptions of species belong to genus *Melodinus* (continued)

Plant Species	Botanical descriptions	Reference
M. tenuicaudatus Tsiang & P.T.Li	Dense montane forests, brushwoods; 800-1800 m. Guangxi, Guizhou, Yunnan. Lianas to 4 m. Branches gray; branchlets gray yellowish. Petiole ca. 5 mm; Leaf blade oblong to narrowly so, 6-15 X 1.5-4 cm, membranous, glabrous, base cuneate or broadly so, apex caudate-acuminate, acumen 1-1.5 cm; lateral veins numerous, subparallel at 70-80 °to midvein, flat on both surfaces. Cymes umbellate, terminal, 4-6 cm, 3-5-flowered; peduncle ca. 1.2 cm, puberulent; bracts and bracteoles narrowly elliptic, 2.5-4 mm. Pedicel ca. 5 mm. Sepals ovate. Corolla white, tube ca. 1.8 cm, glabrous outside, pubescent inside; lobes oblong, as long as tube; Corona scales 10, narrowly elliptic. Filaments puberulent. Berries sub-fusiform, 6.5-7 X 1.8-2.5 cm, acuminate at both ends or base obtuse. Florescence. May-September, Fruit. September -December.	[28-30, 40]

Table 2.2 Botanical descriptions of species belong to genus *Melodinus* (continued)

Plant Species	Botanical descriptions	Reference
M. yunnanensis Tsiang & P. T. Li	Dense montane forests; 1500-2000 m. Guangxi, Yunnan. Lianas to 10 m. Branches dark gray, glabrous; young branchlets and leaves scaly. Petiole 5-10 mm; Leaf blade oblong or narrowly elliptic, 7-18 X 2.5-5.2 cm, papery, base rounded, apex acuminate; lateral veins 10-15 pairs, nearly flat on both surfaces. Cymes umbellate, terminal and axillary, 5-6.5 cm; peduncle 1.5-2 cm, glabrous; bracts and bracteoles 3-7 mm. Pedicel 5-7 mm, pubescent. Flower buds cylindric, ca. 2 cm, glabrous outside. Sepals broadly ovate, ca. 7 5 mm, ciliate, apex acute. Corolla white, tube ca. 1.2 cm, pubescent inside; lobes oblong, ca. 1.1 cm; corona scales linear, decurrent to lower part of corolla tube, included. Ovary glabrous. Style very short. Berries globose, ca. 10.5 cm in diam. Florescence. May.	[28-30]

Figure 2.1 Herbarium samples of species of genus *Melodinus*

2.4 Traditional and medicinal uses of genus *Melodinus*

Some species, such as *Melodinus suaveolens* and *Melodinus henryi*, have been used in Chinese folk medicine for the treatment of meningitis in children and rheumatic heart diseases [41]. They can also "invigorate the circulation of blood", "stimulate sucking and treat fracture" [42]. It has also been used in Chinese folk medicine for the treatment of hernia, infantile malnutrition, dyspepsia and testitis [41].

Melodinus reticulatus has been used to treat cancers as alkaloids were detected in 1983 [43]. Several alkaloids were detected successively from *Melodinus acutiflorus* and *Melodinus tenuicaudatus* [44-46].

2.4.1 *In vitro* pharmacological activities of genus *Melodinus*.

Almost half of species have been studied for their pharmacological activities [40-49]. Extracts and pure compounds derived from *Melodinus* were reported to have a concentrate pharmacological activity of antitumor [40, 45, 47-49].

i. Anticancer activities

Anticancer activities were reported in 1998, demethyltenuicausine (I) [47], a new bisindole alkaloid, was isolated from *Melodinus hemsleyanus* which revealed antitumor activities in pharmacological tests [47].

Antitumor potential was demonstrated by the indole alkaloids from the leaves and twigs of *Melodinus fusiformis* and *Melodinus tenuicaudatus* via the MTT biological assay which showed significant cytotoxicity against five human tumor cell lines: SW480, SMMC-7721, HL-60, MCF-7 and A-549 [40, 45, 48, 57].

Nine isolated from *Melodinus suaveolens* revealed a positive cytotoxicity with IC_{50} all around 10 μM against five human cancer cell lines: human myeloid leukemia HL-60, hepatocellular carcinoma SMMC-7721, lung cancer A-549, breast cancer MCF-7, and colon cancer SW480 cells [49].

ii. Other Pharmacological Activities

A pharmacological test revealed that 11- hydroxyvincadifformine which was isolated from the aerial parts of *Melodinus hemsleyanus* has significant antifertility activity [50].

2.5 The phytochemistry of genus *Melodinus*.

Isolation and structure elucidations of secondary metabolites in *Melodinus* have been carried out since the 80s [40, 59]. The majority of investigations include the aerial part of the species. To date about one hundred compounds have been isolated from about fifteen species, which are *Melodinus acutiflorus, Melodinus aeneus, Melodinus Balansae, Melodinus celastroides, Melodinus fusiformis, Melodinus guillauminii, Melodinus hemsleyanus, Melodinus henryi, Melodinus morsei, Melodinus monobynus, Melodinus reticulatus, Melodinus scandens, Melodinus suaveolens, Melodinus tenuicaudatus,* and *Melodinus yunnanensis* [40, 43-45] [47-61].

The isolation and separation technique is very much dependent on the type of fractions. Chemical substances are separated with liquid chromatography using different solvent mixtures with silica gel [43], charcoal [48] and sephadex [40, 44]. Other types of analytical techniques include thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) [50, 51, 53, 56].

The structures are mainly established by mass spectroscopy (MS), ultra-violet spectroscopy (UV), infrared spectroscopy (IR) and ^{1}H and/or ^{13}C nuclear magnetic resonance (NMR). ^{1}H and/or ^{13}C spectroscopy is

probably the most useful method in structure elucidation [40, 43-45] [47-53] [55-61].

Among the secondary metabolites isolated from members of the genus *Melodinus*, most of them are alkaloids. The main secondary metabolites isolated so far from the genus *Melodinus* consists of 120 compounds. Cognition of these compounds can give a guideline of the isolation and identification of plants belong to the genus *Melodinus*. The names and structures of these compounds, as well as their corresponding plant sources are given in Table 2.3. According to the Table 2.3, plants from genus *Melodinus* should contain several alkaloids which are heterocyclic oxynitrides with comparatively high polarity. This trend should also be indicated on the target plant in this research project.

Table 2.3 Compounds in species of genus *Melodinus*

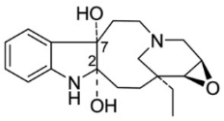
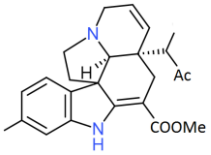
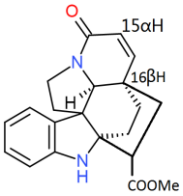
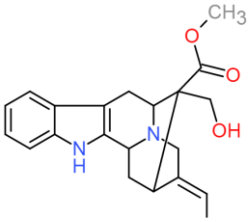
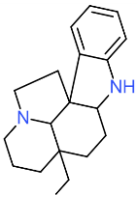
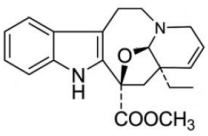
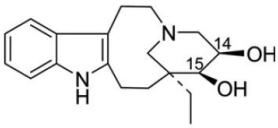
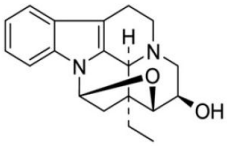
Compound	Structure	Plant species	Reference
2α,7α-Dihydroxy-dihydrovoaphylline		*M. yunnanensis*	[61, 81]
19-acetyltabersonine		*M. fusiformis* *M. morsei*	[48, 51, 53, 58, 62, 63, 75, 77]
15α-hydroxykopsmme		*M. guillauminii*	[64]
Akuammidine		*M. hemsleyanus*	[47, 50]
Aspidospermidine		*M. fusiformis* *M. morsei*	[48, 51, 53, 58, 75, 76]
16β,21β -Epoxy-vincadine		*M. yunnanensis*	[61, 63, 72, 81, 83]
14β,15β -20S-Quebrachamine		*M. yunnanensis*	[61, 78, 81, 82]
14β-Hydroxymeloyunine		*M. yunnanensis*	[61, 81]

Table 2.3 Compounds in species of genus *Melodinus* (continued)

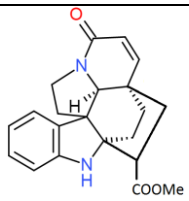
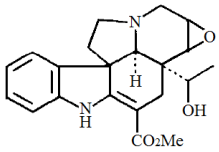
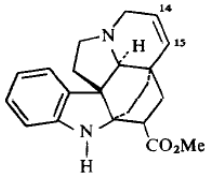
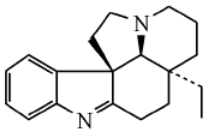
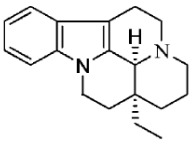
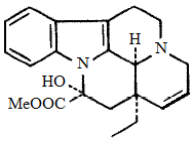
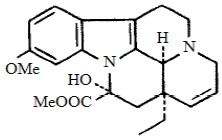
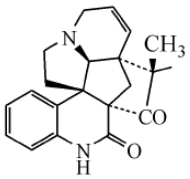
Compound	Structure	Plant species	Reference
19-β-Hydroxyvenalstomne		*M. guillauminii*	[64]
Cathovalinine		*M. suaveolens*	[52, 65]
Dehydro 14-15 venalstonine		*M. balansae*	[66]
Eburenine		*M. fusiformis* *M. morsei* *M. henryi*	[48, 51, 53,58-60, 67, 75]
Eburnamenine		*M. henryi*	[59, 60, 67]
Epi-16 dehydro l4-15 vincamine		*M. aeneus*	[63, 69, 72, 83]
Epi-16 dehydro l4-15 vincine		*M. aeneus*	[69, 83]
19-Epi-meloscandonine		*M. fusiformis* *M. hemsleyanus* *M. morsei*	[47, 48, 50, 51, 53, 58, 75]

Table 2.3 Compounds in species of genus *Melodinus* (continued)

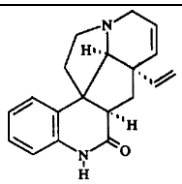
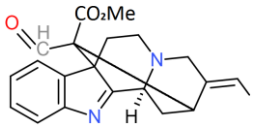
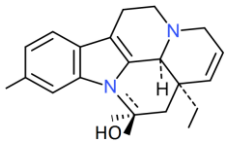
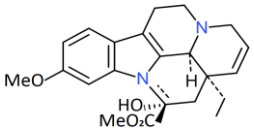
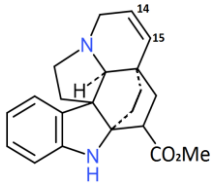
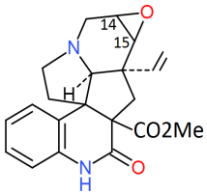
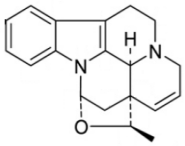
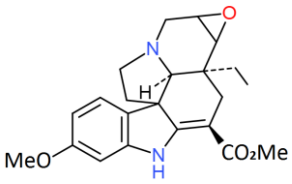
Compound	Structure	Plant species	Reference
Epimeloscine		*M. scandens*	[55]
16-Epi-rhazinaline		*M. acutiflorus*	[44, 70]
16-Epi-Δ^{14}-vmcanol		*M. guillauminii*	[64]
Δ^{14}-16-Eplvmcme		*M. guillauminii*	[64]
Epoxy 14-15α venalstonichne		*M. balansae*	[66]
14,15-Epoxyscandine		*M. hemsleyanus*	[47, 50]
16,19-Epoxy-Δ^{14}-vincanol		*M. yunnanensis*	[61, 63, 72, 81, 83]
Hazuntine		*M. suaveolens*	[52, 65]

Table 2.3 Compounds in species of genus *Melodinus* (continued)

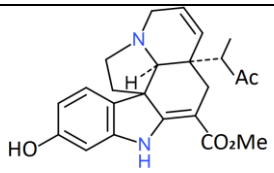
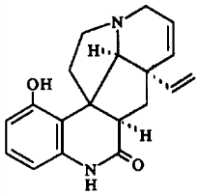
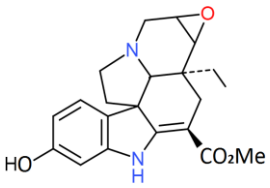
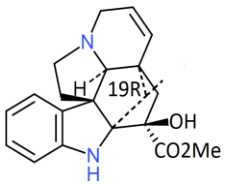
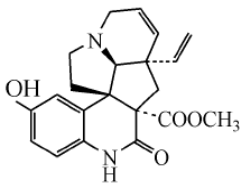
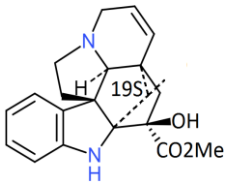
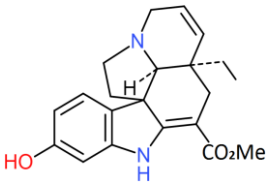
Compound	Structure	Plant species	Reference
11-Hydroxy-19-acetyl-tabersonine		*M. fusiformis* *M. morsei*	[48, 51, 53, 58, 62, 63, 75, 77]
9-Hydroxyepimelos cine		*M. scandens*	[55]
11-hydroxy-14, 15-epoxytabersonine		*M. fusiformis* *M. hemsleyanus* *M. morsei*	[47, 48, 50, 51, 53, 58, 62, 63, 75, 77]
16β -hydroxy-19R-vindolinine		*M. hemsleyanus*	[47, 50, 74, 83]
10-hydroxyscandine		*M. fusiformis* *M. hemsleyanus* *M. morsei*	[47, 48, 50, 51, 53, 58, 75]
16β -hydroxy-19S-vindolinine		*M. hemsleyanus*	[47, 50, 74, 83]
11-hydroxytabersonine		*M. fusiformis* *M. guillauminii* *M. hemsleyanus* *M. morsei* *M. suaveolens* *M. reticulatus* *M. tenuicaudatus*	[40, 43, 45,47-53, 58, 62-65, 75, 77, 80]

Table 2.3 Compounds in species of genus *Melodinus* (continued)

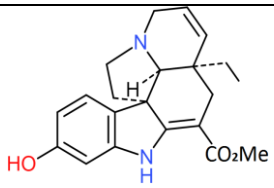
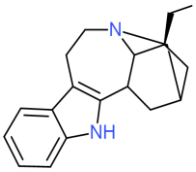
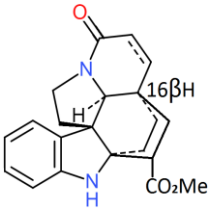
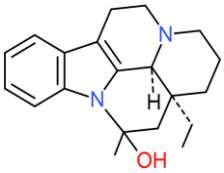
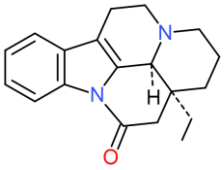
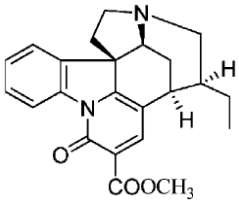
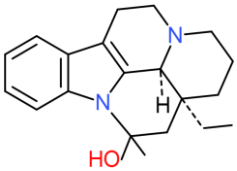
Compound	Structure	Plant species	Reference
11-hydroxyvincadifformine		*M. fusiformis* *M. hemsleyanus* *M. morsei*	[47, 48, 50, 51, 53, 58, 63, 72, 75, 83]
Ibogamine		*M. aeneus*	[69]
Kopsmme		*M. guillauminii*	[64]
Leburnamine		*M. celastroides*	[68]
Leburnamonine		*M. celastroides*	[68]
Leuconotis alkaloid 376		*M. henryi*	[60]
Lisoeburnamine		*M. celastroides*	[68]

Table 2.3 Compounds in species of genus *Melodinus* (continued)

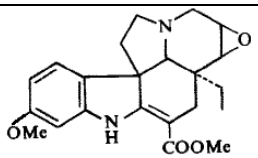
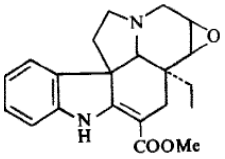
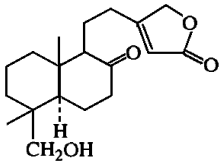
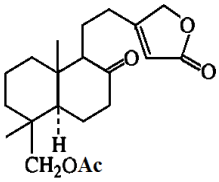
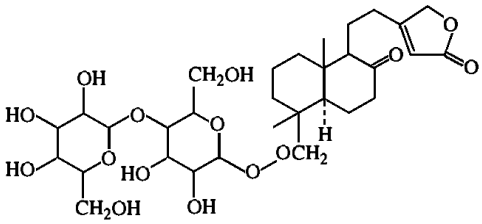
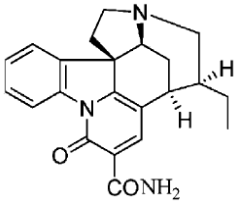
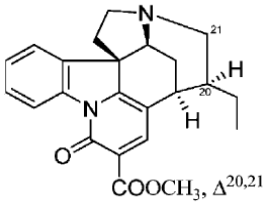
Compound	Structure	Plant species	Reference
Lochneinine		*M. aeneus*	[69, 73]
Lochnericine		*M. aeneus* *M. fusiformis* *M. morsei* *M. suaveolens*	[48, 51, 52, 53, 58, 65, 69,73, 75, 80]
Medigenin		*M. monogynus*	[71, 80]
Medigenin acetate		*M. monogynus*	[71, 80]
Medinin		*M. monogynus*	[71,80]
Melodinine A		*M. henryi*	[60]
Melodinine B		*M. henryi*	[60]

Table 2.3 Compounds in species of genus *Melodinus* (continued)

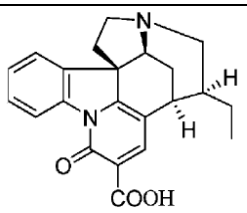
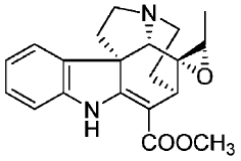
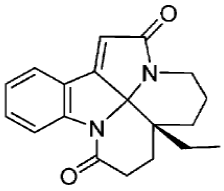
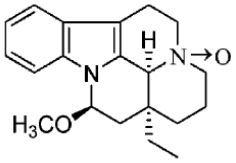
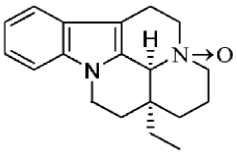
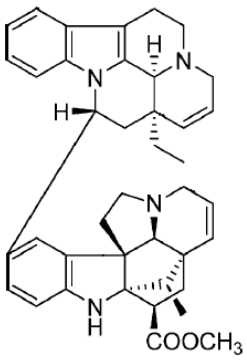
Compound	Structure	Plant species	Reference
Melodinine C		*M. henryi*	[60]
Melodinine D		*M. henryi*	[60]
Melodinine E		*M. henryi*	[60]
Melodinine F		*M. henryi*	[60]
Melodinine G		*M. henryi*	[60]
Melodinine H		*M. tenuicaudatus*	[40]

Table 2.3 Compounds in species of genus *Melodinus* (continued)

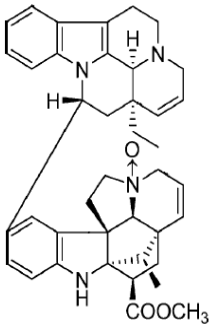
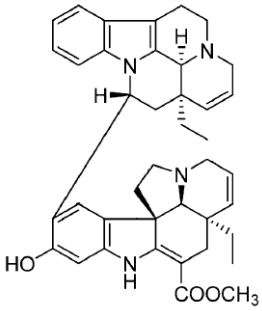
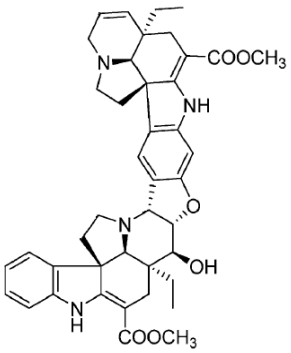
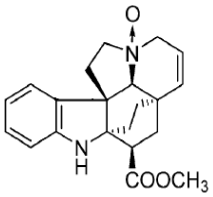
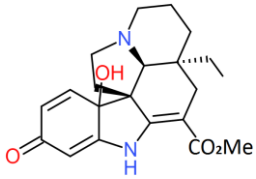
Compound	Structure	Plant species	Reference
Melodinine I		*M. tenuicaudatus*	[40]
Melodinine J		*M. tenuicaudatus*	[40]
Melodinine K		*M. tenuicaudatus*	[40]
Melodinine L		*M. tenuicaudatus*	[40]
Melodinine M		*M. suaveolens*	[49, 52, 65]

Table 2.3 Compounds in species of genus *Melodinus* (continued)

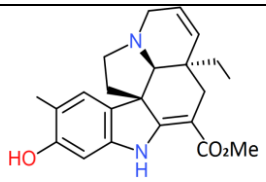
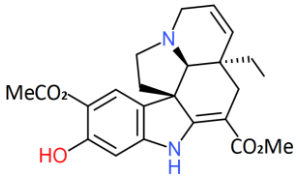
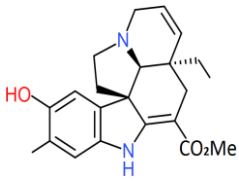
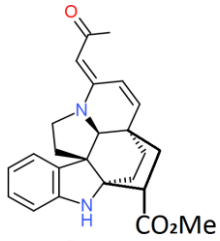
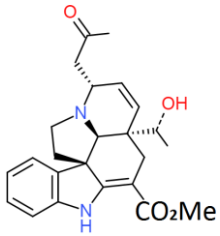
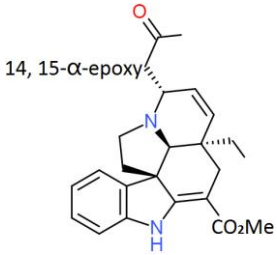
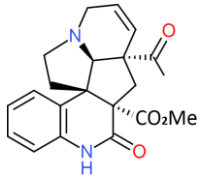
Compound	Structure	Plant species	Reference
Melodinine N		*M. suaveolens*	[49, 52, 65]
Melodinine O		*M. suaveolens*	[49, 52, 65]
Melodinine P		*M. suaveolens*	[49, 52, 65]
Melodinine Q		*M. suaveolens*	[49, 52, 65]
Melodinine R		*M. suaveolens*	[49, 52, 65]
Melodinine S		*M. suaveolens*	[49, 52, 65]
Melodinine T		*M. suaveolens*	[49, 52, 65]

Table 2.3 Compounds in species of genus *Melodinus* (continued)

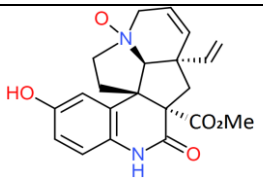
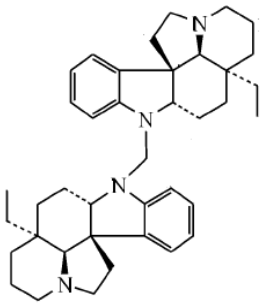
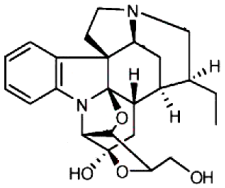
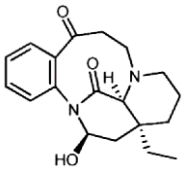
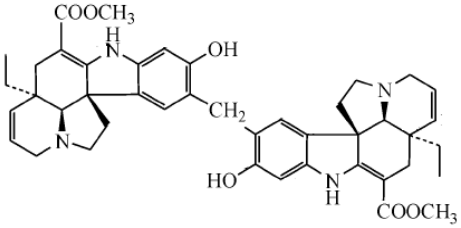
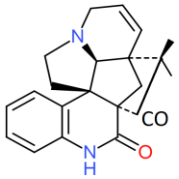
Compound	Structure	Plant species	Reference
Melodinine U		*M. suaveolens*	[49, 52, 65]
Melofusine		*M. fusiformis* *M. morsei*	[48, 51, 53, 58, 75]
Melohenine A		*M. henryi*	[59]
Melohenine B		*M. henryi*	[59]
Melomorsine		*M. fusiformis* *M. morsei*	[48, 51, 53, 58, 75]
Meloscandonine		*M. fusiformis* *M. hemsleyanus* *M. morsei* *M tenuicaudatus*	[40, 45, 47, 48, 50, 51, 53, 58, 75, 80]

Table 2.3 Compounds in species of genus *Melodinus* (continued)

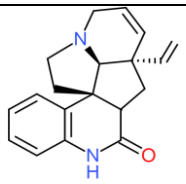
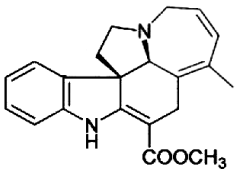
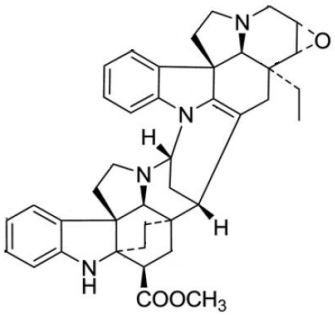
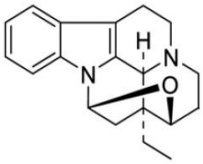
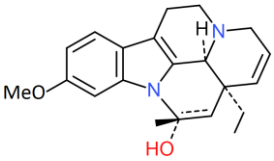
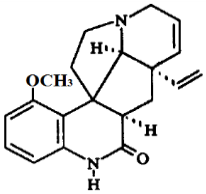
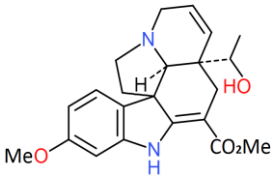
Compound	Structure	Plant species	Reference
Meloscine		*M. hemsleyanus*	[47, 50]
Melotenine A		*M. tenuicaudatus*	[57]
Meloyine		*M. yunnanensis*	[61, 81]
Meloyunine		*M. yunnanensis*	[61, 81]
11-Methoxyd14-vmcanol		*M. guillauminii*	[64, 79]
9-Methoxyepimeloscine		*M. scandens*	[55, 79]
11-Methoxy-19-(R)-hydroxytabersonine		*M. suaveolens*	[49, 52, 62, 63, 65, 77, 79]

Table 2.3 Compounds in species of genus *Melodinus* (continued)

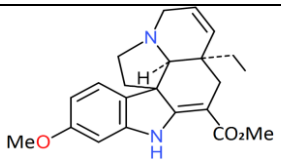
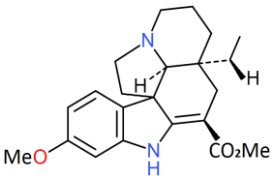
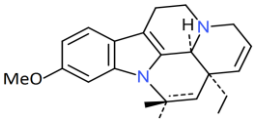
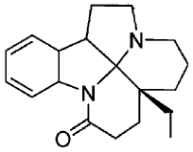
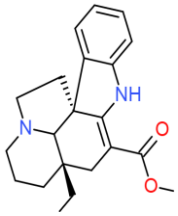
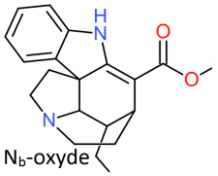
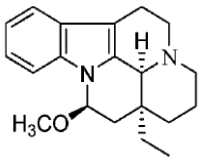
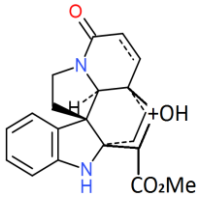
Compound	Structure	Plant species	Reference
11-Methoxytabersonine		*M. aeneus* *M. guillauminii* *M. hemsleyanus* *M. suaveolens* *M. tenuicaudatus*	[40, 45, 47, 49-50, 52, 62-65, 69, 77, 79, 80]
11-Methoxy vincadifformine		*M. suaveolens*	[49, 52, 63, 65, 72, 79, 83]
11-Methoxy-Δ^{14}-vmcamenme		*M. guillauminii*	[64, 79]
N-acyl-indolinique		*M. fusiformis* *M. morsei*	[48, 51, 53, 58, 75, 82]
N_b-oxide vincadifformine		*M. fusiformis* *M. morsei* *M. suaveolens*	[48, 49, 51-53, 58, 63, 65, 72, 75, 83]
N_b-oxyde tubotaiwine		*M. aeneus*	[69]
O-methylepivincanol		*M. henryi* *M. tenuicaudatus*	[40, 45, 59-60, 63, 72, 79, 83]
3-Oxo-hydroxykopsmme		*M. guillauminii*	[64]

Table 2.3 Compounds in species of genus *Melodinus* (continued)

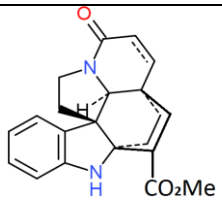
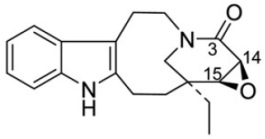
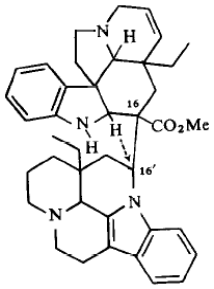
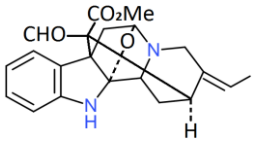
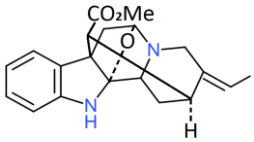
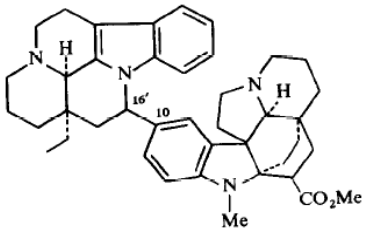
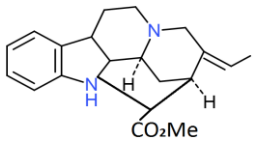
Compound	Structure	Plant species	Reference
3-Oxovenalstomine		*M. guillauminii*	[64]
3-Oxo-voaphylline		*M. yunnanensis*	[61, 81]
Paucivenine		*M. balansae*	[66]
Picralinal		*M. hemsleyanus*	[47, 50]
Picrinine		*M. hemsleyanus*	[47, 50]
Pleiomutine		*M. balansae*	[66]
Plemcarpamme		*M. guillauminii*	[64]

Table 2.3 Compounds in species of genus *Melodinus* (continued)

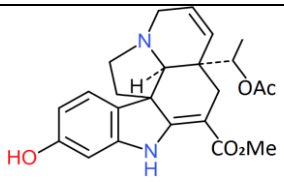
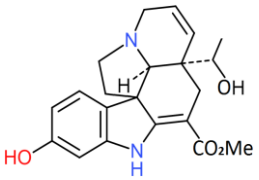
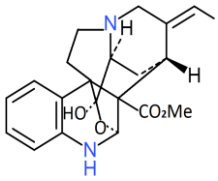
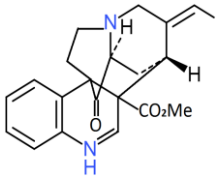
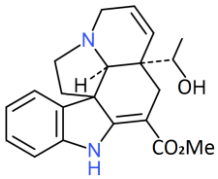
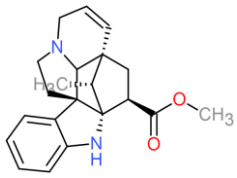
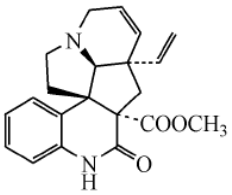
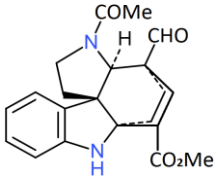
Compound	Structure	Plant species	Reference
19-R-acetoxytabersonine		*M. suaveolens*	[49, 52, 62, 63, 65, 77]
11,19R-dihydroxytabersonine		*M. hemsleyanus* *M. suaveolens*	[47, 49, 50, 52, 62, 63, 65, 77]
Rhaxicine		*M. acutiflorus*	[44, 70]
Rhaximine		*M. acutiflorus*	[44, 70]
19R-hydroxytabersonine		*M. suaveolens*	[49, 52, 62, 63, 65, 77]
19R-Vindolinine		*M. hemsleyanus* *M. morsei* *M. suaveolens* *M. tenuicaudatus*	[40, 45, 47-53, 65, 74, 83]
Scandine		*M. fusiformis* *M. hemsleyanus* *M. morsei* *M. scandens* *M. tenuicaudatus*	[40, 45, 47, 48, 50, 51, 53, 55, 58, 75, 80]
14,15-Seco-3-oxokopsmal		*M. guillauminii*	[64]

Table 2.3 Compounds in species of genus *Melodinus* (continued)

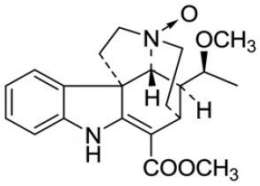
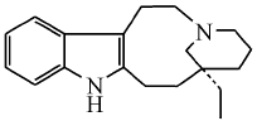
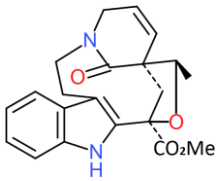
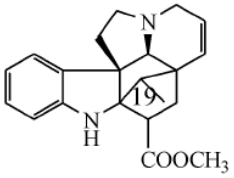
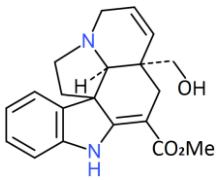
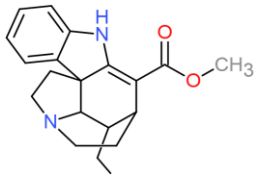
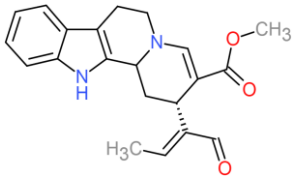
Compound	Structure	Plant species	Reference
19S-Methoxytubotaiwine N_4-oxide		*M. yunnanensis*	[61, 79, 81]
(S)-quebrachamine		*M. fusiformis* *M. morsei*	[48, 51, 53, 58, 75, 78, 82]
Suaveolenine		*M. suaveolens*	[49, 52, 65]
19S-vindolinine		*M. fusiformis* *M. morsei* *M. suaveolens* *M. tenuicaudatus*	[40, 45, 48, 49, 51-53, 58, 65, 74, 75, 83]
Tabersonine		*M. aeneus* *M. celastroides* *M. fusiformis* *M. hemsleyanus* *M. morsei* *M. suaveolens* *M. reticulatus*	[43, 47-53, 58, 62, 63, 65, 68, 69, 75, 77, 80]
Tubotaiwine		*M. aeneus*	[69]
Vallesiachotamine		*M. henryi*	[59, 60]

Table 2.3 Compounds in species of genus *Melodinus* (continued)

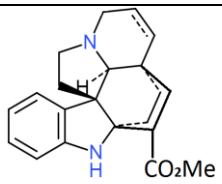
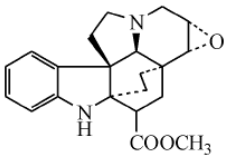
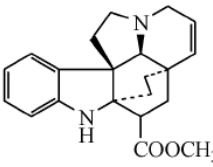
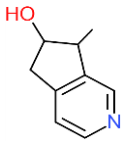
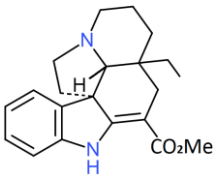
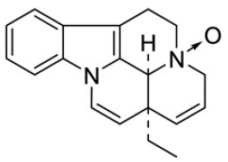
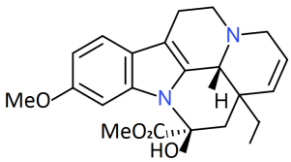
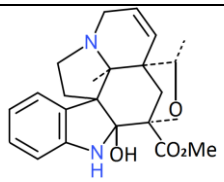
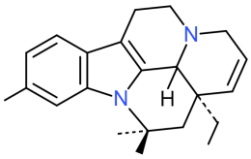
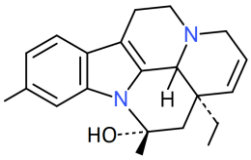
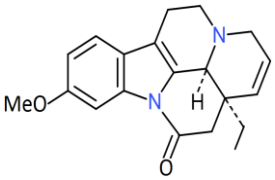
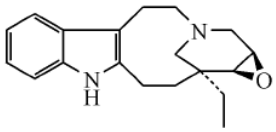
Compound	Structure	Plant species	Reference
Venalstomne		*M. guillauminii*	[64]
Venalstonidine		*M. fusiformis* *M. hemsleyanus* *M. morsei*	[47, 48, 50, 51, 53, 58, 75]
Venalstonine		*M. fusiformis* *M. hemsleyanus* *M. morsei* *M. tenuicaudatus*	[40, 45, 47, 48, 50, 51, 53, 58, 75]
Venoterpine		*M. aeneus*	[69, 76]
Vincadifformine		*M. aeneus* *M. fusiformis* *M. morsei*	[48, 51, 53, 58, 63, 69, 72, 75, 83]
Δ^{14}-Vincamenine N_4-oxide		*M. yunnanensis*	[61, 63, 72, 81, 83]
Δ^{14}-Vincine		*M. suaveolens*	[49, 52, 65, 83]

Table 2.3 Compounds in species of genus *Melodinus* (continued)

Compound	Structure	Plant species	Reference
Vincoline		*M. suaveolens*	[49, 52, 65, 83]
Δ^{14}-Vmcamenme		*M. guillauminii*	[64]
Δ^{14}-Vmcanol		*M. guillauminii*	[64]
Δ^{14}-Vmcmone		*M. guillauminii*	[64]
Voaphylline		*M. fusiformis* *M. morsei*	[48, 51, 53, 58, 75]

2.6 The commercial uses of *Melodinus* produces

There is no relative report mentioned on *Melodinus* products. However, according to its potential, *Melodinus* can be published as a high-value export crop for medicinal use [49-77].

2.7 Selection of plant material

2.7.1. The selection of *Melodinus eugeniifolius.*

The selection of plant material for the screening of biological activity can be based on a random selection or based on ethnopharmacology, where existing knowledge of the particular healing properties have been handed down from generation to generation especially amongst traditional healers. An additional mechanism for the identification of the plants for the study of its chemical constituents is based on chemotaxonomy. Chemotaxonomy is a science focusing on the correlation between related plant species and the occurrence of similar secondary metabolites [21].

2.7.2 The selection of bioassays performed

The origin and design of a screening process incorporates knowledge attained in ethnomedicine, traditional uses of the plant species,

phytochemical evaluation and correlation to specific biological targets as well as the use of natural product libraries and general or targeted literature review. Stable standardized crude extracts are prepared and assayed for the claimed activities for which the particular plant species is traditionally used. The present study is focused on antioxidant and anticancer activities.

2.8 Brief introduction to *Melodinus eugeniifolius* Wall. ex G. Don.

The record of this plant was derived from World Checklist of Seed Plants (WCSP) which reports it as a synonym (record 124204) with original publication details: *Gen. Hist. 4: 101 1837*.

The plant *Melodinus eugeniifolius* Wall. ex G. Don. in Apocynaceae Family is a rare and brand-new plant which has just been discovered by Dr. Christophe Wiart in the Malaysia rain forest [20]. The characteristic description and identification has not been completed yet, not to mention research of its chemical composition. Hence, until now, almost all the research on this plant is brand-new [20]. (1. According to the current *World Checklist of Seed Plants* published by the *International Association for Plant Taxonomy (IAPT)*, there was only a name and photo of the plant, no original plant sample, definite macroscopical identification, microscopic identification, chemical composition research,

standard determination assay; 2. There is no reference or literature report found in SCI (Science Citation Index), or in other database.).

CHAPTER Ⅲ

PLANT COLLECTION AND EXTRACTION

3.1 *Melodinus eugeniifolius* Wall. ex G. Don.

A rare species exists as a stout climber, with white, sticky and poisonous latex. Stems are erect. Leaves are opposite with petiole, 4-16cm long, 1-6cm wide, without stipules, simple, primary nerve is transverse parallel venation 6-14 pairs, raised in both sides of blade, with an intramarginal nerve and there is a nerve around the edge of the leaf; blade elliptical or ovate, apex cuspidate, base acute, edge entire, herbaceous, smooth or light rough. Twigs are often flattened, without tendrils. Flower in cymes, terminal or axillary. Flowers mainly from September to April and fruits from November to May (Figure 3.1) [21].

Figure 3.1: Melodinus eugeniifolius Wall. ex G. Don. (fruits and leaves)

3.2 Methodology

3.2.1 Collection and identification of plant material

The leaves and barks of *Melodinus eugeniifolius* Wall. ex G.Don were collected from one individual vine in August 2012 from Bukit Putih, Selangor, Malaysia (3°5'24" N, 101°46'0" E). The plant was identified and authenticated by Mr. Kamarudin Saleh, Forest Biodiversity Division, Forest Research Institute Malaysia (FRIM). Voucher specimens were prepared and deposited in the Kepong Herbarium (KEP) of FRIM, and the School of Pharmacy, Faculty of Sciences, The University of Nottingham, Malaysia Campus for further reference (Figure 3.2).

Figure 3.2 Herbarium samples of *Melodinus eugeniifolius* Wall. ex G.

3.2.2 Preparation of plant material

The leaves and barks were air dried at ambient temperature (25 ^{o}C) in the laboratory for 15 days. Thereafter the leaves were separated from the barks and any fruit present were removed and stored separately. The leaves and barks were then crushed to a fine powder using a grinder (Philipus HR 2084, China) separately, followed by weighing with a top loading balance (Sartorious AG, Germany) prior to extraction.

Extraction was performed with maceration that involves leaving the pulverized plant to soak in a suitable solvent in a closed container at room temperature. Dried and grinded sample of barks (2.5kg) and leaves (0.3kg) were soaked in hexane (MERCK, Germany) with the ratio of 1:3 parts of sample to solvent for 3 days in a 60^{o} C water bath (Julabo, Germany), then filtered through qualitative filter paper No.1 (Whatman International Ltd., UK) and the collected filtrate was concentrated to dryness under reduced pressure at 40 ^{o} C using a rotary evaporator (Buchi Labortechnik AG, R-200, Switzerland). This was repeated 3 times. Thereafter the leaves and barks were left to air dry completely for 3 days before repeating the whole process with chloroform (MERCK, Germany) and then ethanol (MERCK, Germany) respectively. Eventually, the dried extracts obtained were weighed with analytical balance (Sartorius AG,

Germany) and stored in glass scintillation vials (Kimble, USA) at -20 °C until further use.

3.2.3 Determination of extraction yield

For each extraction, the extraction yields of crude extracts were calculated. The extraction yield was expressed as the weight percentage of the dried plant extract obtained with respect to the dried plant material used, which was given as follows [84, 85]:

$$\text{Extraction yield (\%)} = \frac{\text{Weight of dried plant extract (g)}}{\text{Weight of dried plant material (g)}} \times 100\%$$

3.2.4 Evaluation of organoleptic properties

The organoleptic properties of crude extracts were assessed by their colour, texture and odour [86]. These organoleptic characters were determined using the senses of sight (eyes), touch (skin) and smell (nose) [87].

i. Colour

For colour determination, each crude extract was properly examined under diffuse daylight. An artificial light source with wavelengths similar to those of daylight might be use if necessary [88].

ii. Texture

For texture determination, small quantity of each crude extract was taken and examined by rubbing it between the thumb and index finger [89].

iii. Odour

For odour determination, if the crude extract was expected to be innocuous, small portion of the respective extract was placed in the palm of the hand or in a beaker of suitable size, and examined by slow and repeated inhalation of the air over the extract. If no distinct odour was perceptible, the crude extract was rubbed between the thumb and index finger or between the palms of the hands using gentle pressure. If the crude extract was known to be dangerous, small quantity of boiling water was poured onto the respective extract places in a beaker [90].

3.3 Results

3.3.1 Extraction yields of crude extracts of *Melodinus eugeniifolius*.

The extraction yield in percentage for each crude extract is shown in Figure 3.3 (Appendix A). For different solvent used for extraction, the yield for the hexane, chloroform and ethanol extract of barks were 1.02%, 0.67% and 0.56% respectively. The yields for the hexane, chloroform

and ethanol extract of leaves for were 5.00%, 3.90% and 3.40% (Figure 3.3, Appendix A).

3.3.2 Organoleptic properties of crude extracts of *Melodinus eugeniifolius*.

The colour, texture and odour of crude extracts in different solvent were characterized in Table 3.1. As compared to hexane and chloroform extracts, ethanol extracts were found to be better in retaining in natural fragrance of the plants.

3.4 Discussion

Extraction yield is a measure of the solvent efficiency to extract specific components from the original material [91, 92]. The extraction yield in percentage for each crude extract is shown in Figure 3.3 (Appendix A). For different solvent used for extraction, the leaf hexane extract indicated the highest extraction yield of 5.00%, and the bark ethanol extract indicated the lowest extraction yield of 0.56%. Although the result indicates that the hexane is a superior extraction solvent to chloroform or ethanol in terms of proving a better extraction yield, it does not mean that the hexane extracts have the best chemical capacity.

Organoleptic evaluation refers to the evaluation of individual drugs and formulations by colour, texture and odour [93, 94]. The colour, texture and odour of crude extracts in different solvent were characterized (Table 3.1). As compared to hexane and chloroform extracts, ethanol extracts were found to be better in retaining in natural fragrance of the plants. This may be attributed to the preservative ability of ethanol (reducing breakdown of organic compounds by microorganisms), its enhanced extraction capability (more fragrant components extracted) or a combination of both [86, 95].

Table 3.1 Organoleptic properties of crude extracts of *Melodinus eugeniifolus*.

Organoleptic property	Crude extract					
	Bark Hexane (BH)	Bark Chloroform (BC)	Bark Ethanol (BE)	Leaf Hexane (LH)	Leaf Chloroform (LC)	Leaf Ethanol (LE)
						
Colour	Brownish black	Brownish black	Brownish black	Greenish black	Brownish black	Brownish black
Texture	Waxy	Flaky/Powdery	Sticky	Waxy	Flaky/Powdery	Sticky
Odour	Pungent smell	Pungent smell	Sweet smell	Leafy smell	Pungent smell	Sweet smell

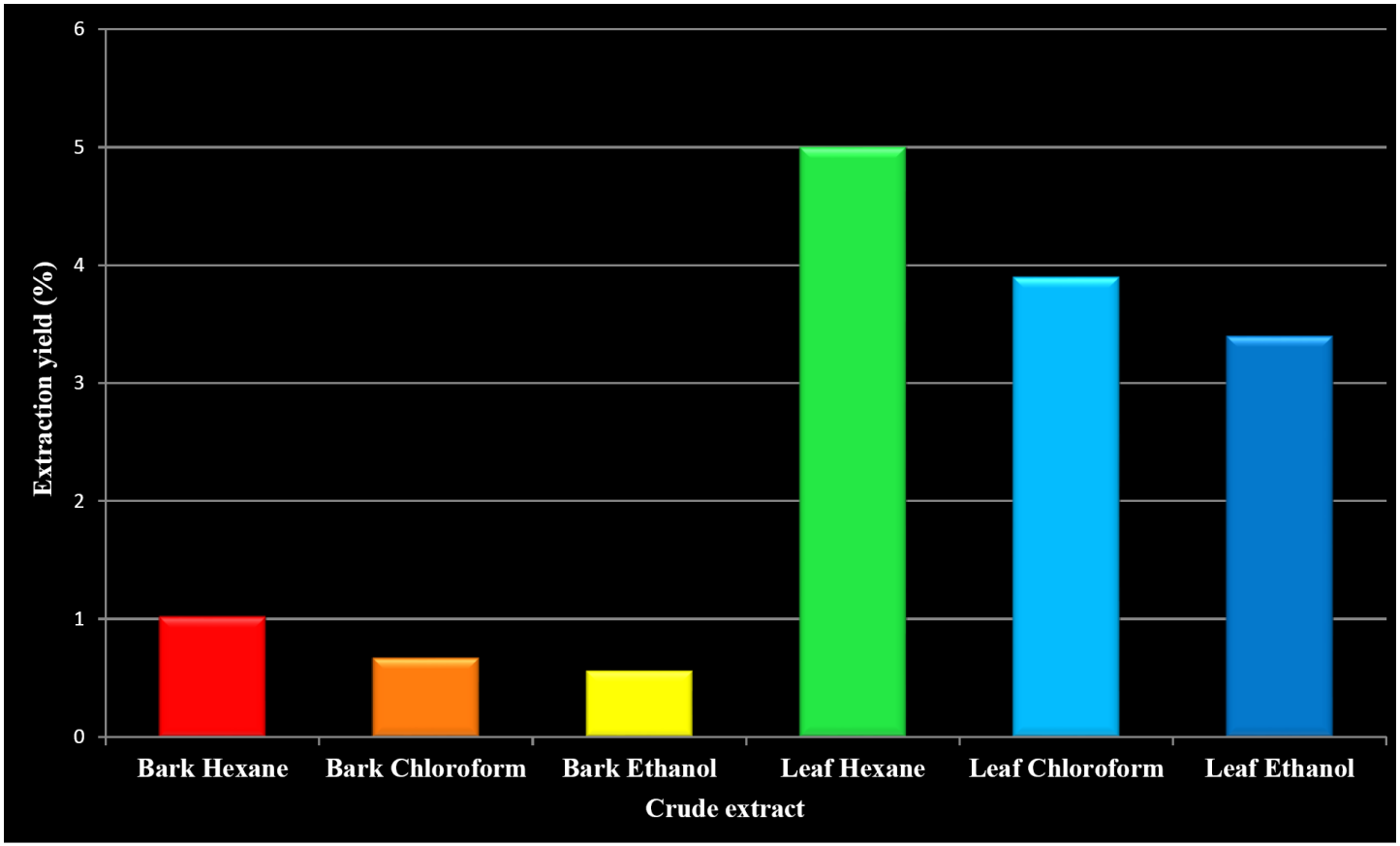


Figure 3.3 Extraction yields of crude extracts of *Melodinus eugeniifolus*.

CHAPTER Ⅳ

PHYTOCHEMICAL SCRENNING OF *MELODINUS EUGENIIFOLIUS*

4.1 Introduction

Phytochemical screenings are basically done to detect the types of secondary metabolites that may be present in the plant extract. A positive reaction should not be taken as proof of presence of a certain type of secondary metabolite because other compound types may give false-positive reactions. Despite this caveat, these detection methods are often effective for generating hyphotheses about what types of secondary metabolites may be present in a mixture of "unknowns" and of monitoring the presence of compounds of interest [96]. Qualitative phytochemical determinations of the crude extracts were expatiated below.

4.2 Phytochemical assay protocol

Qualitative phytochemical analysis of the crude extract was determined as outlined below [96, 97].

4.2.1 Test for alkaloids (Mayer's test , Wagner's test and Dragendorff's test)

200 mg of the extract was dissolved in 10 mL of methanol and heated on a boiling water bath with 2N HCl (5 mL). After cooling, the mixture was filtered and the filtrate was divided into two equal portions. One portion was treated with a few drops of Mayer's reagent and the other with equal amounts of Wagner's reagent. The samples were then observed for the presence of turbidity or precipitation. A (+) score was recorded if the reagent produced only a slight opaqueness; a (++) score was recorded if a definite turbidity, but no flocculation was observed and a (+++) score was recorded if a definite heavy precipitate or flocculation was produced [89, 97].

Mayer's reagent consists of 2 solutions described here as solution I and solution II. Solution I: 1.36g $HgCl_2$ was dissolved in 60mL water. Solution II: 5g KI was dissolved in 10mL water. Both solutions are combined and diluted with water to 100mL. If alkaloids are present a white to yellowish precipitate will appear [97].

Wagner's reagent consists of 1.27g I_2 (sublimed) and 2g KI was dissolved in 20mL water and water is made up to 100mL. A brown precipitate indicates the presence of alkaloids [97].

Dragendorff's test: Prior to detection of alkaloids, solution A was prepared by dissolving 1.7 g of bismuth subnitrate (Mallinckrodt, USA) in 100 mL of 4:1 (v/v) of distilled water and acetic acid (Systerm, Malaysia), whereas 40 g of potassium iodide (R & M Chemicals, UK) was dissolved in 100 mL of distilled water as solution B. To prepare Dragendorff's reagent, 5 mL of solution A and B was added in 20 mL of acetic acid and topped up with distilled water to 100 mL [98].

Approximately 20 mg of each crude extract was mixed with 4 mL of methanol (MERCK, Germany). The mixture was filtered and the filtrate was divided into two test tubes with one portion as the control. Another portion of the filtrate was tested with 1 mL of 1% (v/v) of hydrochloric acid (HCl) (Systerm, Malaysia) and warmed on steam bath, followed by addition of a few drops of Dragendorff's reagent. Reddish orange precipitation indicated the presence of alkaloids [99].

4.2.2 Test for flavonoids (Shinoda test)

40mg plant material was dissolved in 2mL ethanol and filtered. The filtrate was treated with a few drops of concentrated HCl and magnesium turnings (0.5 g). The presence of flavonoids was indicative if pink or magenta-red colour developed within 3 min [97, 100].

4.2.3 Test for saponins (Frothing test)

About 2.5 g of the plant material was extracted with boiling water. After cooling, the extract was shaken vigorously to froth and was then allowed to stand for 15-20 min and classified for saponin content as follows: (no froth = negative; froth less than 1 cm = weakly positive; froth 1.2 cm high = positive; and froth greater than 2 cm high = strongly positive) [97, 101].

4.2.4 Test for tannins (Gelatine-salt test)

Approximately 10mg of each crude extract was mixed with 4 mL of hot distilled water. The mixture was filtered and the filtrate was divided in three test tubes. To the first portion of the filtrate, 1 mL of 1% (w/v) of sodium chloride (NaCl) (R & M Chemical, UK) solution was added as the control. The second portion of the filtrate was added with 1 mL of 1% (w/v) of sodium chloride and 1 mL of 5% (w/v) of gelatine (R & M Chemical, UK), whereas a few drops of 5% (w/v) of $FeCl_3$ $6H_2O$ were added to the third portion of the filtrate. Formation of a precipitate in the second treatment suggests the presence of tannins, and a positive response after addition of $FeCl_3$ $6H_2O$ to the third portion supports this inference [97, 103].

4.2.5 Test for sterols (Salkowski test).

40mg of extract was dissolved in 2mL of chloroform and filtered. The filtrate was then added to 1mL of concentrated H_2SO_4. The presence of sterols was indicated by the 2 phase formation with a red color in the chloroform phase [96, 97].

4.2.6 Test for cardiac glycosides (Keller-Kiliani test).

40mg extract was dissolved in 2 mL ethanol and filtered. The filtrate was treated with 1mL glacial acetic acid, then added to a few drops of $FeCl_3$ and concentrated H_2SO_4. The presence of cardiac glycosides was indicated by the green-blue colour indicates [96, 97].

4.3 Results

Phytochemical analysis results exhibits the presence of alkaloids, flavonoids, sterols and cardiac glycosides in all the crude extracts as shown in Table 4.1, Figure 4.1- 4.6. Saponins were detected in all the chloroform and ethanol extracts. Tannins were not detected in any of crude extract (Table 4.1, Figure 4.1- 4.6).

Table 4.1: Preliminary phytochemical analysis of the crude extract of *Melodinus eugeniifolus*.

Sample	Alkaloids	Flavonoids	Saponins	Tannins	Sterols	Cardiac glycosides
BH	++	++	-	-	++	+++
BC	+++	+	+	-	++	++
BE	++	+	+	-	++	++
LH	++	++	-	-	++	+++
LC	+++	+	+	-	++	+++
LE	+	++	+	-	++	++

BH: bark hexane extract, BC: bark chloroform extract, BE: bark ethanol extract, LH: leaf hexane extract, LC: leaf chloroform extract, LE: leaf ethanol extract, and -: negative, +: trace, ++: positive, and +++: strongly positive.

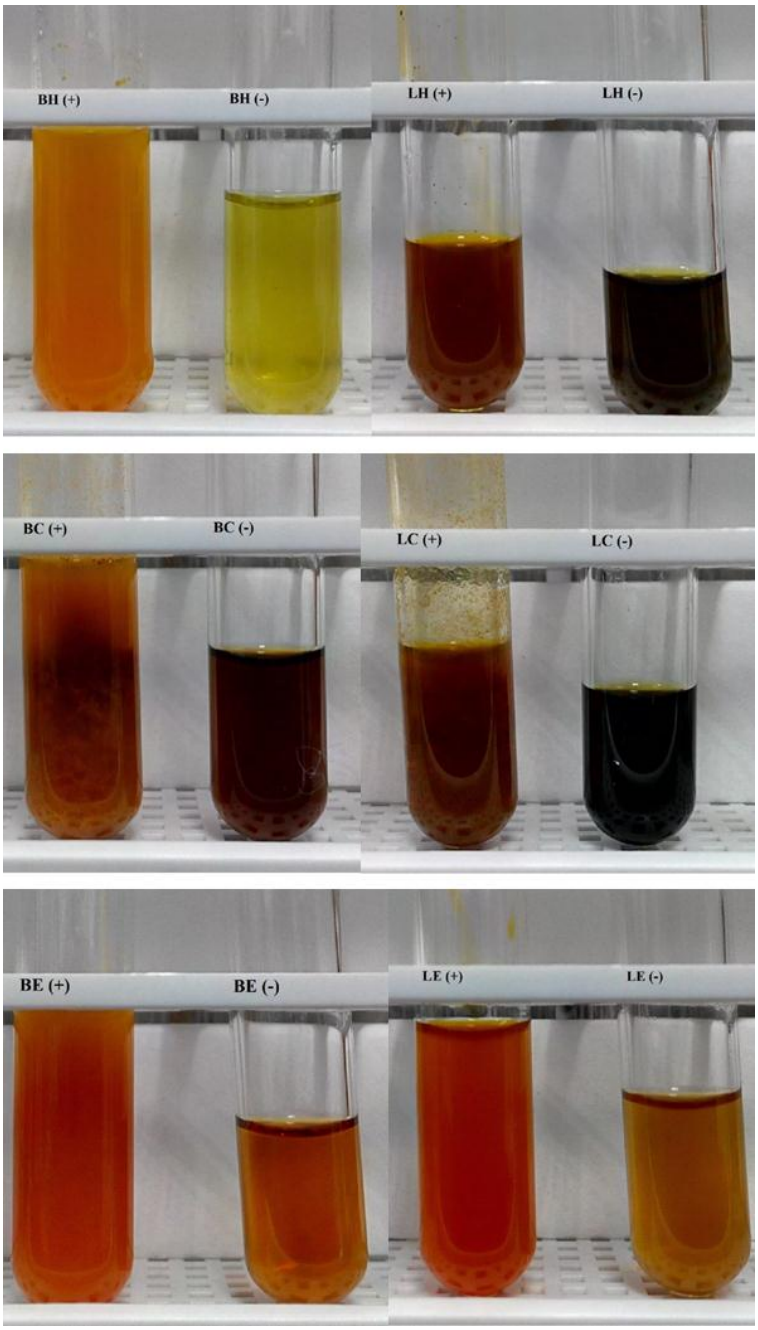


Figure 4.1: Positive alkaloid result on crude extracts of *Melodinus eugeniifolius.*

BH: bark hexane extract, BC: bark chloroform extract, BE: bark ethanol extract, LH: leaf hexane extract, LC: leaf chloroform extract, LE: leaf ethanol extract, and (-): blank, (+): tested.

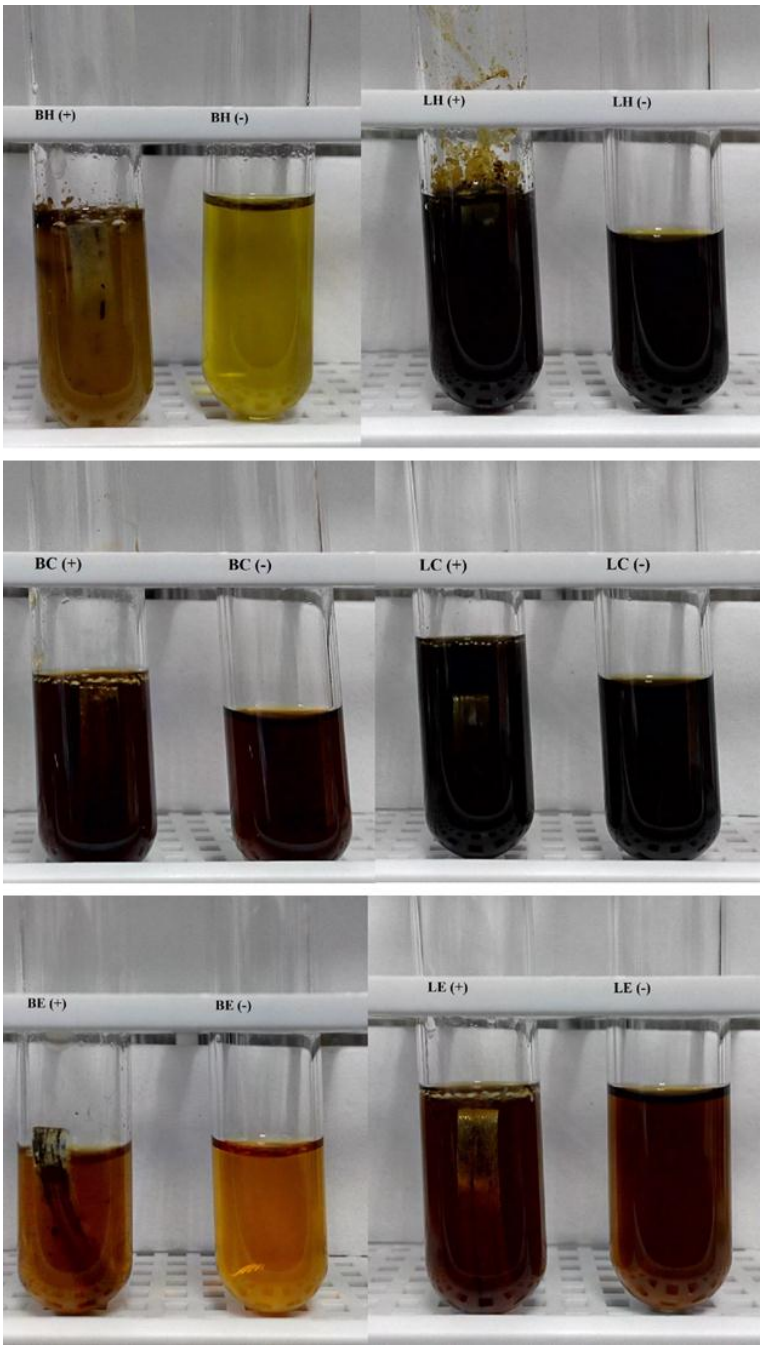


Figure 4.2: Positive flavonoid result on crude extracts of *Melodinus eugeniifolius.*

BH: bark hexane extract, BC: bark chloroform extract, BE: bark ethanol extract, LH: leaf hexane extract, LC: leaf chloroform extract, LE: leaf ethanol extract, and (-): blank, (+): tested.

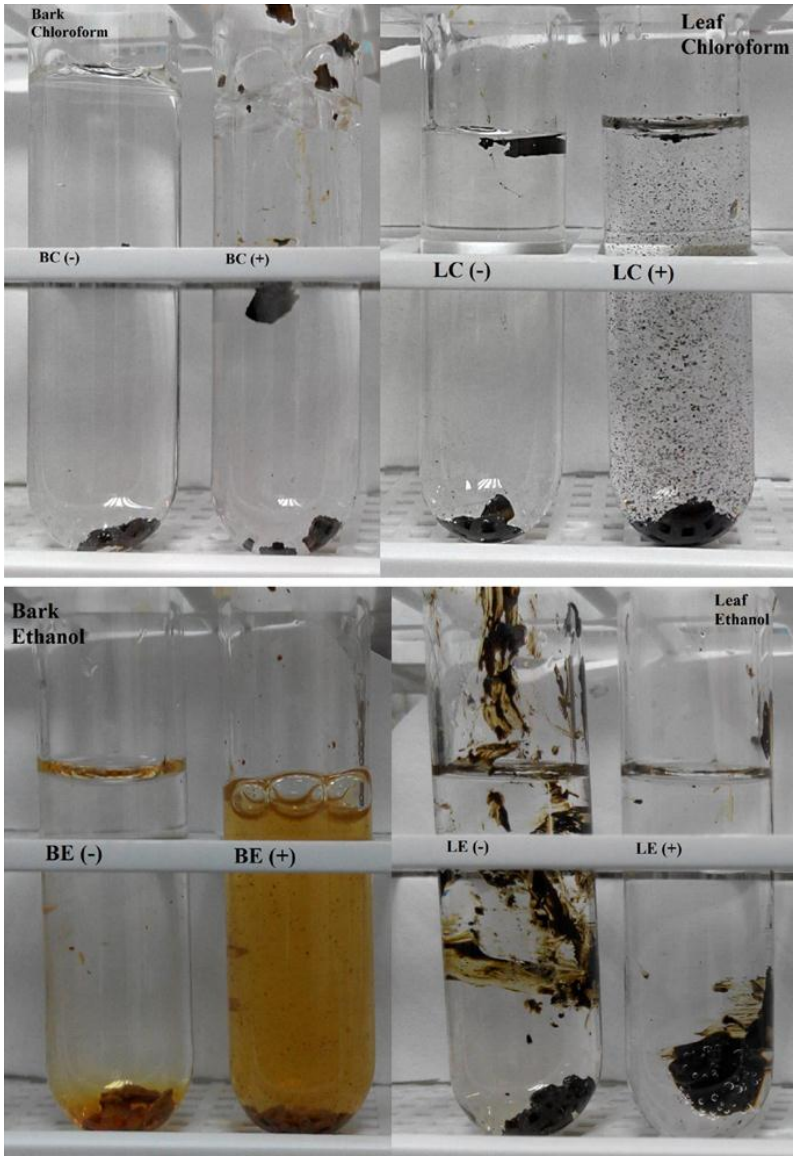


Figure 4.3: Positive saponin result on crude extracts of *Melodinus eugeniifolius.*

BC: bark chloroform extract, BE: bark ethanol extract, LC: leaf chloroform extract, LE: leaf ethanol extract, and (-): blank, (+): tested.

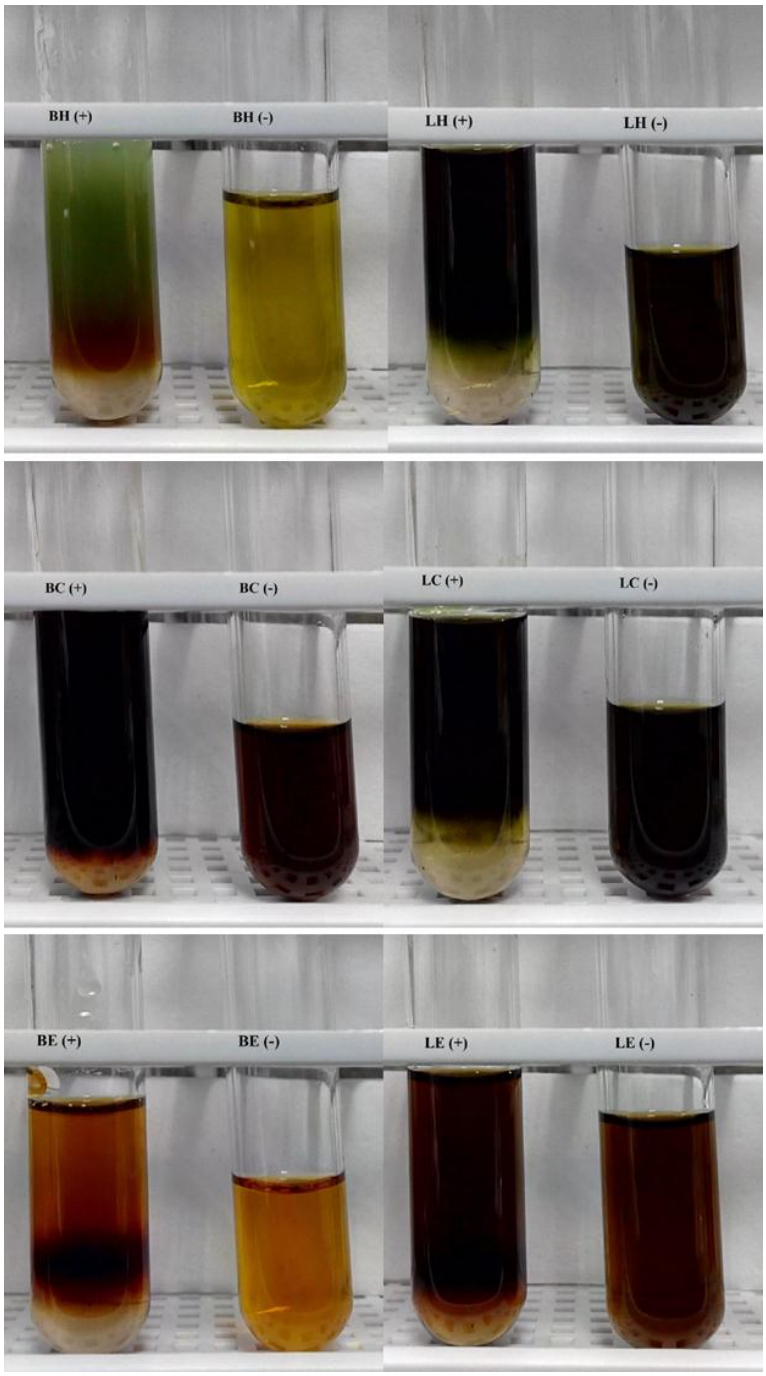


Figure 4.4: Positive sterol result on crude extracts of *Melodinus eugeniifolius.*

BH: bark hexane extract, BC: bark chloroform extract, BE: bark ethanol extract, LH: leaf hexane extract, LC: leaf chloroform extract, LE: leaf ethanol extract, and (-): blank, (+): tested.

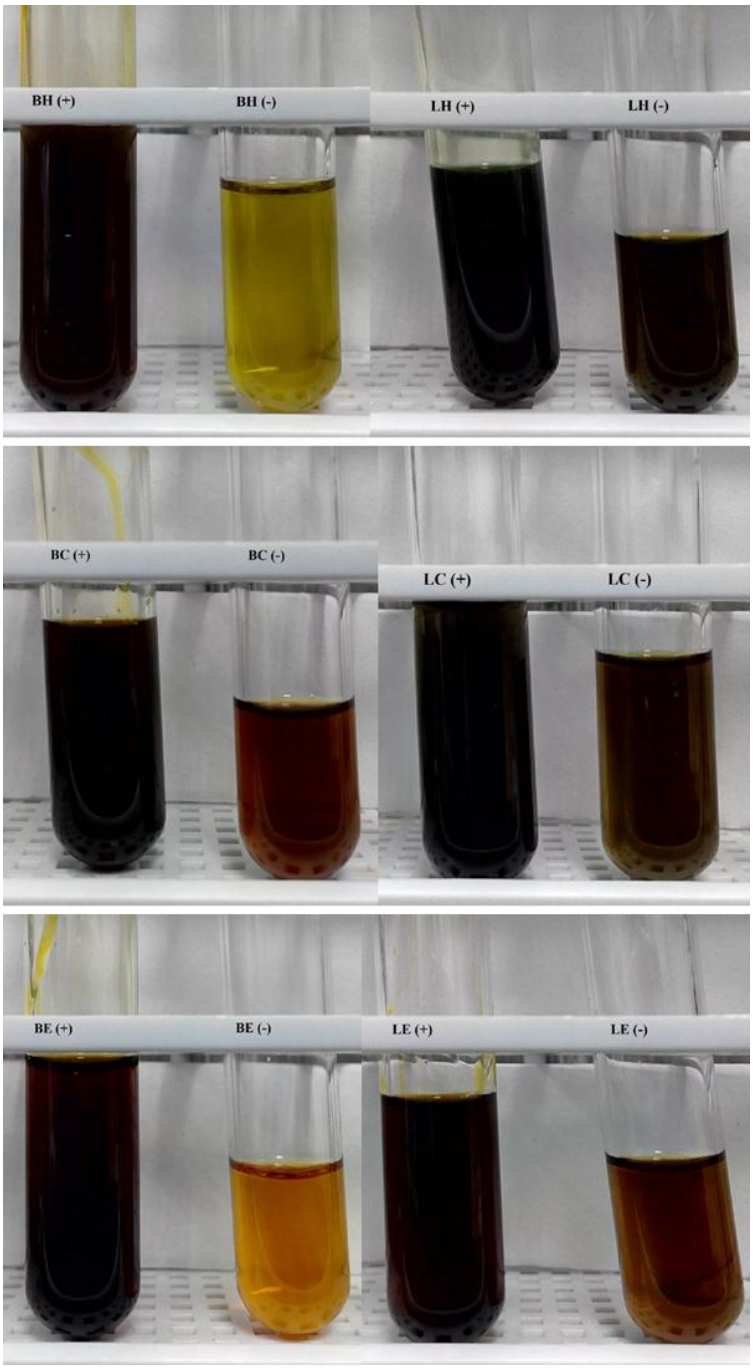


Figure 4.5: Positive cardiac glycoside result on crude extracts of *Melodinus eugeniifolius.*

BH: bark hexane extract, BC: bark chloroform extract, BE: bark ethanol extract, LH: leaf hexane extract, LC: leaf chloroform extract, LE: leaf ethanol extract, and (-): blank, (+): tested.

Figure 4.1 indicated that all the crude extracts of *Melodinus eugeniifolius* contain alkaloids, and the chloroform extracts of barks and leaves revealed the best result. Figure 4.2 showed the ethanol extract of leaves, the hexane extracts of barks and leaves had been observed certain flavonoids, the rest extracts may not contain too much flavonoids. Figure 4.3 indicated that only the chloroform and ethanol extracts contained few saponins. Figure 4.4 indicated all the crude extracts had been detected certain sterols. Figure 4.5 showed that all the crude extracts of *Melodinus eugeniifolius* contain cardiac glycoside, and the chloroform extract of leaves and the hexane extracts indicated the best result.

4.4 Discussion

Phytochemical analysis of *Melodinus eugeniifolius* (Table 4.1) revealed that the chloroform extract of leaves and barks accumulate substantial amounts of alkaloids and cardiac glycosides which could be well correlated with the activities measured. Triterpenoids can be divided into at least four groups of compounds namely true triterpenes, steroids, saponins and cardiac glycosides [104]. Flavonoids and steroids were detected in all extracts.

Phytochemical analysis is applied for the component catalogues or types of secondary metabolites which may indicate different pharmacological

avtivities. The presence of alkaloids and cardiac glycosides indicate the plant may work on antioxidant or anticancer aspects. Flavonoids components indicate there may be antitumor and antipatasitic capacity inside. Saponins and steroids indicate the plant may have potential antioxidant or antimicrobial activities [104, 105]. According to the result 4.3, the crude extracts of *Melodinus eugeniifolius* should be next tested on their antioxidant, antimicrobial, antiparasitic and anticancer potentials. And among all the pharmacological activity tests, the antioxidant and anticancer tests should be the main focus.

4.5 Conclusion

Results from our phytochemical analysis revealed that the ethanol extract of leaves and barks of *Melodinus eugeniifolius* accumulate substantial amounts of alkaloids and cardiac glycosides which could be possibly result mainly in anti-cancer and antioxidant activity [105].

CHAPTER Ⅴ

IN VITRO ANTIOXIDANT ACTIVITIES OF *MELODINUS EUGENIIFOLIUS*

5.1 Introduction

5.1.1 Free radicals and their scavengers

Oxygen is important for life processes to occur, however, an excess of oxygen could result in oxidative damage, which may even lead to death. The damage is not due to the presence of oxygen, but rather due to its role in the reduction of certain products to toxic free radicals. These free radicals are produced within living cells and are part of the cell's normal metabolic processes, including detoxification processes and immune system defences. It is the excessive generation of the free radicals, reactive oxygen species (ROS), such as superoxide anions, hydroxyl radicals and hydrogen peroxide that contribute to the development of various diseases such as cancer, rheumatoid arthritis, certain neurodegenerative diseases, tissue damage and also ageing, especially if free radical production exceeds the capacity of tissues to remove them [106].

In aerobic organisms, the defence system against these free radicals is provided by free radical scavengers which act as antioxidants. Free radical scavengers function by donating an electron to the free radical, the latter of which pairs with the unpaired electron and thereby stabilising it. Antioxidant defence involves both enzymatic mechanisms, which utilise specific enzymes such as superoxide dismutase, catalase and glutathione peroxidase, as well as non-enzymatic mechanisms, which utilise nutrients and minerals [107].

Free radicals are atomic or molecular species that can exist independently with one or more unpaired electrons in their outermost shell [108]. They are generated as by-products during normal cellular metabolism [109]. Due to their highly reactive and unstable properties in nature [110], they are capable of inducing oxidative damage to all the major classes of biomolecules including carbohydrates, lipids, proteins, and nucleic acids [111]. These damages are further implicated in the pathogenesis of atherosclerosis, cancer, diabetes mellitus, ischemia and reperfusion injury, neurodegenerative diseases, obstructive sleep apnea, rheumatoid arthritis as well as senescence [112].

Although the human body possesses the comprehensive network of antioxidant defence and repair systems, these endogenous protective mechanisms are inadequate to counteract the damaging effects of free

radicals completely [113]. More importantly, the application of currently available synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), gallic acid esters and tert-butylhydroquinone (TBHQ) are often restricted because of their low solubility, moderate antioxidant activity and possible toxicity [114]. Therefore, the exploration of alternative antioxidants from natural sources is highly desirable.

5.1.2 Natural antioxidants

The term, antioxidant is used to describe a component that can function to decrease tissue damage by reactive oxygen [115]. Defence provided for by the anti-oxidant systems is crucial to survival and can operate at different levels within the cells through the prevention of radical formation, intercepting formed radicals, repairing oxidative damage, increasing the elimination of damaged molecules, and recognition of excessively damaged molecules, which are not being repaired but rather eliminated to prevent mutations from occurring during replication.

Non-enzymatic anti-oxidants are classified as being either water-soluble or lipid-soluble, depending on whether they act primarily in the aqueous phase or in the lipophilic region of the cell membranes. The hydrophilic anti-oxidants include vitamin C (ascorbic acid) and certain polyphenol

flavonoid groups, while the lipophilic anti-oxidants include ubiquinols, retinoids, carotenoids, apocynin, procyanidins, certain polyphenol flavonoid groups and tocopherols [116]. Other non-enzymatic anti-oxidants include antioxidant enzyme cofactors, oxidative enzyme inhibitors and transition metal chelators such as ethylene diamine tetra-acetic acid (EDTA).

5.1.3 Therapeutic potential of phenolic substances

The establishment of an inverse correlation between the intake of fruits and vegetables and the occurrence of diseases such as cancer, age-related disorders, inflammation and cardiovascular disease is derived from clinical trials and epidemiological studies [115, 116].

Phenolic substances, which are known to possess high antioxidative activity, are actually common phytochemicals in fruits and leafy vegetables. Plants containing phenolic compounds have been reported to possess strong antioxidant properties. Most of these phenolics are classified into two principal groups of phenol; carboxylic acids and flavonoids, the latter being the most significant [115].

Phenolic compounds are found abundantly in all parts of the plant, such as wood, bark, stems, leaves, fruit, root, flowers, pollen and seeds.

Antioxidative activity of phenolic compounds is based on their ability to donate hydrogen atoms to free radicals. Many phenolic compounds, particularly flavonoids, exhibit a wide range of biological effects, including antioxidant activity, antibacterial, antiviral, anti-inflammatory, antiallergic, anticancer, anti-thrombotic, vasodilatory actions and the ability to lower the risk of coronary heart diseases [115].

Knowledge of the potential antioxidant compounds present within a plant species does not necessarily indicate its antioxidant capacity, as the total anti-oxidant effect may be greater than the individual antioxidant activity of compounds, due to synergism between different antioxidant compounds. Methods to measure the antioxidant activity in plant material generally involve both the generation of radicals (and their related compounds), and the addition of anti-oxidants, the latter resulting in the reduction of the radical and consequent disappearance [117, 118].

The role of free radicals and active oxygen is becoming increasingly recognized in the pathogenesis of the many human diseases, including cancer, neurodegenerative diseases, ageing and atherosclerosis [119]. Free radicals can also cause lipid peroxidation in foods that leads to their deterioration. Synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), have restricted use in food as they are suspected of carcinogenicity and of hormonal perturbation

[120, 121]. In search for sources of novel anti-oxidants with low toxicity, medicinal plants have over the past few years been studied extensively for their radical scavenging activity [122]. As plants produce a large number of anti-oxidants to control the oxidative stress caused by sunbeams and oxygen, it is clear that plants may represent a source of new compounds with antioxidant activity [123].

5.1.4 Antioxidant potential of genus *Melodinus*.

No reference reported the antioxidant assay in genus *Melodinus.*

5.2 Methodology

Phytochemical analysis in CHAPTER IV indicates the plant *Melodinus eugeniifolius* may have antioxidant potential. In this chapter, six crude extracts of *Melodinus eugeniifolius* would be tesed the antioxidant activity. Since the antioxidant tests are easily to gain the false positive result, to avoid the false positive reaction, three different matnods were used in this chapter.

5.2.1 Ferric reducing antioxidant potential (FRAP)

The FRAP assay is employed to estimate the antioxidant capacity of samples *in vitro*. The antioxidant capacity was determined following the procedure described by Benzie and Strain [124] with slight modifications.

5.2.1.1 Principle of the assay

The FRAP assay is always the first option for *in vitro* antioxidant capacity determination of samples. The FRAP reagent was prepared fresh by adding 10 mM of 2,4,6- Tris (2-pyridyl) -1,3,5 -triazine (TPTZ) (dissolved with 40 mM of HCl), 20 mM of $FeCl_3$ in water and 300 mM of acetate buffer (pH 3.6) in ratio of 1:1:10. A blank containing sample and solvents only was used for colour correction [125, 126].

5.2.1.2 Colorimetric spectrophotometric assay

Aliquots of plant extract dissolved in dimethyl sulfoxide (DMSO, R&M) were plated out in triplicate in a 96-well microtiter plate at different concentrations and repeated each concentration for three times. The 96-well plates were then incubated at 37°C for 90 minutes before absorbance were recorded at 593 nm. Vitamin C (L-ascorbic acid), gallic acid and quercetin were used as antioxidant standards and positive controls. The absorbance of the samples were compared to a $FeSO_4$ standard curve and the FRAP values were expressed as Ferrous Equivalent (FE), the concentration of extract or chemical which gives the same absorbance as 1 mmol ferrous ion (Fe^{2+}).

5.2.1.3 Statistical analysis

Data are presented as mean ± standard error of the mean (SEM) of triplicates in three independent experiments. The data were analyzed by one-way ANOVA, followed by Tukey's multiple comparison test for each paired data. A $p < 0.05$ was regarded as indicating a significant difference.

Concentration–response curves were calculated using the Prism software package 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com

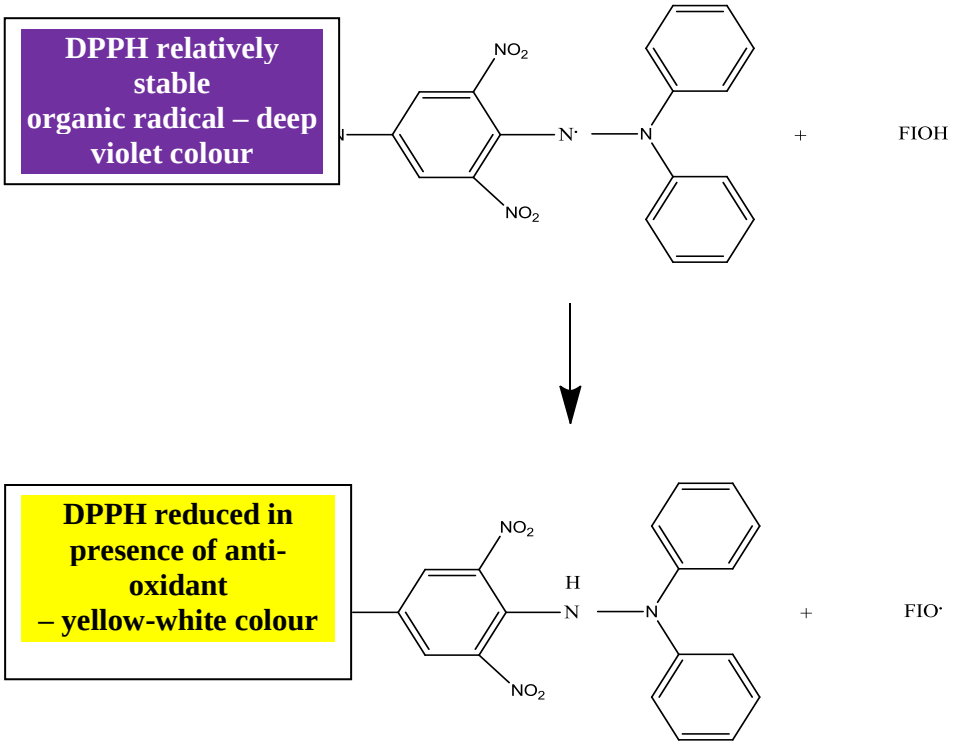
5.2.2 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

The antioxidant activity of each of the plant extracts was determined using the colorimetric DPPH assay, as described by Alothman and Juan Badaturuge [127, 128], was employed to determine the radical scavenging activity of the plant extracts.

5.2.2.1 Principle of the assay

DPPH is a stable free radical which accepts an electron or hydrogen radical to become a stable diamagnetic molecule, which is widely used to investigate radical-scavenging activity. In DPPH radical-scavenging assay, antioxidants react with DPPH, and convert it to yellow coloured α,α-diphenyl-β-picryl hydrazine. The degree of discolouration indicates the radical-scavenging potential of the antioxidant activities [129] (Figure 5.1).

The hydrogen donating capacity of test samples is quantified in terms of their ability to scavenge the relatively stable, organic free radical DPPH and by consequent reduction thereof. The absorption of the deep violet DPPH solution is measured at 517 nm, after which absorption decreases due to decolourisation to a yellow-white colour, in the event of reduction. This decrease in absorption is stoichiometric according to the degree of reduction. The remaining DPPH is measured at a time interval of 30 min after the addition of the DPPH, which corresponds inversely to the radical scavenging activity of the sample extract or anti-oxidant.



FIOH – flavonoid compound, FIO· – flavonoid having donated a hydrogen

Figure 5.1: Diagrammatic representation of chemical reaction of the reaction of DPPH in the presence of an electron donating antioxidant [130].

5.2.2.2 Colorimetric spectrophotometric assay

Aliquots of plant extract dissolved in dimethyl sulfoxide (DMSO, R&M) were plated out in triplicate in a 96-well microtiter plate at different concentrations, prepared as serial dilutions ranging from 10 µg/mL to 0.3125 µg/mL. The 0.1mM DPPH solution (Aldrich^{R}) was added to alternating columns of the test samples and methanol for control of test samples, in the remaining columns. The plate was shaken for 2 min and incubated for 30 min in the dark. The percentage decolourisation was obtained spectrophotometrically at 517 nm using the Thermo Scientific Varioskan Flash microtiter plate reader, linked to a computer equipped with (SkanIt Software 2.4.3). Percentage decolourisation was plotted against the concentration of the sample and the EC_{50} values were determined using Prism 5.00 software. Vitamin C (L-ascorbic acid), gallic acid and quercetin were used as positive controls. At least three independent tests were performed for each sample. The DPPH absorbance decreases with an increase in DPPH radical scavenging activity. This activity is given as percent DPPH radical scavenging, which is calculated with the equation:

DPPH radical scavenging activity (%) = $[(Abs_{control} - Abs_{sample})] / (Abs_{control})] \times 100$

$Abs_{control}$ is the absorbance of DPPH radical + methanol;

Abs_{sample} is the absorbance of DPPH radical + sample extract /standard.

5.2.2.3 Statistical analysis

Concentration–response curves were calculated using the Prism software package 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com (GraphPad, San Diego, USA) and data were reported as mean and SD values obtained from a minimum of three determinations. Non-linear best fit was plotted with SD and 95% confidence interval.

5.2.3 β-Carotene bleaching (BCB) assay

To appraise lipid peroxidation activity of the samples, β-carotene bleaching assay is carried out according to the method of Barreira *et al*. [131], Velioglu *et al*. [132] and Lu and Foo [133].

5.2.3.1 Principle of the assay

β-carotene was dissolved with chloroform, and linoleic acid and Tween 80 were added in. After the chloroform had been evaporated, distilled water was added in to form an emulsion. The emulsion is tested with

different concentrations of samples and standards. Incubations were done at 50°C for 4 hours. Vitamin C (L-ascorbic acid), gallic acid and quercetin were used as antioxidant standards and positive controls. The oxidation of the β-carotene emulsion was monitored by measuring the absorbance at 490 nm via a Dynex microplate reader. Degradation rates are calculated according to first order kinetics.

Degradation rate (DR) = ln $a/b \times 1/t$

Whereby 'a' is the initial absorbance, 'b' is the absorbance at 240 min and t is time (min). The antioxidant activity was presented as % inhibition using the following formula:

Antioxidant activity (%) = (DR control – DR sample) / DR control × 100.

IC_{50} -- concentration where 50% inhibition of the β-Carotene bleaching radical is obtained.

5.2.3.2 Colorimetric spectrophotometric assay

The oxidation of the β-carotene emulsion was monitored by measuring the absorbance at 490 nm via a Dynex microplate reader. Aliquots of plant extract dissolved in dimethyl sulfoxide (DMSO, R&M) were plated out in triplicate in a 96-well microtiter plate at different concentrations.

5.2.3.3 Statistical analysis

Data are presented as mean ± standard error of the mean (SEM) of triplicates in three independent experiments. The data were analyzed by one-way ANOVA, followed by Tukey's multiple comparison test for each paired data. A $p < 0.05$ was regarded as indicating a significant difference. Concentration–response curves were calculated using the Prism software package 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com

5.2.4 Reagents and materials

2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteau reagent, ferric chloride ($FeCl_3$), ferrous sulphate ($FeSO_4$), β-carotene, Vitamin C (l-ascorbic acid) and quercetin were purchased from Sigma, Germany. Gallic acid was purchased from Tokyo Chemical Industry, Japan; dimethyl sulfoxide (DMSO, R&M). All other chemicals were of analytical grade.

96-well TC Plate (Orange Scientific), kit, pipette and tips are all from Dragon Med.

5.3 Results

5.3.1 Ferric reducing antioxidant potential (FRAP)

The method is based on the principle of the reduction of the ferric-tripyridyltriazine complex to the ferrous form, upon which an intense blue color develops, and the change of absorbance is measured at 593 nm (kinetic method) [124]. The FRAP assay is employed to estimate the antioxidant capacity of samples in vitro. In this test, the result (Figure 5.2) revealed that a good linearity of ferrous sulfate ($FeSO_4$) and was obtained within the range of 0.15—1.40 mM (R^2 = 0.9999). The antioxidant activities were highest for the ethanol extract of barks (Table 5.1; 5.2) (2.40 ± 0.0018 mmol/g), even better than gallic acid (2.17 ± 0.0036 mmol/g), followed by the ethanol extract of leaves (1.28 ± 0.0020 mmol/g) and the chloroform extract of barks (1.22 ± 0.0014 mmol/g).

Table 5.1: Ferric reducing antioxidant capacity of extract

SAMPLE	Concentration (mg/mL)	Ferrous Equivalent (mM)	Antioxidant Capacity (mmol/g)
BH	1.00	0.77 ± 0.0011	0.77 ± 0.0011
BC	0.50	0.61 ± 0.0007	1.22 ± 0.0014
BE	0.50	1.20 ± 0.0009	2.40 ± 0.0018
LH	1.00	0.24 ± 0.0012	0.24 ± 0.0012
LC	0.50	0.44 ± 0.0011	0.88 ± 0.0022
LE	0.50	0.64 ± 0.0010	1.28 ± 0.0020
AA	0.25	1.20 ± 0.0007	4.79 ± 0.0028
GA	0.25	0.54 ± 0.0009	2.17 ± 0.0036
QC	0.25	1.39 ± 0.0008	5.56 ± 0.0032

(Probability: $P < 0.01$)

BH: bark hexane extract, BC: bark chloroform extract, BE: bark ethanol extract, LH: leaf hexane extract, LC: leaf chloroform extract, LE: leaf ethanol extract, AA: Ascorbic Acid, GA: Gallic Acid, QC: Quercetin.

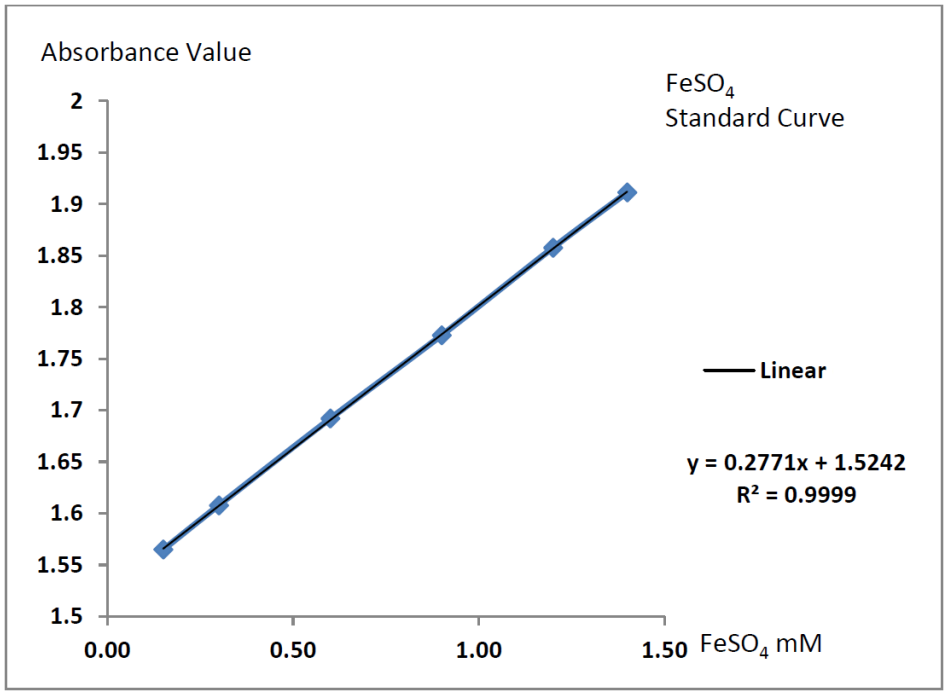


Figure 5.2: $FeSO_4$ Standard Curve

Standard equation: $y = 0.2771x + 1.5242$; $R^2 = 0.9999$;

R-squared (R^2) -- A decision coefficient worked to measure the explained variables that predicted by the regression within the scope of sample. The larger the coefficient is, the more close to 1, shows that the better the regression fitting. R^2 is coefficienting the liner regression.

Table 5.2: Samples & Control in FRAP.

SAMPLE	C (mg/mL)	FE (mM)	Antioxidant Capacity (mmol/g)
QC	0.25	1.389455	5.55782
AA	0.25	1.195936	4.783746
GA	0.25	0.543125	2.172501
BE	**0.5**	**1.20071**	**2.401419**
LE	**0.5**	**0.639986**	**1.279971**
BC	**0.5**	**0.608781**	**1.217563**
LC	0.5	0.436214	0.872429
BH	1.0	0.77742	0.77742
LH	1.0	0.237506	0.237506

(Probability: $P < 0.05$)

BH: bark hexane extract, BC: bark chloroform extract, BE: bark ethanol extract, LH: leaf hexane extract, LC: leaf chloroform extract, LE: leaf ethanol extract, AA: Ascorbic Acid, GA: Gallic Acid, QC: Quercetin.

C -- Concentration; FE -- Ferrous Equivalent

5.3.2 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

In this test, the ethanol extract of barks and leaves exhibited profound antioxidant activities (Table 5.3; 5.4; Figure 5.3). The antioxidant activities were highest for the ethanol extract of barks (IC_{50}: 1.90 ± 0.01 μg/mL), followed by the ethanol extract of leaves (IC_{50}: 11.30 ± 0.02 μg/mL) and the chloroform extract of leaves (IC_{50}: 19.90 ± 0.01 μg/mL) (Table 5.3; 5.4; Figure 5.3).

Table 5.3: DPPH radical scavenging activity at 10 μg/mL of extract

Extracts	% DPPH radical scavenging
BH	2.30 ± 0.02
BC	27.10 ± 0.01
BE	76.70 ± 0.02
LH	6.20 ± 0.02
LC	31.20 ± 0.01
LE	46.60 ± 0.02
AA	97.60 ± 0.00
GA	91.50 ± 0.01
QC	81.60 ± 0.01

(Probability: $P < 0.05$)

BH: bark hexane extract, BC: bark chloroform extract, BE: bark ethanol extract, LH: leaf hexane extract, LC: leaf chloroform extract, LE: leaf ethanol extract, AA: Ascorbic Acid, GA: Gallic Acid, QC: Quercetin.

Table 5.4: Concentration of extract at DPPH radical scavenging activity 50% (IC_{50}).

Extracts	DPPH IC_{50} (μg/mL)
BH	289.70 ± 0.02
BC	37.50 ± 0.02
BE	**1.90 ± 0.01**
LH	179.90 ± 0.02
LC	19.90 ± 0.01
LE	11.30 ± 0.02
AA	0.60 ± 0.01
GA	1.50 ± 0.02
QC	1.00 ± 0.01

(Probability: $P < 0.05$)

BH: bark hexane extract, BC: bark chloroform extract, BE: bark ethanol extract, LH: leaf hexane extract, LC: leaf chloroform extract, LE: leaf ethanol extract, AA: Ascorbic Acid, GA: Gallic Acid, QC: Quercetin and IC_{50}: concentration where 50% inhibition of the DPPH radical is obtained.

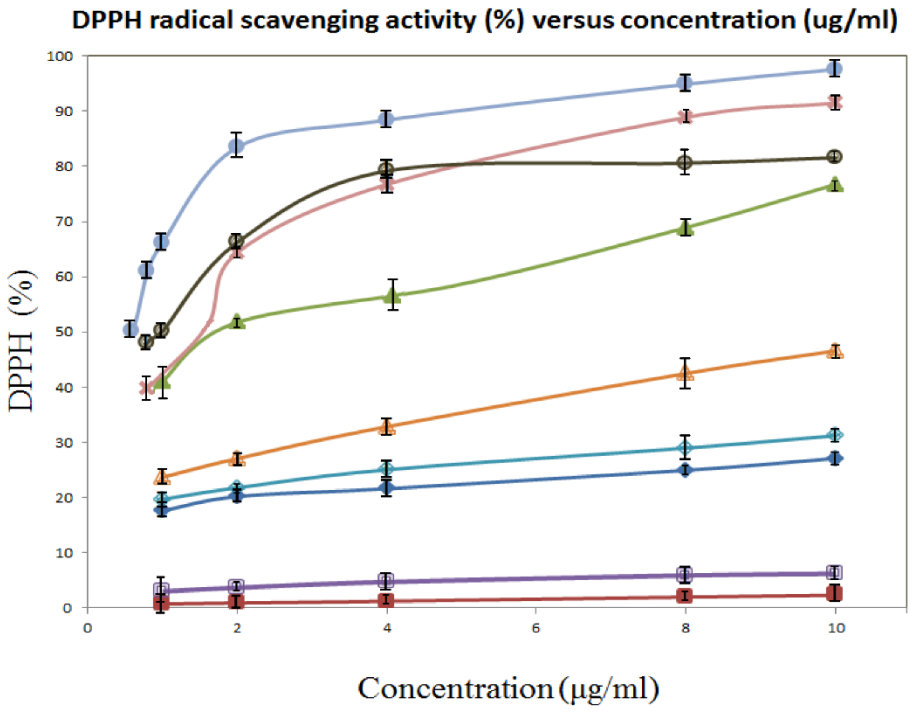


Figure 5.3: DPPH radical scavenging activity (%) versus concentration(µg/mL)

: bark hexane extract, : bark chloroform extract, : bark ethanol extract, : leaf hexane extract, : leaf chloroform extract, : leaf ethanol extract, : Ascorbic Acid, : Gallic Acid, : Quercetin.

5.3.3 β-Carotene bleaching (BCB) assay

In this assay, the antioxidant activities were highest for the ethanol extract of barks (IC_{50}: 9.90 ± 0.01 μg/mL), even better than gallic acid (IC_{50}: 10.50 ± 0.02 μg/mL) and quecetin (IC_{50}: 25.10 ± 0.01 μg/mL), followed by the ethanol extract of leaves (IC_{50}: 24.30 ± 0.02 μg/mL) and the hexane extract of leaves (IC_{50}: 24.90 ± 0.02 μg/mL) (Table 5.5).

Table 5.5: Antioxidant activity of extract at 50% Inhibition of β-Carotene (IC_{50}).

Extracts	50% Inhibition of β-Carotene (IC_{50}) (μg/mL)
BH	50.70 ± 0.02
BC	49.50 ± 0.02
BE	**9.90 ± 0.01**
LH	24.90 ± 0.02
LC	48.90 ± 0.01
LE	24.30 ± 0.02
AA	9.60 ± 0.01
GA	10.50 ± 0.02
QC	25.10 ± 0.01

(Probability: $P < 0.05$)

BH: bark hexane extract, BC: bark chloroform extract, BE: bark ethanol extract, LH: leaf hexane extract, LC: leaf chloroform extract, LE: leaf ethanol extract, AA: Ascorbic Acid, GA: Gallic Acid, QC: Quercetin.

5.4 Discussion

The antioxidant activity reflected by the FRAP, DPPH, and β-carotene bleaching assay were clearly observed in the ethanol extracts of bark and leaves among the six crude extracts of *Melodinus eugeniifolius*. Results were followed by the chloroform extract of barks and leaves. Hexane extract of barks and leaves had the lowest result.

In the FRAP test, the result (Figure 5.2) revealed that a good linearity of ferrous sulfate ($FeSO_4$) and was obtained within the range of 0.15—1.40 mM (R^2 = 0.9999). The antioxidant activities were highest for the ethanol extract of barks (Table 5.1; 5.2) (2.40 ± 0.001 mmol/g), even better than gallic acid (2.17 ± 0.002 mmol/g), followed by the ethanol extract of leaves (1.28 ± 0.002 mmol/g) and the chloroform extract of barks (1.22 ± 0.002 mmol/g).

The DPPH radical has a deep purple colour and absorbs strongly at a wavelength of 550nm, whereas the yellowish reduction product $DPPH_2$ does not. The radical scavenging potential of *Melodinus eugeniifolius.* is summarised in Table 5.3; 5.4 and depicted in Figure 5.3. The antioxidant activities were highest for the ethanol extract of barks (IC_{50}: 1.90 ± 0.01

μg/mL), followed by the ethanol extract of leaves (IC_{50}: 11.30 ± 0.02 μg/mL) and the chloroform extract of leaves (IC_{50}: 19.90 ± 0.01 μg/mL).

The radical scavenging potential against DPPH organic radical directly depends on the number of hydroxyl groups present in ring B of flavonoids, with an increase in the number of hydroxyl groups resulting in an increase in radical scavenging activity [134]. Phenols, amino and thiophenol groups are commonly known to be the active groups for DPPH scavenging. The mechanism by which DPPH is scavenged, aids in elucidating the structure-activity relationship (SAR) of the antioxidant and in doing so, may be beneficial in the rational design of novel flavonoid-derived antioxidants with improved pharmacological profiles [135].

Pronounced radical scavenging activity has been reported in plants with phenolic moieties, the presence of which is common in natural antioxidants. These phenolic moieties include substances such as tannins, flavonoids, tocopherol, and cathecheses. Tannins are, at least in part, responsible for the free radical scavenging activities working synergistically with other antioxidant substances [136].

In *β*-Carotene bleaching assay, the antioxidant activities were highest for the ethanol extract of barks (IC_{50}: 9.90 ± 0.01 μg/mL), even better than

gallic acid (IC_{50}: 10.50 ± 0.02 μg/mL) and quecetin (IC_{50}: 25.10 ± 0.01 μg/mL), followed by the ethanol extract of leaves (IC_{50}: 24.30 ± 0.02 μg/mL) and the hexane extract of leaves (IC_{50}: 24.90 ± 0.02 μg/mL) (Table 5.5).

Comparing with the three different assays, the FRAP assay was employed to estimate the antioxidant capacity of the samples *in vitro* [137]. This method is based on the reduction of colourless ferric complex (Fe^{3+}-tripyridyltriazine) to blue-coloured ferrous complex (Fe^{2+}-tripyridyltriazine) in the presence of electron-donating antioxidants under acidic conditions [138]. DPPH assay is widely used for the evaluation of radical scavenging activity of plant extracts [139]. In the DPPH radical scavenging assay, antioxidants react with purple-coloured DPPH radical (2,2-diphenyl-1-picrylhydrazyl), and convert it to the yellow coloured DPPH (2,2-diphenyl-1-picrylhydrazine) [140, 141]. The degree of discolouration indicates the radical-scavenging potential of the sample [134-136]. The *β*-Carotene bleaching method is based on the loss of the yellow colour of *β*-carotene due to its reaction with radicals which are formed by linoleic acid oxidation in an emulsion. The rate of *β*-carotene bleaching can be slowed down in the presence of antioxidants [131-133].

5.5 Conclusion

The antioxidant activity reflected by the FRAP, DPPH, and β-carotene bleaching assay was clearly observed in the ethanol extracts of bark and leaves among the six crude extracts of *Melodinus*. Results were followed by the chloroform extract of barks and leaves. The hexane extract of barks and leaves had the lowest result in the antioxidant activity tests.

CHAPTER Ⅵ

IN VITRO ANTIBACTERIAL ACTIVITIES OF *MELODINUS EUGENIIFOLIUS*

6.1 Introduction

Bacteria are unicellular prokaryotic microorganisms that exhibit different cellular sizes and shapes ranging from spheres to rods and spirals [142]. In the human body, most of the bacteria are rendered harmless or beneficial by the protective effects of the immune system [143]. Nevertheless, some species of bacteria are pathogenic and capable of causing infectious diseases such as anthrax, bubonic plague, cholera, leprosy, syphilis and tuberculosis [144].

Since their introduction, antimicrobials (antibiotics) have played an essential role in decreasing morbidity due to infectious diseases. Antimicrobials are used for treatment of infections and for prophylaxis against infections in humans and animals, for growth promotion in food animal rearing and in agriculture. However infectious diseases remain the leading cause of death worldwide and bacteria have become more resistant to conventional antibiotic in recent years.

In spite of the widespread availability of antibacterial therapies, bacterial infections continue to pose a significant threat to public health worldwide [145, 146]. The widespread use of these compounds is thought to further encourage the emergence of antimicrobial resistance [147]. The number of resistant pathogenic bacteria is increased in an alarming rate worldwide and the search for novel antimicrobial agents from medicinal plants to combat such pathogens has become crucial for avoiding the emergence of untreatable bacterial infections [148, 149]. More importantly, the clinical efficacy of many existing antibacterial drugs is declining precipitously due to the emergence and dissemination of multiple drug resistant pathogens [150]. These bacteria are endowed with the ability to become resistant to antibiotics through mutation or gene transfer [151, 152], which further leads to higher morbidity, prolonged length of stay, increased mortality, and costly healthcare as compared to antibiotic-susceptible microorganisms [153]. Therefore, alternative antibacterial agents with diverse chemical structures as well as novel mechanisms of actions are urgently required to combat the new and re-emerging bacterial infections.

6.1.1 Chemotherapeutic agents: Factors affecting their effectiveness

The ideal chemotherapeutic agent has a high therapeutic index with selective toxicity, thereby resulting in lethal damage to pathogens

through the inhibition of cell wall synthesis, protein synthesis or nucleic acid synthesis, as well as through the disruption of the cell membrane and the inhibition of certain essential enzymes. This results in selective disruption of the specific structure and/or function essential to bacterial growth and survival, without causing similar effects to its eukaryotic host [154].

The efficacy of antimicrobial agents is influenced by a number of factors. Firstly, it is an obvious importance that the antimicrobial agent reaches the site of the infection. This greatly depends on the stability of the drug, its lipophilic or hydrophilic nature, its absorption from a specific site and the presence of blood clots or necrotic tissue, the latter of which may protect the pathogen against the antibiotic. Secondly, the susceptibility of the pathogen to the particular chemotherapeutic agent is of utmost importance, as well as the specific growth phase in which the pathogen is in at that particular stage [154].

6.1.2 Drug resistance

Bacteria have evolved many different mechanisms of resistance. These can be classified as: a) alteration in, or addition of, the target site of antimicrobial binding; b) alteration in access to the target site for instance the decreased permeability of cell wall or efflux mechanisms;

and c) inactivation of the antimicrobial binding. Furthermore resistance may arise through mutation or by acquisition of resistance genes by horizontal transmission from another bacterial species [147].

On the other hand, drug resistance may be brought about by the limited drug diffusion into the biofilm matrix, enzyme-mediated resistance, genetic adaptation, efflux pumps, as well as through the adaptation of the outer microbial membrane, the latter occurring either through the lack of or through the overexpression of certain membrane proteins [155].

This phenomenon of increased drug resistance, combined with the multiplicity of side effects by existing agents and the emergence of diseases for which no treatment yet exists, makes the search for the new antimicrobial agents a highly relevant and important subject for research. For centuries, plants have been used in the traditional treatment of microbial infections. This assembly of knowledge by indigenous peoples about plants and their products continue to play an essential role in health care of a great proportion of the population [156].

6.1.3 Natural products and their role in drug discovery

Natural products have played a pivotal role in the discovery of antimicrobial drugs, with the drug either being completely derived from

the natural product, or serving as a lead for novel drug discovery. Most antimicrobials discovered during the past six to seven decades have been discovered through screening of soil samples, of which the antimicrobial efficacies were determined first *in vivo* and later *in vitro* [156].

Plants synthesize a diverse array of secondary metabolites, which play a key role in the natural defence mechanisms employed by the plant against predation by microorganisms and insects. It is not surprise that these aromatic compounds have, in numerous instances, been found to be useful antimicrobial phytochemicals and, as a result, these compounds are now divided into different chemical categories: phenolics, terpenoids and essential oils, alkaloids, lectins and polypeptides, as well as polyacetylenes [5]. An increase in the isolation and identification of such compounds may thus contribute greatly to the success in antibiotic discovery.

6.2 Methodology

Phytochemical analysis in CHAPTER IV indicates the plant *Melodinus eugeniifolius* may have antimicrobial potential. Therefore, *in vitro* antimicrobial activity of the hexane, chloroform and ethanol extracts of *Melodinus eugeniifolius* was examined. The following bacterial strains were employed in the screening:

Gram-positive bacteria such as *Staphylococcus aureus* (ATCC 11632), *Bacillus cereus* (ATCC 10876), methicillin-resistant *Staphylococcus aureus* (ATCC 43300) and Gram-negative bacteria such as *Escherichia coli* (ATCC 10536) and *Pseudomonas aeruginosa* (ATCC 10145). The microorganisms used in the current study are shown in Table 6.1 [154, 155, 157, 158].

Thirty bacterial strains including American Type Culture Collection (ATCC) and clinical strains were produced from the Biochemical Laboratory, Faculty of Science, University of Nottingham Malaysia Campus, and the Bacteriology Unit, Department of Medical Microbiology and Immunology, University Kebangsaan Malaysia Medical Centre (UKMMC) respectively. Table 6.1 includes the types of culture media required for the growth of the respective bacteria.

Table 6.1 Type of microorganisms and culture media

Micro-organism	Bacterial Strain	Culture medium
	ATCC Strain	
Gram-positive bacteria	*Bacillus cereus* (ATCC 10876) *Bacillus subtilis* (ATCC 10876) *Listria monocytogenes* (ATCC 21332) *Micrococcus luteus* (ATCC 10240) *Proteus hauseri* (ATCC 13315) *Rhodococcus equi* (ATCC 33701)	Tryptic soy broth (Difco Labora-tories, USA)

	Staphylococcus aureus (ATCC 11632)	
	Staphylococcus epidermidis (ATCC 12228)	Tryptic
Methicillin-resistant	*Staphylococcus aureus* subsp. aureus (ATCC 43300)	soy agar (HiMedia,
Gram-negative bacteria	*Citrobacter freundii* (ATCC 22636)	India)
	Escherichia coli (ATCC 10536)	
	Klebsiella pneumoniae (ATCC 13883)	
	Pseudomonas aeruginosa (ATCC 10145)	
	Salmonella enteritidis (ATCC 13076)	
	Salmonella typhimurium (ATCC 14028)	
	Clinical isolate	
Gram-positive bacteria	Enterococcus faecalis	Tryptic soy broth
	Methicillin-resistant *Staphylococcus aureus* (MRSA)	
	Methicillin-sensitive *Staphylococcus aureus* (MSSA)	Tryptic soy agar
	Oxacillin-resistant coagulase negative staphylococcus (ORCNS)	
	Oxacillin- sensitive coagulase negative staphylococcus (ORCNS)	
	Streptococcus agalactiae (Group B *Streptococcus*, GBS)	
	Streptococcus pneumoniae	
Gram-negative bacteria	*Actinobacillus sp*.	
	Enterobacter sp.	
	Escherichia coli	
	Extended-spectrum beta-lactamase-producing *Escherichia coli* (ESBL-EC)	
	Extended-spectrum beta-lactamase-producing *Klebsiella pneumonia* (ESBL-KP)	
	Klebsiella sp.	
	Moraxella sp.	
	Serratia sp.	

6.2.1 Disc diffusion assay

6.2.1.1 Principle of the assay

The principles of determining the effectivity of a noxious agent to a bacterium were well enumerated by Rideal, Walker and others at the turn of the century, the discovery of antibiotics made these tests (or their modification) too cumbersome for the large numbers of tests necessary to be put up as a routine [157]. The ditch plate method of agar diffusion used by Alexander Fleming was the forerunner of a variety of agar diffusion methods devised by workers in this field. The Oxford group used these methods initially to assay the antibiotic contained in blood by allowing the antibiotics to diffuse out of reservoirs in the medium in containers placed on the surface [157].

With the introduction of a variety of antimicrobials it became necessary to perform the antimicrobial susceptibility test as a routine. For this, the antimicrobial contained in a reservoir was allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. Even now a variety of antimicrobial containing reservoirs are used but the antimicrobial impregnated absorbent paper disc is by far the commonest type used. The disc diffusion method of antimicrobial susceptibility testing (AST) is the most practical method and is still the

method of choice for the average laboratory. Automation may force the method out of the diagnostic laboratory but in this country as well as in the smaller laboratories of many countries, it will certainly be the most commonly carried out microbiological test for many coming years [157].

The Kirby-Bauer and Stokes' methods are usually used for antimicrobial susceptibility testing, with the Kirby-Bauer method being recommended by the National Committee for Clinical Laboratory Standards (NCCLS). The accuracy and reproducibility of this test are dependent on maintaining a standard set of procedures.

NCCLS is an international, interdisciplinary, non-profit, non-governmental organization composed of medical professionals, government, industry, healthcare providers, educators etc. It promotes accurate antimicrobial susceptibility testing (AST) and appropriate reporting by developing standard reference methods, interpretative criteria for the results of standard AST methods, establishing quality control parameters for standard test methods, provides testing and reporting strategies that are clinically relevant and cost-effective [157].

Interpretative criteria of NCCLS are developed based on international collaborative studies and well correlated with MIC’s and the results have corroborated with clinical data. Based on study results NCCLS

interpretative criteria are revised frequently. NCCLS is approved by FDA-USA and recommended by WHO [157, 158].

6.2.1.2 Protocol

i. Preparation of Mueller Hilton Agar

Mueller Hilton Agar (MHA) was prepared from a commercially available dehydrated base according to the manufacturer's instructions. The agar solution was then autoclaved. It was then allowed to cool in a 45-50 ℃ water bath. The agar was then poured into plastic flat bottomed petri dishes on a level, horizontal surface to give a uniform depth of approximately 4 mm. This corresponds to 25 mL for plates with a diameter of 100 mm. The agar medium was then allowed to cool to room temperature and unless plate is used the same day, stored in a refrigerator (2 to 8 ℃). A representative sample of each batch of plates was examined for sterility by incubation at 30-35 ℃ for 24 hrs or longer.

ii. Preparation of dried filter paper discs / cotton swabs / tryptic soy broth / tryptic soy agar / normal saline solution

Whatman filter paper no.1 was used to prepare discs approximately 6 mm in diameter. They were then placed in a bijou bottle and autoclaved

for sterility. Same was done for cotton swabs. Tryptic soy broth (TSB) was prepared as the manufacturer's instructions. About 30 g of TSB powder in 1 L of sterile distilled water. The solution was mixed thoroughly and warmed slightly to completely dissolve the powder before dispensing into universal bottles. After autoclaved at 121 ℃ for 15min, the bottle was allowed to cool down to room temperature before storing at 4 ℃ until further use. Tryptic soy agar (TSA) was prepared by suspending 40 g of TSA powder in 1 L of sterile distilled water. The solution was mixed thoroughly and heated to boiling to dissolve the powder completely, followed by autoclaving at 121 ℃ for 15min. The autoclaved medium was allowed to cool down by immersing into a 45 ℃ to 50 ℃ water bath before pouring into sterile Petri dishes in laminar flow cabinet. After pouring, the molten agar was allowed to solidify and dried for 30 minutes before covering the plates to prevent formation of water on the agar surface. The prepared agar medium was stored in a 4 ℃ chiller until further use.

iii. Preparation of sample extracts, negative control and standard antibiotic solution.

Sample extracts were dissolved in DMSO at a concentration of 100 mg/mL and filtered. Negative control was DMSO. Positive controls used

were ampicillin and streptomycin which were prepared at the concentration of 100 µg/mL.

iv. Preparation of fresh / pure colonies of bacteria

Tryptic soy agar (TSA) was prepared according to the manufacturers intructions. Agar was autoclaved and poured on petri dishes to 4 mm, approximately 25 mL to a 100 mm Sterile Petri dish. Bacteria were streaked on the TSA plates in 4 density level around the plates. Plates are then sealed with a parafilm and incubated for 18hrs to obtain single colonies.

v. Preparation of disc with extracts, control and standards

About 10 µl of sample extracts was pipetted onto the disc in triplicates. Same was done for controls and standards. Impregnated discs are left to air dry overnight for 12 hrs.

vi. Inoculum Preparation

At least 3-5 well isolated colonies of the same morphological type were selected from an agar plate culture. The top of each colony was touched with a sterilized loop and the growth was transferred into the Bijou bottle

containing TSB. This was repeated for each bacterium into each Bijou bottle. The broth culture was incubated at 35 ℃ for about 2 hours till it achieved the turbidity of 0.5 Mcfarland standard / 625 nm to yield 1 X 10^8 cfu / mL. Using optical density at 625 nm, the reading below must be obtained for different bacteria, turbidity was adjusted with sterile normal saline.

vii. Inoculation of test plates

The bacterial broth was used within 15 minutes after the turbidity of the inoculum suspension was adjusted. Sterile cotton swab was dipped into the suspension and rotated several times and pressed firmly on the inside wall of the tube above the fluid level. This was to remove any excess inoculum from the swab. The dried surface of a Mueller Hilton agar plate was inoculated by streaking the swab over the entire agar at every 60^o. As a final step, the rim of the agar was swabbed.

viii. Application of discs to inoculated agar plates

Each disc was pressed down on the agar to ensure complete contact according to prepared template. Plates were placed in 35 ^{o}C incubator within 15 minutes after discs are applied.

ix. Reading plates and interpretation of results

After incubation, each plate was examined. Zone of inhibition was measure using sliding calipers to the nearest millimeters. The inhibition zones would thus include disc size of 6 mm. The experiment was done three times and the mean values are presented. Two samples with the best inhibition zone were chosen and test was repeated in triplicate with the concentration of 2 mg/disc using the same negative and positive controls to test dose/inhibition zones correlation [158-160].

6.2.1.3 Reagents and materials

Mueller Hilton Agar (MHA) is from Difco Laboratories, USA. Dimethyl sulfoxide (DMSO, R & M Chemicals, UK), Penicillin-Streptomycin (liquid, 100 ml/pk) (Gibco). Incubator (Binder, Germany), Whatman filter paper no.1, minisart syringe filter (SSI) are all from Dragon Med. Water bath is from Julabo, Germany. Sterile Petri dishes are from Facorit, Malaysia, laminar flow cabinet is from Esco Micro, Malaysia.

6.3 Results

The antibacterial activity of six extracts of *Melodinus eugeniifolius* was evaluated (Table 6.2). The extracts were screened for activity against Gram-positive bacteria (*Staphylococcus aureus*, MRSA and *Bacillus*

cereus) and Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) by agar disc diffusion method. All the extracts displayed diverse antibacterial activity against Gram-positive bacteria and Gram-negative bacteria respectively. The best sensitivity to the extracts at 2 mg/disc was respectively obtained against *Staphylococcus aureus*, MRSA, *Bacillus cereus* and *Escherichia coli*.

Table 6.2. Antibacterial activity of *Melodinus eugeniifolus*. (1 mg/disc and 2 mg/disc) vs. ampicillin and streptomycin (1µg/disc) against 30 bacterial species tested by disc diffusion assay.

Bacterial Strain	**Zone of inhibition (mm)** [a]					
ATCC Strain	Plant extract					
	BH	BC	BE	LH	LC	LE
Bacillus cereus (ATCC 10876)	9.00±0.25	-	7.00±0.09	12.00±0.35	8.00±0.42	-
Bacillus subtilis (ATCC 10876)	-	-	-	-	-	-
Citrobacter freundii (ATCC 22636)	7.00±0.31	-	-	7.00±0.56	-	7.00±0.26
Escherichia coli (ATCC 10536)	14.00±0.17	8.00±0.19	7.00±0.52	7.00±0.28	7.00±0.38	-
Klebsiella pneumoniae (ATCC 13883)	-	-	-	-	-	-
Listria monocytogenes (ATCC 21332)	-	-	-	-	-	-
Micrococcus luteus (ATCC 10240)	-	-	-	-	-	-
Proteus hauseri (ATCC 13315)	-	-	-	-	-	-
Pseudomonas aeruginosa (ATCC 10145)	-	-	-	-	-	-
Rhodococcus equi (ATCC 33701)	-	-	-	-	-	-
Salmonella enteritidis (ATCC 13076)	-	-	-	-	-	-
Salmonella typhimurium (ATCC 14028)	-	-	-	-	-	-
Staphylococcus aureus (ATCC 11632)	12.00±0.25	-	10.00±0.58	14.00±0.25	7.00±0.29	8.00±0.21
Staphylococcus epidermidis (ATCC 12228)	-	-	-	-	-	-
Staphylococcus aureus subsp. aureus (ATCC 43300)	12.00±0.25	-	10.00±0.58	14.00±0.25	7.00±0.29	8.00±0.21

Table 6.2. Antibacterial activity of *Melodinus eugeniifolus*. (1 mg/disc and 2 mg/disc) vs. ampicillin and streptomycin (1µg/disc) against 30 bacterial species tested by disc diffusion assay (continued).

Bacterial Strain	**Zone of inhibition (mm)** [a]					
Clinical isolate	Plant extract					
	BH	BC	BE	LH	LC	LE
Enterococcus faecalis	-	-	-	-	-	-
Methicillin-resistant *Staphylococcus aureus* (MRSA)	12.00±0.45	-	-	-	-	12.00±0.25
Methicillin-sensitive *Staphylococcus aureus* (MSSA)	-	-	-	-	-	-
Oxacillin-resistant coagulase negative staphylococcus (ORCNS)	-	-	-	-	-	-
Oxacillin- sensitive coagulase negative staphylococcus (ORCNS)	-	-	-	-	-	-
Streptococcus agalactiae (Group B *Streptococcus*, GBS)	-	-	-	-	-	-
Streptococcus pneumoniae	-	-	-	-	-	-
Actinobacillus sp.	-	-	-	-	-	-
Enterobacter sp.	-	-	-	-	-	-
Escherichia coli	12.00±0.21	8.00±0.13	7.00±0.22	7.00±0.38	7.00±0.15	7.00±0.31
Extended-spectrum beta-lactamase-producing *Escherichia coli* (ESBL-EC)	8.00±0.14	-	-	7.00±0.13	-	-
Extended-spectrum beta-lactamase-producing *Klebsiella pneumonia* (ESBL-KP)	-	-	-	-	-	-

Table 6.2. Antibacterial activity of *Melodinus eugeniifolus*. (1 mg/disc and 2 mg/disc) vs. ampicillin and streptomycin (1µg/disc) against 30 bacterial species tested by disc diffusion assay (continued).

Bacterial Strain	**Zone of inhibition (mm)** [a]					
Clinical isolate	Plant extract					
	BH	BC	BE	LH	LC	LE
Klebsiella sp.	-	-	-	-	-	-
Moraxella sp.	-	-	-	-	-	-
Serratia sp.	-	-	-	-	-	-

BH: bark hexane extract, BC: bark chloroform extract, BE: bark ethanol extract, LH: leaf hexane extract, LC: leaf chloroform extract, LE: leaf ethanol extract.

a: inhibition zones are the mean including disc (6 mm). b: ampicillin and streptomycin at 100µg/mL = 1 µg/disc.

-: no activity noted i.e. inhibition zone of 6 mm.

6.4 Discussions

Plants constitute a vast untapped source of medicines with great therapeutic values [161]. The prospects for the development of antibacterial drugs from medicinal plants appear to be rewarding as they can mitigate the adverse effects that are often associated with synthetic antibiotics [162, 163]. In the present study, disc diffusion assay was conducted to evaluate the antibacterial activities of crude extracts against ATCC and clinical strains.

This qualitative method is extensively used for antibiotic susceptibility testing in which filter paper discs impregnated with antibacterial agents are applied on the inoculated agar plate [164]. The efficacy of these agents can subsequently determined by measuring the diameter of the zones of inhibition that resulting from their diffusion into the agar medium around the discs [165].

According to Gislene et al. (2000) [166], any chemicals that have antibacterial activity with zones of inhibition of 7 mm and above can be considered as potential antimicrobial candidates.

In the present study, antibacterial screening of six extracts of *Melodinus eugeniifolius*. All the extracts displayed different antibacterial activity against *Staphylococcus aureus*, *Bacillus cereus* and *Escherichia coli*.

Accordin to the methodology in 6.2.1, the result is the inhibition zones which include the paper disc size of 6 mm. Therefore, out of six extracts, the hexane extract of barks and leaves exhibited certain antibacterial activity against both Gram-positive and Gram-negative bacteria. The best sensitivity to the hexane extracts at 2 mg/disc was respectively obtained against *Staphylococcus aureus* (14 mm), MRSA (12 mm), *Bacillus cereus* (12 mm) and *Escherichia coli* (14 mm). This is of special interest since most Gram-negative bacteria are more resistant to plant extracts [167]. Results from our phytochemical analysis revealed that the hexane extract of barks of *Melodinus eugeniifolius* accumulate amounts of flavonoids which could be well correlated with the activities measured.

The current result indicated the plant *Melodinus eugeniifolius* have no inhibition for most of the bacteria strains. Ideally, a chemotherapeutic agent should have a high therapeutic index and selective toxicity to pathogens by inhibition of cell wall synthesis, protein synthesis or nucleic acid synthesis and disruption of the cell membrane and enzyme inhibition. These effects should reduce bacterial survival without killing

the host [154]. According to this, the plant *Melodinus eugeniifolius* may not have these toxicity to those pathogens, or not enough.

The ability of an antimicrobial agent to reach the site of infection, which is determined by its stability and solubility, state of the infected area and absorption from where it was applied, among other things, will affect its efficacy. How susceptible the pathogen is to the antimicrobial agent will also determine the success of the treatment [154].

Natural products are a valuable source of antimicrobial drugs, and can lead to the development of drugs that have been sourced directly from a natural product or the discovery of compounds that have potential to be developed into drugs. Many antimicrobials were discovered by screening soil samples for their antimicrobial activity *in vitro* and *in vivo* [156].

In this study, almost all the crude extracts of the plant *Melodinus eugeniifolius* show the inhibition on the bacterial strain *Staphylococcus aureus. Staphylococcus aureus* is a gram positive coccus bacterium that is a member of the Firmicutes. It is found in the human respiratory tract and on the skin. Strains associated with disease can produce protein toxins, and express cell-surface proteins that bind and inactivate antibodies [168-170]. The plant *Melodinus eugeniifolius* may contain

some of the compounds which can damage the cell-surface protein expression of the pathogen.

The plant *Melodinus eugeniifolius* also indicate certain antibacterial capacity against *Bacillus cereus. Bacillus cereus* is a soil-dwelling, Gram-positive, rod-shaped bacterium. Some strains can cause foodborne illness in humans, while others are probiotics for animals [171-173]. There may be several components with selective toxicity existed in plant *Melodinus eugeniifolius*.

Among the results on all of the baterials, the bark exane extract of the plant *Melodinus eugeniifolius* the best antibacterial activity (zone of inhibition 14 mm) on the strain *Escherichia coli*. *Escherichia coli* is a gram-negative, facultatively anaerobic, rod shaped bacterium of the genus Escherichia that is commonly found in the lower intestine of warm-blooded organisms (endotherms) [174-177]. The plant *Melodinus eugeniifolius* may contain compounds which can inhibit the growth of the pathogen and defense the infection.

In general, Gram-negative bacteria are more resistant to plant-based antibacterial agents in comparison to Gram-positive bacteria [178]. The susceptibility differences between these two groups of bacteria can be attributed to their distinct cell wall structures [179].

Gram-negative bacteria are characterised by an outer membrane that enclose a comparatively thin layer of peptidoglycan [180]. The outer membrane has a phospholipid-rich inner leaflet of similar composition to the cytoplasmic membrane, while the outer leaflet facing the extracellular environment is composed primarily of lipopolysaccharides (LPS) [181], which provide an effective permeability barrier against hydrophobic compounds [182]. In addition to these structural components, the asymmetric lipid bilayer also contains porins, which from water-filled channels that selectively facilitate the passage of hydrophilic compounds based on their molecular weight and ionic charge [183, 184]. This may explain the reason of the crude extracts of the plant *Melodinus eugeniifolius* indicate better result on Gam-positive bacterias but not on Gram-negative bacterias.

On the other hand, Gram-positive bacteria posses a relatively thick peptidoglycan layer with lipoteichoic acids (LTA) anchored to the cytoplasmic membrane [185]. Nonetheless, they are devoid of a highly impermeable outer membrane, making them more susceptible to antibacterial compounds [186, 187]. These chemical composition and organisation of bacterial cell wall may rationalise the variations in the sensitivity of the ATCC and clinical bacterial strains to the crude extracts.

Plants synthesize a variety of secondary metabolites, which play a key role in the natural defense mechanisms employed by the plant against predation by microorganisms and insects. With regard to the phytochemical screening of crude extracts [188], the occurrence of alkaloids or cardiac glycosides in hexane extracts of barks may explain their activity as compared to the other crude extracts studied. For this reason, plants are able to provide us with a diverse array of antimicrobials that can be categorized into groups such as phenolics, terpenoids and essential oils, alkaloids, lectins and polypeptides, as well as polyacetylenes [5]. The isolation and identification of such compounds may contribute greatly to the success in antibiotic discovery.

Nature can provide us with a great number of novel structures, with varying complexity. Natural products are a promising source of potential drugs [189].

Flavones are phenolic structures containing one carbonyl group (as opposed to the two carbonyls in quinones). The addition of a 3-hydroxyl group yields a flavonol. Flavonoids are also hydroxylated phenolic substances but occur as a C6- C3 unit linked to an aromatic ring. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls. More lipophilic flavonoids may also disrupt microbial membranes [190].

According to all these analysis, the plant *Melodinus eugeniifolius* may have limited or even no toxicity to pathogens by inhibition of cell wall synthesis, protein synthesis or nucleic acid synthesis and disruption of the cell membrane and enzyme inhibition. Hense, the result on antibacterial aspect indicate the limited activities.

6.5 Conclusion

All crude extracts of *Melodinus eugeniifolius* exist certain antimicrobial activity against both Gram-positive and Gram-negative bacteria. The most promising activity was from hexane extracts which were displayed against Gram-positive bacteria *Staphylococcus aureus* and Gram-negative bacteria *Escherichia coli*. This serves as a clear indication that only a few potential of these extracts for further chemical studies as antimicrobial agents.

CHAPTER Ⅶ

IN VITRO ANTIPARASITIC ACTIVITIES OF *MELODINUS EUGENIIFOLIUS*

7.1 Introduction

Parasitic infection is becoming a severe diseases that threaten human health in the world. Around one fourth of the population of the world may be suffering from parasitic disease, and those who come from underdeveloped agricultural and rural areas in the tropical and subtropical regions have the highest prevalence. During the evolution of humans a broad set of parasites have evolved, that use us as a host organism [191]. Usually a parasite will not kill the host, or not kill the host immediately, most parasites weaken our health. However, some parasitic infections can be deadly if the patients are not treated with adequate therapeutics. Because humans usually live in close proximity and often without good hygienic conditions the transmission of parasites within a human population is often facilitated [192].

Many parasitic infections are the cause of tropical diseases, such as malaria, trypanosomiasis, leishmaniasis, Chagas disease, schistosomiasis, onchocerciasis, lymphatic filariasis, and helminthiases. Parasites are

responsible for probably more than one to two billion infections, which lead to several million deaths every year [193].

Leishmaniasis is a group of disease, caused by *Leishmania* species. The disease is considered a major public health problem in almost a hundred countries in the world causing morbidity and mortality [194]. Different modes of treatment are used in the treatment of cutaneous leishmaniasis. Pentavalent antimonial compunds are the first line treatment but generally are toxic so several significant advances in the chemotherapy of the leishmaniasis have occurred in the last 10 years [191]. Leishmaniasis is caused by protozoan parasites of the genus *Leishmania* which invade macrophages of the host organism. A distinction is made between cutaneous, visceral and diffuse leishmaniasis, of which visceral leishmaniasis is a fatal disease causing approximately sixty thousand death per year [195]. In some cases, leishmaniasis and HIV infections co-occur and these patients usually have a poor prognosis. Patients are treated with the synthetic drugs stibogluconate, meglumine and pentamidine, developed seventy years before, which have severe side effects and have failed to work in North Bihar [196, 197]. Besides, the macrolide antibiotic amphotericin B has been employed, which can also be toxic for patients. New developments include the anticancer drug miltefosine, the aminoglycoside antibiotic paronomycin, and the 8-aminoquinoline sitamaquine [195]. Human have used medicinal plant for

several thousands of years to treat illness and health disorders [198]. Until now, natural products still play an important role in therapy and new drugs with functional pharmacological capacity have been derived from natural products continuously [199]. Promising anti-leishmanial activities exist among natural products and berberine which occurs in many TCM plants [196, 197].

7.2 Methodology

Phytochemical analysis in CHAPTER IV indicates the plant *Melodinus eugeniifolius* may have antiparasitic potential. In this chapter, six crude extracts of *Melodinus eugeniifolius* would be tesed the antiparasitic activity.

7.2.1 Principle of method

The antileishmanial activity of the compounds was performed according to the standard methods as described by Mosman [200].

7.2.2 Protocol

In vitro antileishmanial activity against *Leishmania donovani* promastigotes.

Antileishmanial activity of tested plant extract, against *Leishmania donovani* (strain MHOM / IN / 1983 / AG 83) promastigotes, was evaluated by a quantitative colourimetric assay using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide)] [201]. Amphotericin B (IC_{50} = 0.4 ± 0.1 µM) was used as the positive control in all the experiments. Promastigotes (5×10^5 cells/mL; 300 µl), were treated with and without tested samples at concentrations of 100 and 500 µg/mL, and incubated at 22 ± 2 °C. After 72 hrs, cells were harvested, and re-suspended in PBS (500 µl) containing MTT (0.3 mg/mL). Purple formazan crystals were dissolved in DMSO and the optical density (O.D.) was measured at 570 nm in an ELISA reader (BIO-RAD; model 680, USA). The number of viable cells was directly proportional to the amount of formazan produced through the reduction of yellow MTT by the dehydrogenase enzymes present in the inner mitochondrial membrane of the living cells.

The percentage of Growth inhibition was calculated as follows:

Inhibition % = [(O.D. of untreated control – O.D. of treated set) / O.D. of untreated control] × 100

7.2.3 Statistical analysis

The IC_{50} values (concentration of drug which inhibited at least 50 % cell growth) for each compounds were determined from respective dose-responsive percentage inhibition curves with the help of Microsoft Excel and data were reported as mean and SD values obtained from a minimum of three determinations. Graph was plotted with SD and 95% confidence interval.

7.2.4 Reagents and materials

Dimethyl sulfoxide (DMSO, R&M Chemicals, UK), MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Life Technologies), FCS (fetal calf serum) (for cell culture, Sigma, Germany), RPMI-1640 medium (for cell culture, Sigma), defined keratinocyte-SFM (Life Technologies), PBS(dulbecco's phosphate buffered saline) (Sigma), trypan blue (for cell culture, Sigma), Trypsin EDTA (0.05% Trypsin, 0.53mM EDTA.4Na) (Gibco), Penicillin-Streptomycin (liquid, 100 mL/pk) (Gibco).

96-well TC Plate (for mammalian cell culture, Orange Scientific), centrifuge tube (Orange Scientific), TC flask (Orange Scientific),

minisart syringe filter (SSI), kit, pipette and tips are all from Dragon Med.

7.3 Result

All the plant extracts, except the chloroform fractions of bark and leaf of *Melodinus eugeniifolius*, showed strong antileishmanial effect on the promastigotes of *Leishmania donovani* (IC_{50} < 500 µg/mL). Among the different extracts tested, the ethanol and hexane extract of barks showed significant antileishmanial activities with IC_{50} values of 159.9 µg/mL and 270.3 µg/mL (Table 7.1, Figure 7.1).

Table 7.1: MTT assay of crude plant extracts against *Leishmania donovani* (AG 83) promastigotes.

Extracts	Inhibition% at	
	500 µg/mL	1000 µg/mL
BH	**73.3**	**79.9**
BC	46.1	52.8
BE	**74.4**	**82.5**
LH	67.8	76.3
LC	23.2	36.2
LE	52.1	80.2

BH: bark hexane extract, BC: bark chloroform extract, BE: bark ethanol extract, LH: leaf hexane extract, LC: leaf chloroform extract, LE: leaf ethanol extract.

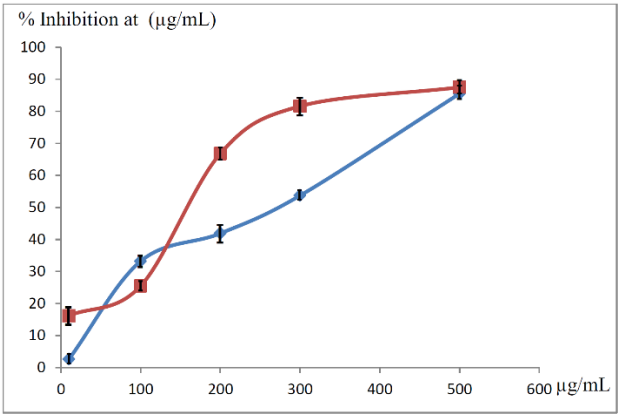


Figure 7.1: Detailed assay against *Leishmania donovani* (AG 83) promastigotes on BH & BE extracts.

BH: bark hexane extract, BE: bark ethanol extract

7.4 Discussion

According to the introduction, parasites are eukaryotes and share a large amount of molecular and biochemical properties with their eukaryotic hosts, and it is always difficult to find antipatasitic medicines which are both non-toxic and effective for humans. This limitation often has to be kept in mind when discussing the numerous findings that some drug or extract from a medicinal plant is active against a parasite *in vitro*. In order to be medicinally functional, a qualified medicine should have bioavailablity and cannot poison the patient. A first guidance is the determination of a selectivity index (SI) which compares the cytotoxicity of a drug against a parasite and a library of human cells [192].

In the present study, six different extractions of *Melodinus eugeniifolius.* (Apocynaceae) leaves and barks were screened for their *in vitro* antileishmanial activity, among the different extracts tested, the ethanol and hexane extract of barks showed significant antileishmanial activities with IC_{50} values of 159.9 µg/mL and 270.3 µg/mL.

Most of the antiparasitic properties of extracts and isolated natural products have been tested *in vitro* only. Translation of the *in vitro* research results into *in vivo* trials is urgently required. Small lipophilic secondary metabolites, such as terpenoids or phenylpropanoids as found in the essential oil of many plants, can dissolve in biomembranes and disturb their fluidity and the function of membrane proteins [203]. Therefore, many of the lipophilic mono- and sesquiterpenes, phenylpropanoids and isothiocyanates have a certain degree of antimicrobial and antiparasitic properties [204]. Furthermore, even if animal experiments were successful, we would need clinical trials of the new compounds alone or in combination with established parasiticidal drugs to prove their efficacy and safety. These developments are costly and it is presently difficult to attract the pharmaceutical industries into these fields for various reasons [198, 199]. TCM and other traditional medicine systems employ several thousand of medicinal plants; some of which have known antiparasitic properties [199]. They offer a unique opportunity to identify natural products which could be potentially used to treat parasitic infections [199, 202]. A number medicinal plants and secondary metabolites isolated from them have been screened for anti-trypanosomal activity [205].

According to the analysis above mentioned, it can be explained that crude extracts of *Melodinus eugeniifolius* indicate certain but not satisfied antileishmanial activities. Based on the phytochemical analysis result, lipophilic secondary metabolites, such as terpenoids or phenylpropanoids should be existed in the crude extracts of the plant *Melodinus eugeniifolius*, but not too much. Therefore, limited activities of dissolving in biomembranes of parasites and disturbing their fluidity and the function of membrane proteins.

7.5 Conclusion

Plant substances continue to serve as a wellspring of drugs for the world population and several plant-based drugs are in extensive clinical use. Preliminary antiparasitic screening of six extracts of *Melodinus eugeniifolius* leaves and barks were screened for their *in vitro* antileishmanial activity, among the different extracts tested, the ethanol and hexane extract of barks showed significant antileishmanial activities.

CHAPTER Ⅷ

IN VITRO ANTICANCER ACTIVITIES OF *MELODINUS EUGENIIFOLIUS*

8.1 Introduction

Cancer, a cellular malignancy that results in the loss of normal cell-cycle control, such as unregulated growth and the lack of differentiation, can develop in any tissue of any organ, and at any time [206]. Cancer is a group of diseases characterised by uncontrolled growth and spread of abnormal cells, which can lead to death if left untreated [207]. The etiology of cancer can be associated with both external factors, including tobacco, infectious organisms, chemicals and radiation, as well as internal factors, such as inherited mutations, hormones, immune conditions and mutations occurring from metabolism [208]. These causal factors may act synergistically or sequentially to initiate or promote carcinogenesis [209].

Carcinogenesis is the transformation of a normal cell to a cancerous cell through many stages, which occur over a number of years or even decades. The first stage of carcinogenesis is the initiation stage, which involves the reaction between the carcinogens and the DNA of the cells. Inhibiting this early stage of cancer is an important strategy in cancer

prevention or treatment. Promotion is the second stage and may occur slowly over an extended period of time, ranging from several months to years. Beneficial effects may arise from a change in lifestyle and diet, which may result in the individual not developing cancer during his or her lifetime. The third stage is the progressive stage, involving the spread of the cancer. It is evident that, upon entering into this stage, preventative factors such as diet have less of an impact [210].

Despite the therapeutic advances made in understanding the processes involved in carcinogenesis, cancer remains one of the foremost causes of morbidity and mortality, the worldwide mortality rate increases annually, with more than seven million deaths occurring per year [211]. More significantly, the most common cancer treatments are restricted to surgery, radiation and chemotherapy [212], which are severely fraught with challenges concerned with adverse side effects of drugs [213] due to their non-specific systemic distribution [214], inadequate drug concentrations reaching the tumour [215], intolerable toxicity [216], and development of multiple drug resistance acquired upon repeated chemotherapeutic cycles [217]. Hence, cancer chemotherapy has become a major focus area of research, and it is necessary to develop novel chemotherapeutic agents with enhanced potency and specificity. Different lifestyles, risk factors (such as age, gender, race, genetic disposition) and the exposure to different environmental carcinogens,

lead to the varying patterns of cancer incidence [206]. At least 35% of all cancers worldwide result from an incorrect diet, and in the case of colon cancer, diet may account for 80% of these cases [210]. One of the most important contributions to the development of cancer is the oxidative damage to DNA [218]. Permanent genetic alterations may occur in those cells where DNA is damaged and where division of this DNA occurs before it can be repaired. These cells may begin to divide more rapidly and result in carcinogenesis [210].

8.1.1 Natural products and carcinogenesis defence

"Chemoprevention" is defined as a process to delay or prevent carcinogenesis in humans through the ingestion of dietary or pharmaceutical agents. This also implies the identification of chemical entities (specifically cytotoxic entities) that are effective against a range of cancer cell lines, although less active or non-toxic against the normal (healthy) cell population. The search for such anticancer agents from plant sources started in the 1950's, and plant products have proven to be an important source of anticancer drugs [219]. This directly results from the biological and chemical diversity of nature, which allows for the discovery of completely new chemical classes of compounds.

The discovery and development of plant-derived compounds led to the first cures of human cancer, specifically upon administration of these compounds in combination with synthetic agents. At present more than 450 different compounds have been isolated from active plants that have shown *in vitro* and/or *in vivo* antitumor activity. Virtually every major class of natural chemical compound is represented in the list of active constituents [220]. Of the 121 medications being prescribed for use in cancer treatment, 90 are sourced from plants. It was also determined that approximately 74% of these discoveries were as a result of an investigation into the claims made by folkloric medicine [221]. A number of natural products are used as chemoprotective agents against commonly occurring cancers Examples of these compounds used as cytotoxic drugs are shown in Table 8.1.

A number of mechanisms exist by which phytochemicals aid in the prevention of cancer. This preventative action most probably results from the additive or synergistic effects of a number of phytochemicals, since cancer is a multi-step process. Proposed mechanisms by which phytochemicals may prevent cancer include: (i) anti-oxidant and free radical scavenging activity; (ii) antiproliferative activity; (iii) cell-cycle arresting activity; (iv) induction of apoptosis; (v) activity as enzyme cofactors; (vi) enzyme inhibition; (vii) gene regulation; (viii) activity as hepatic phase I enzyme inducers, and (ix) activity as hepatic phase II

enzyme inducers. Oxidative damage to DNA, proteins and lipids, resulting from an increase in oxidative stress, is considered to be one of the most important mechanisms contributing to the development of cancer [222].

The phytochemicals that most often appear to be protective against cancer are curcumin, genistein, resveratrol, diallyl sulfide, (S)-allyl cystein, allicin, lycopene, ellagic acid, ursolic acid, catechins, eugenol, isoeugenol, isoflavones, protease inhibitors, saponins, phytosterols, vitamin C, lutein, folic acid, beta carotene, vitamin E and flavonoids, to name but a few [210]. These phytochemicals suppress the inflammatory processes that lead to transformation, hyperproliferation, and initiation of carcinogenesis. The inhibitory influences of these phytochemicals may ultimately suppress the final steps of carcinogenesis viz. angiogenesis and metastasis.

The present study was undertaken to screen the anticancer potential *Melodinus eugeniifolius.* No anticancer studies have been reported on this species to date.

Table 8.1: Cytotoxic drugs developed from plant sources.

Therapeutic agent	Plant source	Mechanism of action	Treatment of cancer type	Reference
Vincristine, vinblastine	*Catharanthus roseus*	Inhibition of tubulin polymerisation	Hodgkin's disease	[223]
Etoposide, teniposide	*Podophyllum peltatum*	Inhibition of topoisomerase II	Testicular cancer, and small cell lung carcinoma, leukaemias, lymphomas	[223-225]
Paclitaxel, docetaxel	*Taxus brevifolia*	Promotion of tubulin stabilization	Ovarian and breast carcinoma	[223]
Irinotecan, topotecan, 9-aminocampothecin, 9-nitrocamptothecin	*Camptotheca acuminata*	Inhibition of topoisomerase I	Advanced colorectal cancer, also active in lung, cervix and ovarian cancer	[224]
Homoharingtonine	*Harringtonia cephalotaxus*	Inhibition of DNA polymerase	Various leukaemias	[223]

Table 8.1: Cytotoxic drugs developed from plant sources (continued).

Therapeutic agent	Plant source	Mechanism of action	Treatment of cancer type	Reference
4-Ipomeanol	*Ipomoea batatas*	Cytochrome P-450-mediated conversion into DNA-binding metabolites	Lung cancer	[223]
Elliptinium	*Bleekeria vitensis*	Inhibition of topoisomerase II	Advanced breast cancer	[223]
Flavopiridol	*Amoora rohituka, Dysoxylum binectariferum*	Inhibition of cyclindependent kinases	Encouraging results noted in a variety of solid and haematological malignancies, in patients with colorectal, prostate, lung, renal carcinoma, non-Hodgkin's lymphoma and chronic lymphocytic leukaemia	[223]

8.2 Methodology

Phytochemical analysis in CHAPTER IV indicates the plant *Melodinus eugeniifolius* may have anticancer potential. In this chapter, six crude extracts of *Melodinus eugeniifolius* would be tesed the anticancer activity.

8.2.1 Principle of method

MTT assay is dependent on the reduction of the tetrazolium salt MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide) by the mitochondrial dehydrogenase of viable cells to form a blue formazan product. The assay measures cell respiration and the amount of formazan produced is proportional to the number of living cells present in culture which will result in lower optical density (OD) [226]: Biological in vitro assay was determined as follows [227].

8.2.2 Protocol

8.2.2.1 Cell lines and cell culture

Human derived cell lines were routinely cultivated at 37 ℃ in an atmosphere of 5 % CO_2 in RPMI 1640 medium supplemented with 2

mM L-glutamine and 10% fetal calf serum and subcultured twice weekly to maintain continuous logarithmic growth. Cells were seeded into 96-well microtiter plates at a density of 3-5 $\times 10^3$ per well and allowed 24 hrs to adhere before extracts were introduced (final concentration 200 μg/mL to 1 μg/mL).

8.2.2.2 Preparation of plant samples

Extracts were prepared as 500 ug/mL top stock solutions, dissolved in DMSO, and stored at 40 ℃, protected from light for a maximum period of 4 weeks. Serial drug dilutions were prepared in medium immediately prior to each assay.

8.2.2.3 The MTT assay

The remainder of the cells were syringed through a 23 G needle to attain a single cell suspension. The cells were counted and seeded at a density of 3 x 10^3 cells per well in a 96-well plate. The cells were suspended in RPMI tissue culture medium per well. The outer columns of the plate were filled with medium to prevent evaporation from treatment wells in addition to providing blank readings for the plate reader. Cells were additionally seeded in a time zero (t_0) plate in the same manner as the experimental treatment plate. The cells were incubated overnight at 37

^{o}C in a 5 % CO_2 environment to allow for attachment. Cells were then treated with serial dilutions of various concentrations the following day. A volume of 20 μL of each dilution was added to respective treatment wells (total volume 200 μL) to yield final concentrations of 0.1 μg/mL, 0.5 μg/mL, 1 μg/mL, 5 μg/mL, 10 μg/mL, 50 μg/mL, 100 μg/mL, 500 μg/mL and 1 mg/mL. A separate DMSO control trial was done to ensure that it did not affect any of the results obtained. A t_0 measurement was taken to obtain a measurement of cell viability at the time of treatment. Treated cells were incubated for 72 hrs at 37 ^{o}C. At the time of extract addition and following 72 hrs exposure, MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide) was added to each well. Incubation at 37 ℃ for 4 hrs allowed reduction of MTT by viable cells to an insoluble formazan product. Well contents were aspirated and formazan solubilized by addition of DMSO: glycine buffer (pH 10.5) (4:1). The plate was then placed in an orbital plate shaker for 2-3 minutes to aid formazan dissolution before obtaining the absorbance reading at 550-570 nm on microplate readers (Anthos Labtec, Perkin Elmer Envision, Biohit plc). The cell viability rate was calculated as follows:

$$Cell\ viability\ (\%) = \frac{(Absorbance\ of\ the\ treated\ wells)}{(Absorbance\ of\ the\ control\ wells)} \times 100$$

8.2.3 Statistical analysis

Concentration–response curves were calculated using the Prism software package 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com (GraphPad, San Diego, USA) and data were reported as mean and SD values obtained from a minimum of three determinations. Non-linear best fit was plotted with SD and 95% confidence interval.

To calculate IC_{50}, It would need a series of dose-response data and growth inhibition or % cell viability. The values of y are in the range of 0-100.

Linear Regression: The simplest estimate of IC_{50} is to plot x-y and fit the data with a straight line (linear regression). IC_{50} value is then estimated using the fitted line, i.e., $Y = a * X + b$, $IC_{50} = (50 - b)/a$.

Log transformation: Frequently, linear regression is not a good fit to dose-response data. The response-curve fits better to a straight line if the x-axis is logarithm-transformed.

8.2.4 Reagents and materials

Dimethyl sulfoxide (DMSO, R&M Chemicals, UK), MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Life Technologies), FCS (fetal calf serum) (for cell culture, Sigma, Germany), RPMI-1640 medium (for cell culture, Sigma), defined keratinocyte-SFM (Life Technologies), PBS(dulbecco's phosphate buffered saline) (Sigma), trypan blue (for cell culture, Sigma), Trypsin EDTA (0.05% Trypsin, 0.53mM EDTA.4Na) (Gibco).

96-well TC Plate (for mammalian cell culture, Orange Scientific), centrifuge tube (Orange Scientific), TC flask (Orange Scientific), minisart syringe filter (SSI), kit, pipette and tips are all from Dragon Med.

8.3 Results

8.3.1 HT-29 human colon cancer cell line

A range of extracts were evaluated in MTT assays following a 3-day exposure against a panel of human colon cancer cell lines, HT-29. The best growth inhibition was observed with the chloroform extracts of

leaves and barks, GI_{50} values of 99.5 µg/mL and 124.2 µg/mL respectively (Table 8.2, Figure 8.1).

Table 8.2: Growth inhibition of HT-29 human cancer cell lines by plant extracts

Cell lines/extract	**GI_{50} µg/mL**		
	Chloroform	Ethanol	Hexane
HT 29			
L	99.5±4.23	154.6±10.16	>200
B	124.2±7.95	>200	>200

L-leaves, B-barks

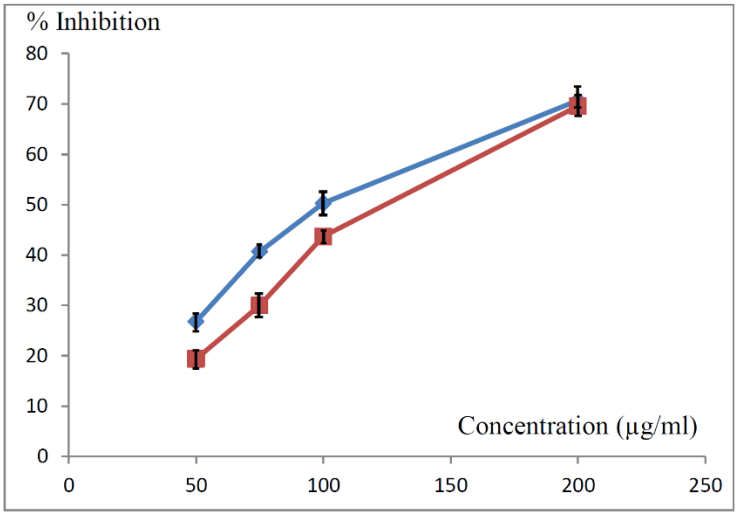


Figure 8.1: Effects of extracts on HT 29 growth.

LC: leaves chloroform extract;

BC: bark chloroform extract.

8.3.2 HCT-116 human colon cancer cell line

All the plant extracts of *Melodinus eugeniifolius.* showed anti-cancer activity on the HCT-116 cell line. The most extraordinary result was revealed by the leaf hexane and chloroform extract, GI_{50} values of 50.22 µg/mL and 50.84 µg/mL respectively against the human colon cancer cell HCT-116. (Table 8.3, Figure 8.2, 8.3).

Table 8.3: MTT assay against HCT-116 cell line.

Extracts	GI_{50} value (µg/mL)
BH	517.71 ±22.15
BC	66.65± 10.31
BE	475.70± 37.38
LH	50.22±8.23
LC	50.84± 12.02
LE	480.17± 24.78

BH: bark hexane extract, BC: bark chloroform, BE: bark ethanol extract, LH: leaf hexane extract, LC: leaf chloroform extract, LE: leaf ethanol extract.

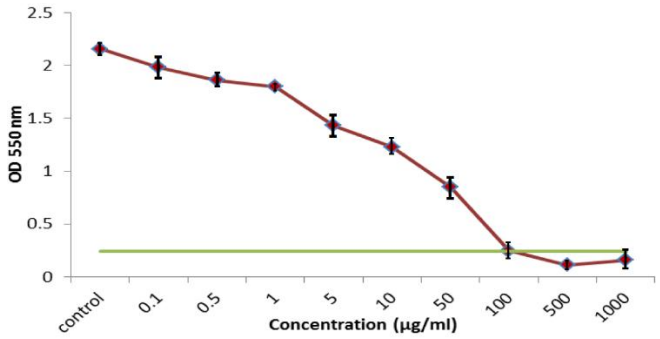


Figure 8.2: Effect of leaf hexane extract on HCT-116 cell line growth.

Extract Time Zero

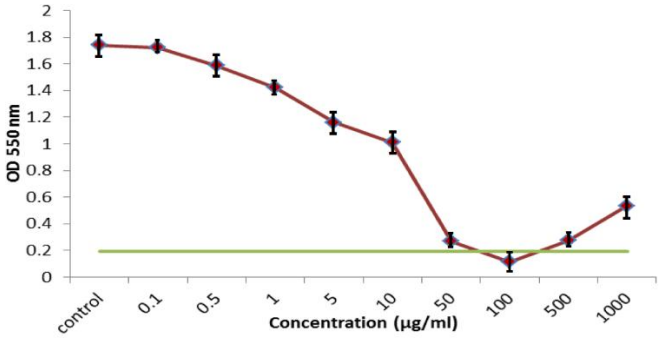


Figure 8.3: Effect of leaf chloroform extract on HCT-116 cell line growth.

Extract Time Zero

8.3.3 MCF-7 human breast cancer cell line

Six crude extracts of *Melodinus eugeniifolius* had obvious antitumor activity against the MCF-7 human breast cancer cell line. Among all the extracts, hexane and chloroform extract of leaves revealed the most significant antitumor activities with GI_{50} values of 53.93 µg/mL and 63.80 µg/mL respectively (Table 8.4, Figure 8.4, 8.5).

Table 8.4: GI_{50} values against MCF-7 cell line.

Extracts	GI_{50} value (µg/mL)
BH	489.14 ±35.03
BC	64.54± 21.25
BE	459.62± 28.65
LH	53.93±9.61
LC	63.80± 9.86
LE	80.42± 4.62

BH: bark hexane extract, BC: bark chloroform, BE: bark ethanol extract, LH: leaf hexane extract, LC: leaf chloroform extract, LE: leaf ethanol extract.

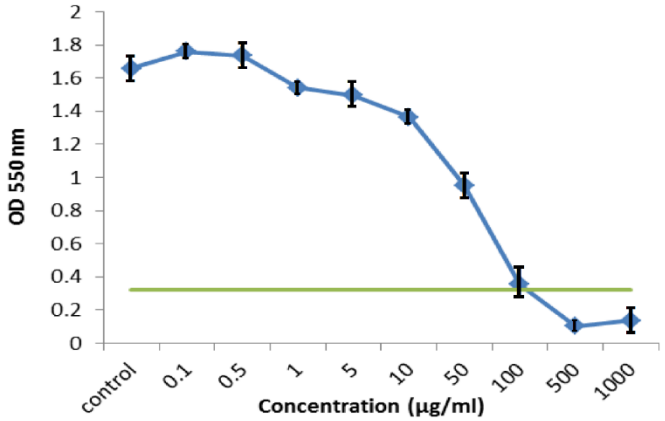


Figure 8.4: Effect of leaf hexane extract on MCF-7 cell line growth.

Extract Time Zero

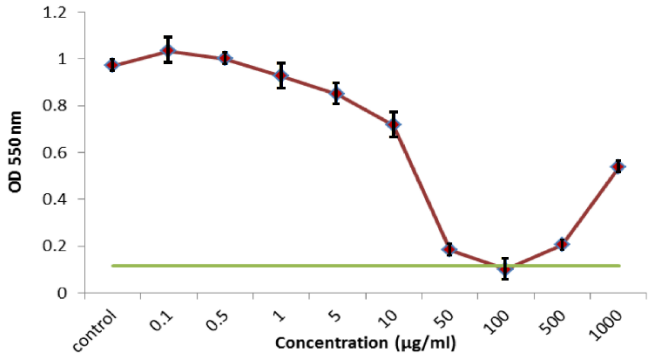


Figure 8.5: Effect of leaf chloroform extract on MCF-7 cell line growth.

Extract Time Zero

8.3.4 Caco-2 human colon adenocarcinoma cell line

Extracts were evaluated by MTT assays against a panel of Caco-2 human colon adenocarcinoma cell line. The bark chloroform and leaf ethanol extract showed strong antitumor activities with GI_{50} values of 193.7 µg/mL and 167.1 µg/mL respectively against the human colon cancer cell CaCo-2 (Table 8.5, Figure 8.6, 8.7).

Table 8.5: GI_{50} values against CaCo-2 cell line.

Extracts	GI_{50} value (μg/mL)
BH	>500
BC	193.7
BE	>500
LH	>500
LC	>500
LE	167.1

BH: bark hexane extract, BC: bark chloroform, BE: bark ethanol extract, LH: leaf hexane extract, LC: leaf chloroform extract, LE: leaf ethanol extract.

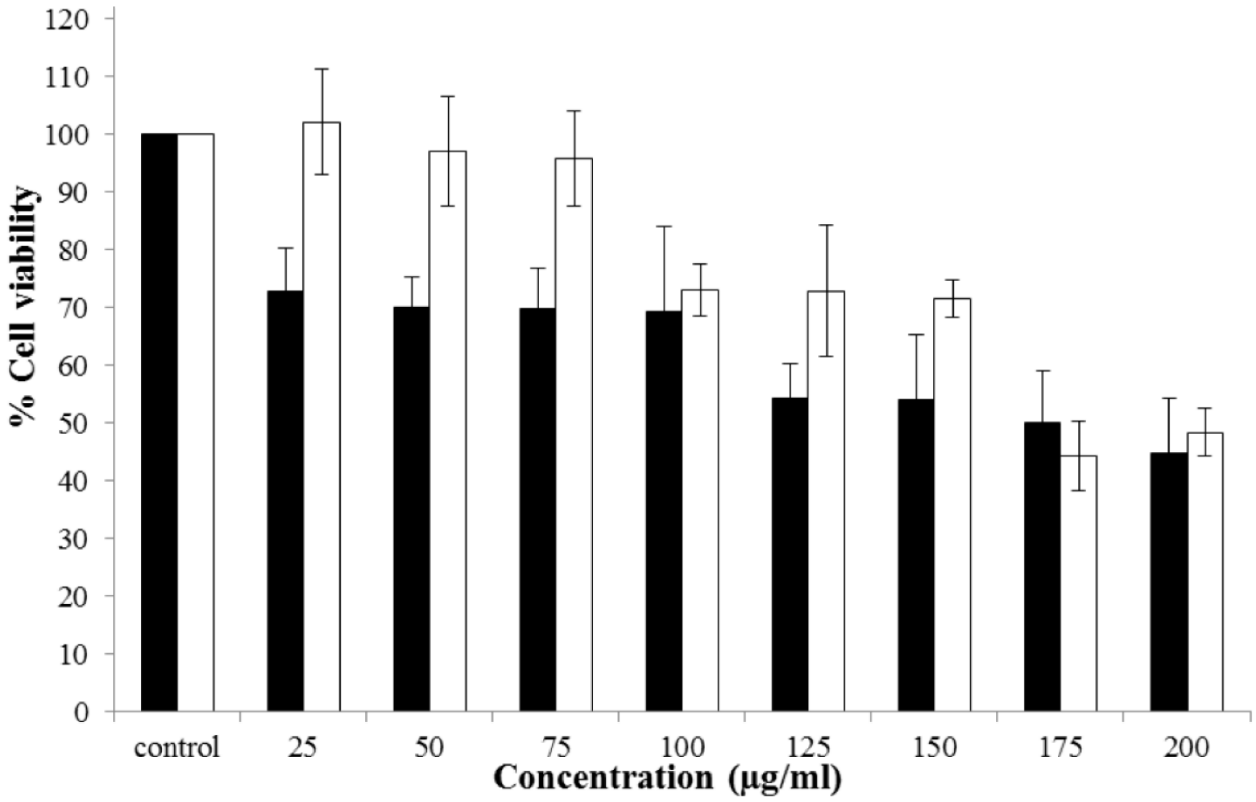


Figure 8.6: Effect of leaf ethanol and bark chloroform extracts on CaCo-2 cell line growth.

□BC: bark chloroform extract, ■LE: leaf ethanol extract.

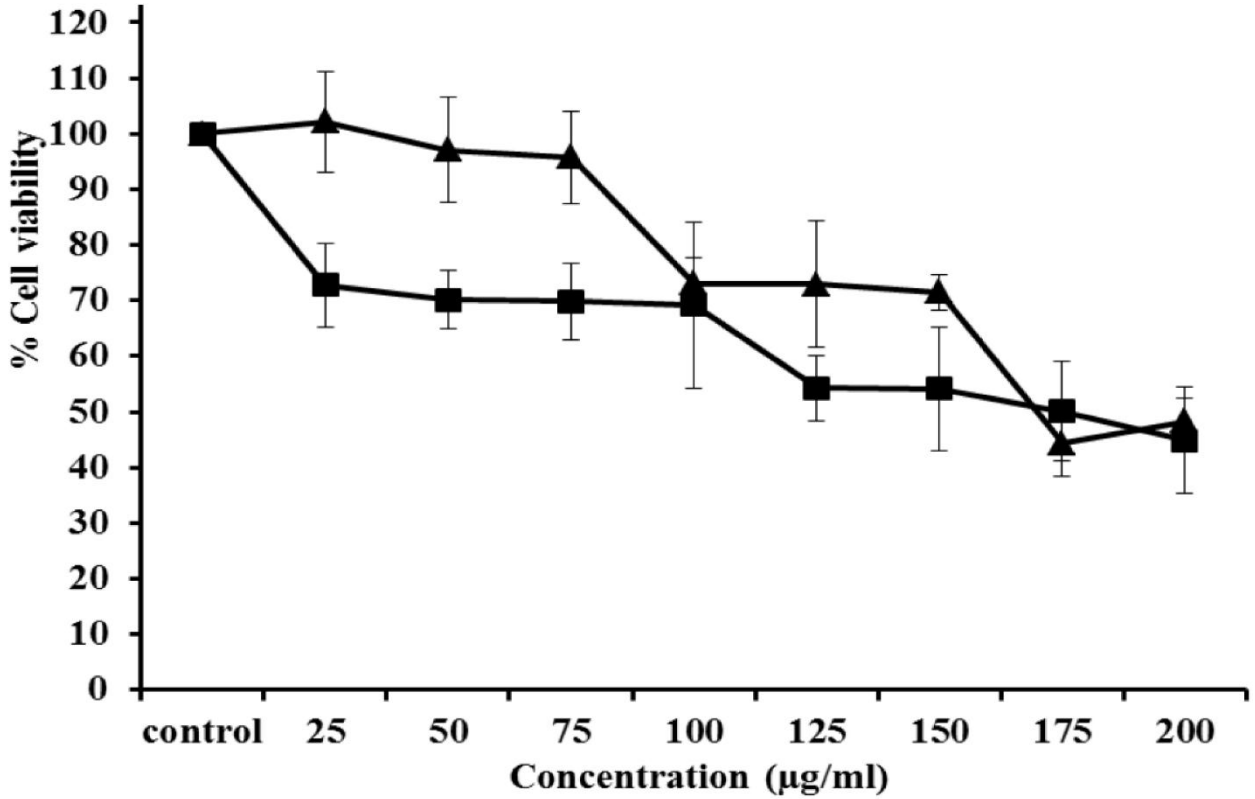


Figure 8.7: Effect of leaf ethanol and bark chloroform extracts on CaCo-2 cell line growth (2).

BC: bark chloroform extract, LE: leaf ethanol extract.

8.3.5 HK-1 human nasopharyngeal carcinoma cell line

Six crude extracts of *Melodinus eugeniifolius* were observed obvious to have antitumor activity against the HK-1 human nasopharyngeal cancer cell line. Among all the extracts, only the bark chloroform extract revealed the most significant anticancer activity with a GI_{50} value of 66.7 µg/mL, besides, the leaf chloroform and ethanol extracts also shown certain antitumor capacity with GI_{50} values of 170.7 µg/mL and 159.2 µg/mL respectively (Table 8.6, Figure 8.8, 8.9).

Table 8.6: GI_{50} values against HK-1 cell line.

Extracts	GI_{50} value (μg/mL)
BH	>500
BC	66.7
BE	>500
LH	>500
LC	170.7
LE	159.2

BH: bark hexane extract, BC: bark chloroform, BE: bark ethanol extract, LH: leaf hexane extract, LC: leaf chloroform extract, LE: leaf ethanol extract.

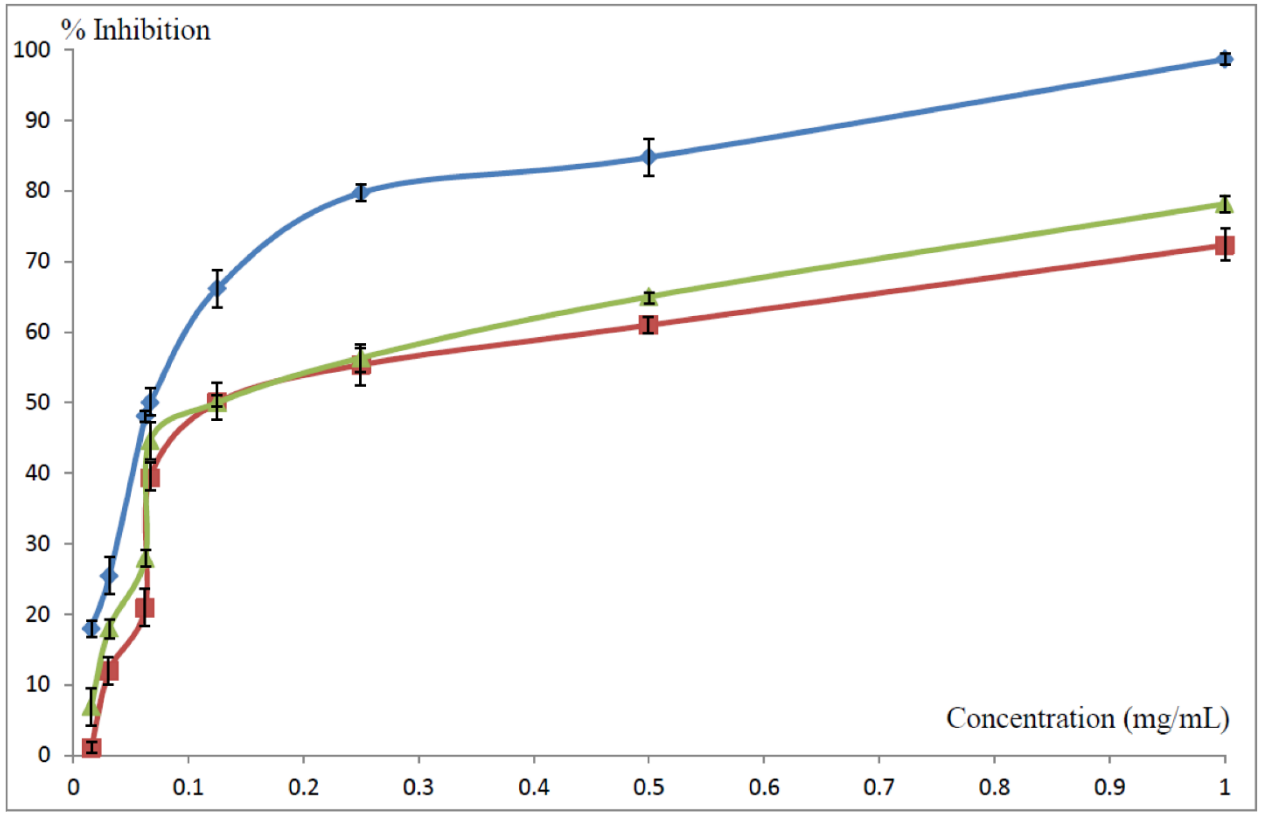


Figure 8.8: Effect of crude extracts on HK-1 cell line growth.

BC: bark chloroform extract, LC: leaf chloroform extract, LE: leaf ethanol extract.

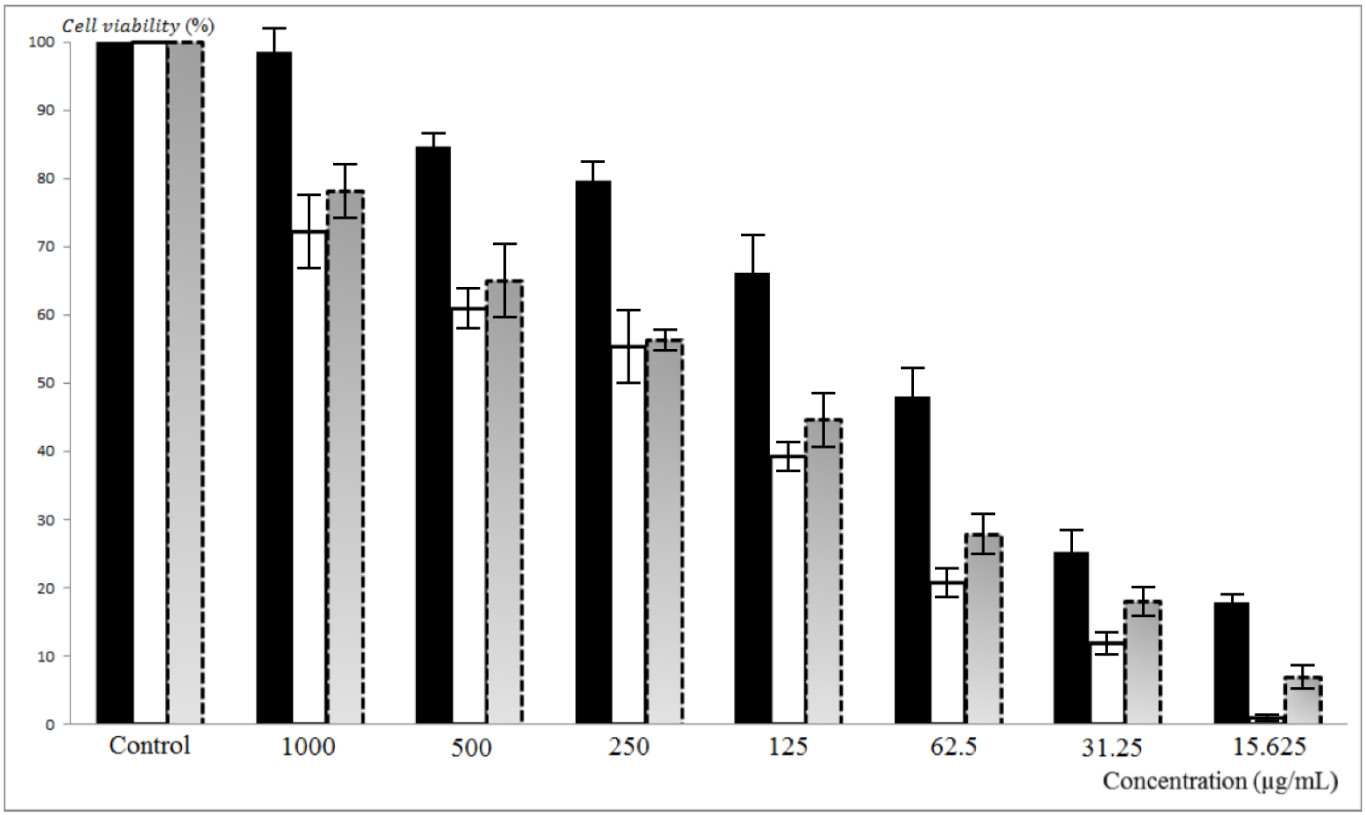


Figure 8.9: Effect of crude extracts on HK-1cell line growth (2).

■BC: bark chloroform extract, ◘LC: leaf chloroform extract, ◘LE: leaf ethanol extract.

8.3.6 MRC-5 human fetal lung fibroblast cell line

Six crude extracts of *Melodinus eugeniifolius* had been observed to have obvious antitumor activity against the MRC-5 human lung fibroblast cell line. None of the fractions from extracts revealed inhibition activity as all of the GI_{50} values were higher than 1000 μg/mL (Table 8.7, Figure 8.10-8.17).

Table 8.7: GI_{50} values against MRC-5 cell line.

Extracts	GI_{50} value (μg/mL)
BH	>1000
BC	>1000
BE	>1000
LH	>1000
LC	>1000
LE	>1000

BH: bark hexane extract, BC: bark chloroform, BE: bark ethanol extract, LH: leaf hexane extract, LC: leaf chloroform extract, LE: leaf ethanol extract.

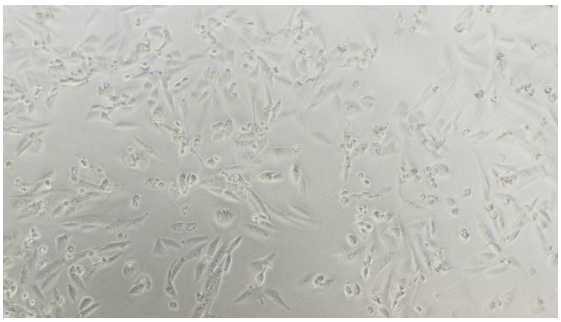


Figure 8.10: MRC-5 human lung fibroblast cells (Blank)

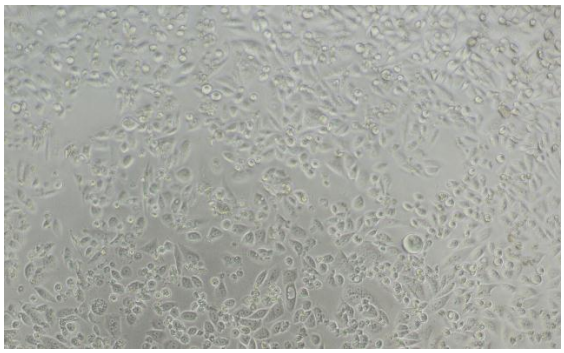


Figure 8.11: MRC-5 human lung fibroblast cells (Control)

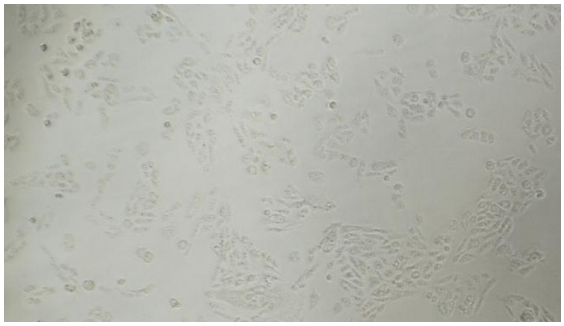


Figure 8.12: MRC-5 human lung fibroblast cells (Bark Hexane Extract)

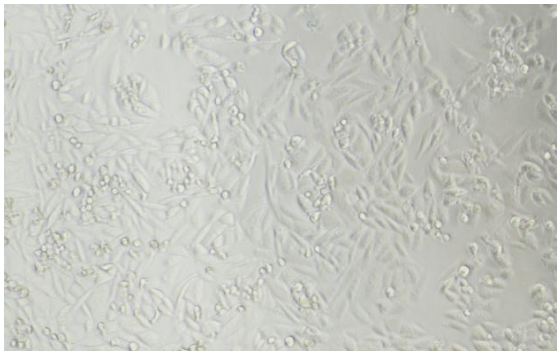


Figure 8.13: MRC-5 human lung fibroblast cells (Leaf Hexane Extracts)

Figure 8.14: MRC-5 human lung fibroblast cells (Bark Chloroform)

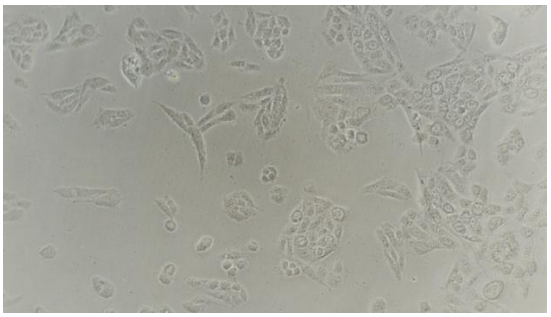


Figure 8.15: MRC-5 human lung fibroblast cells (Leaf Chloroform)

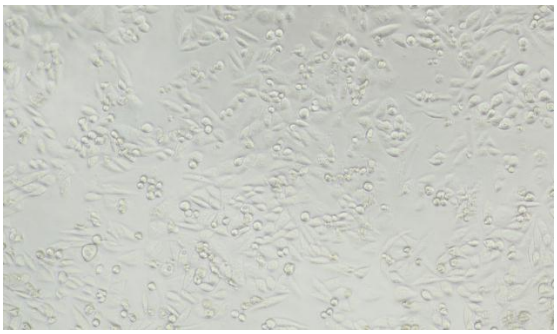


Figure 8.16: MRC-5 human lung fibroblast cells (Bark Ethanol)

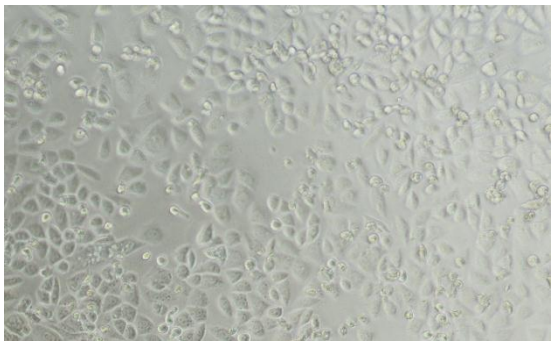


Figure 8.17: MRC-5 human lung fibroblast cells (Leaf Ethanol)

8.4 Discussion

The method used for the MTT (3-(4,5-dimethylazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was adapted from Mosmann [200]. The MTT assay is colorimetric in nature and is can be used to assess cell viability from treatment of therapeutic agents or toxic compounds. MTT (yellow) is reduced to a purple formazan in living cells by the activity of cellular enzymes, specifically mitochondrial dehydrogenases, and the intensity of the dye can be quantified by a spectrophotometer. The assay is rapid, economical, and reproducible.

HT-29 is a human colorectal adenocarcinoma cell line with epithelial morphology. These cells are sensitive to the chemotherapeutic drugs 5-fluorouracil and oxaliplatin, which are standard treatment options for colorectal cancer. In addition to being a xenograft tumor model for colorectal cancer, the HT-29 cell line is also used as an in-vitro model to study absorption, transport, and secretion by intestinal cells. Under standard culture conditions, these cells grow as a nonpolarized, undifferentiated multilayer. Altering culture conditions or treating the cells with various inducers, however, results in a differentiated and polarized morphology, characterized by the redistribution of membrane antigens and development of an apical brush-border membrane. This cell line was established in 1964 from the primary tumor of a 44-year-old

Caucasian female with colorectal adenocarcinoma [201, 228-229]. HT-29 cells are human intestinal epithelial cells which produce the secretory component of Immunoglobulin A (IgA), and carcinoembryonic antigen (CEA). Cells are used for tumourigenicity studies (Figure 8.18).

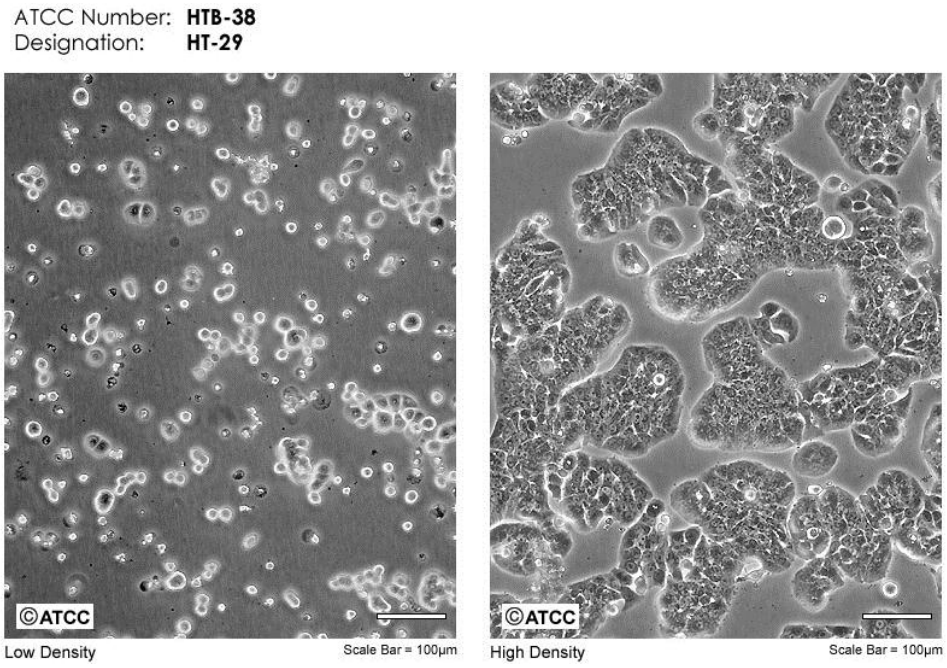


Figure 8.18: HT-29 human colon cancer cells.

In the present study, six extracts of *Melodinus eugeniifolius* leaves and barks were screened for their *in vitro* antitumor activities, among the different extracts tested, the chloroform extracts of leaves and barks revealed significant antitumor activities with GI_{50} values of 99.5 µg/mL and 124.2 µg/mL against the human colon cancer cell HT-29.

Since the carcinogenesis of HT-29 cells is mainly focused on the production of Ig A and CEA which made by the intestinal epithelium. The antitumor mechanism of the crude extracts of the plant *Melodinus eugeniifolius* on HT-29 cells had been predicted the induction of apoptosis of intestinal epithelium.

The current study indicated the leaf extracts of the plant *Melodinus eugeniifolius* viewed the best antitumor capacity on HCT-116 cell line among all of the five cacer cell lines tested in this study. Independent cell lines derived from the colonic epithelial cell line HCT-116 were selected for resistance to bile salt-induced apoptosis. These cell lines were developed as tissue culture models of apoptosis resistance. Selection was carried out for resistance to apoptosis induced by sodium deoxycholate (NaDOC), the bile salt found in highest concentrations in human fecal water. Cultures of HCT-116 cells were serially passaged in the presence of increasing concentrations of NaDOC. The resulting apoptosis resistant cells were able to grow at concentrations of NaDOC (0.5 mM) that cause apoptosis in a few hours in unselected HCT-116 cells. These cells were then analyzed for changes in gene expression [230]. Molecular and cellular analyses of these resistant cell lines has suggested potential mechanisms by which apoptosis resistance may develop in the colonic epithelium in response to high concentrations of hydrophobic bile acids that are associated with a Western-style diet. These analyses provide the

rationale for the development of hypothesis-driven intermediate biomarkers to assess colon cancer risk on an individual basis [231, 232] (Figure 8.19).

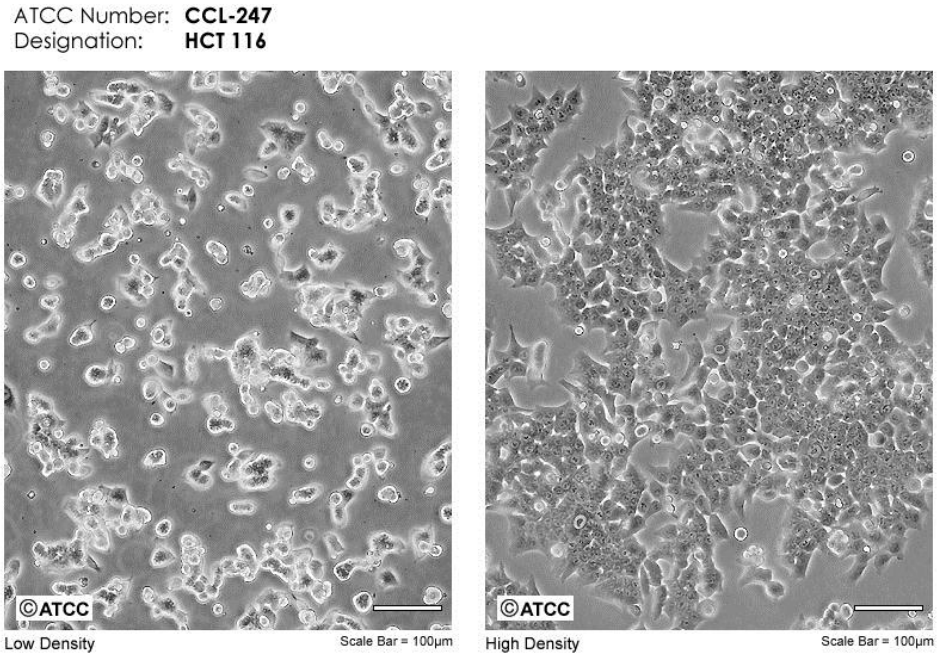


Figure 8.19: HCT-116 human colon cancer cells.

The HCT-116 (wildtype p53; mutant RAS) cell line is one of three malignant cells (HCT-116a and HCT-116b) isolated from a male with colonic carcinoma. These cells are adherent, have a short doubling time of 17.4 hours and possess a modal chromosome number of 46. They are also reported to have higher levels of colony formation capability. According to our assay, leaf hexane and chloroform extract showed the excellent antitumor activities with GI_{50} values of 50.22 µg/mL and 50.84 µg/mL against the human colon cancer cell HCT-116.

The cytology characteristics of HCT-116 cells not just explaine the carcinogenic mechanism, but also help to conjecture the inhibition mechanism of the crude extracts of the plant *Melodinus eugeniifolius* on HCT-116 cells. The mechanism may present in the gene regulation and the cell-cycle arresting activity.

MCF-7 is an ER+ breast cancer cell line isolated in 1970 from a 69-year-old Caucasian woman. MCF-7 is the acronym of Michigan Cancer Foundation-7, referring to the institute in Detroit where the cell line was established in 1973 by Herbert Soule and co-workers [233]. The Michigan Cancer Foundation is now known as the Barbara Ann Karmanos Cancer Institute. Prior to MCF-7, it was not possible for cancer researchers to obtain a mammary cell line that was capable of living longer than a few months. The patient, whose name, Frances Mallon, is unknown to the vast majority of cancer researchers, and died in 1970. Her cells were the source of much of current knowledge about breast cancer [233, 234]. At the time of sampling, she was a nun in the convent of Immaculate Heart of Mary in Monroe, Michigan under the name of Sister Catherine Frances. MCF-7 and two other breast cancer cell lines, named T-47D and MDA-MB-231, account for more than two-thirds of all abstracts reporting studies on mentioned BCC lines, as concluded from a Medline-based survey [235] (Figure 8.20).

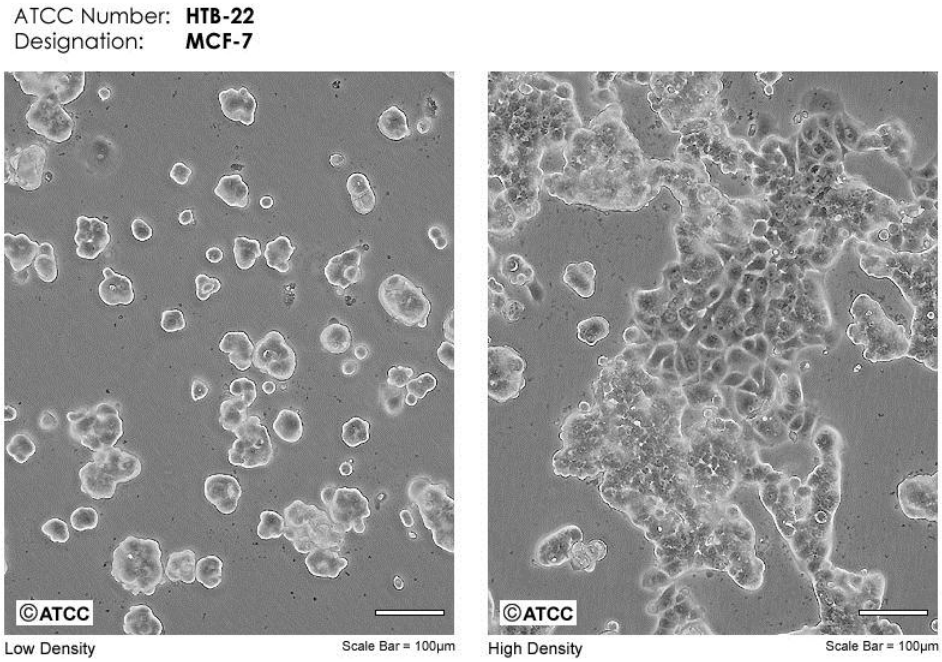


Figure 8.20: MCF-7 human breast cancer cells.

MCF-7 cells form tightly cohesive structures thereby illustrating robust cell to cell adhesions and display a luminal epithelial phenotype. This cell line retained several characteristics of differentiated mammary epithelium, including the ability to process estradiol via cytoplasmic estrogen receptors and the capability of forming domes [236]. Tumor necrosis factor alpha (TNF alpha) inhibits the growth of MCF-7 breast cancer cells. Treatment with anti-estrogens can modulate the secretion of insulin-like growth factor binding proteins. PIK3CA helical mutations were identified in MCF-7, but with low AKT activation [237, 238]. In this study, leaf hexane and chloroform extract revealed significant

antitumor activities with GI_{50} values of 53.93μg/mL and 63.80μg/mL against the human breast cancer cell MCF-7.

According to the characters of the MCF-7 cells and their nosogeneses, the growth of MCF-7 cells can be inhibited by proventing the conjunction with the estrogen receptor, or by an anti-estrogen agent [233-238]. Therefore, the conceivable anticancer mechanism of the crude extracts of the plant *Melodinus eugeniifolius* on MCF-7 cells can be ranged into four aspects: ①: the induction of apoptosis of differentiated mammary epithelium and stop the combination with estrogen receptor; ②: the increase in oxidative stress and free redical scavenging activity; ③: gene regulation; ④: work anti-estrogen directly.

The CaCo-2 cell line is an immortalized line of heterogeneous human epithelial colorectal adenocarcinoma cells, developed by the Sloan-Kettering Institute for Cancer Research by Dr. Jorgen Fogh [239]. Although derived from a colon (large intestine) carcinoma, when cultured under specific conditions the cells become differentiated and polarized such that their phenotype, morphologically and functionally, resembles the enterocytes lining the small intestine. Caco-2 cells express tight junctions, microvilli, and a number of enzymes and transporters that are characteristic of such enterocytes: peptidases, esterases, P-

glycoprotein, uptake transporters for amino acids, bile acids carboxylic acids, etc. They are commercially available through the American Type Culture Collection (ATCC; Manassas, VA, USA) [240, 241]. When looking at Caco-2 cell cultures microscopically, it is evident even by visual inspection that the cells are heterogeneous. As a result, over the years the characteristics of the cells used in different laboratories around the world have diverged significantly, which makes it difficult to compare results across labs [242]. Caco-2 cells are most commonly used not as individual cells, but as a confluent monolayer on a cell culture insert filter. When cultured in this format, the cells differentiate to form a polarized epithelial cell monolayer that provides a physical and biochemical barrier to the passage of ions and small molecules [241, 243]. The Caco-2 monolayer is widely used across the pharmaceutical industry as an in vitro model of the human small intestinal mucosa to predict the absorption of orally administered drugs. The correlation between the in vitro apparent permeability across Caco-2 monolayers and the in vivo fraction absorbed is well established [244]. The versatility of Caco-2 cells is demonstrated by the fact that, even to this day, they are serving as the basis for the creation of innovative new models that are contributing to our understanding of drug efflux transporters such as P-glycoprotein (ABCB1) and BCRP (ABCG2). RNA interference has been used to silence the expression of individual

efflux transporters, either transiently or long-term [245-247] (Figure 8.21).

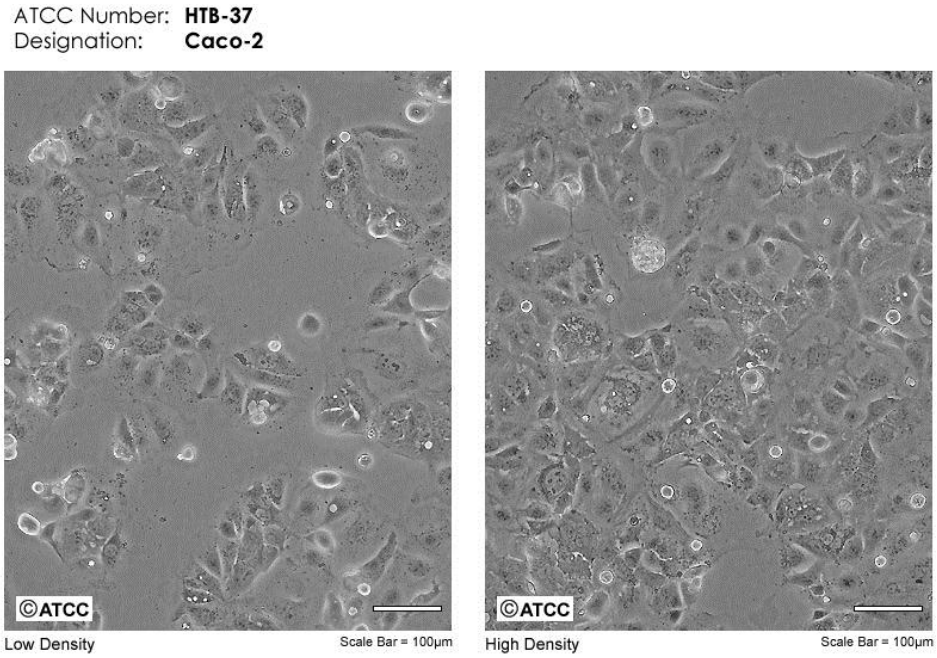


Figure 8.21: Caco-2 human colon adenocarcinoma cells.

The CaCo-2 cell line is widely used with in vitro assays to predict the absorption rate of candidate drug compounds across the intestinal epithelial cell barrier. The assay requires that drug absorption rates be determined 21 days after CaCo-2 cell seeding to allow for monolayer formation and cell differentiation. CaCo-2 may also refer to a cell monolayer absorption model. Cell-based functional assays, such as the Caco-2 drug transport model for assessing intestinal transport, are extremely valuable for screening lead compounds in drug discovery. Bark chloroform and leaf ethanol extract showed significant antitumor

activities with GI_{50} values of 193.7 µg/mL and 167.1 µg/mL against the human colon cancer cell CaCo-2.

According to the introduction of CaCo-2 cells, the carcinogenesis is the cells become differentiated and polarized by the transportation of enzymes. Therefore, the antitumor mechanism of the crude extracts of the plant *Melodinus eugeniifolius* on CaCo-2 cells had been predicted the induction of apoptosis of large intestinal epithelium and the enzyme inhibition.

Nasopharynx cancer or nasopharyngeal carcinoma (NPC) is the most common cancer originating in the nasopharynx, the uppermost region of the pharynx ("throat"), behind the nose where the nasal passages and auditory tubes join the remainder of theupper respiratory tract [248, 249]. It is vastly more common in certain regions of East Asia and Africa than elsewhere, with viral, dietary and genetic factors implicated in its causation. It is most common in males [249]. HK-1 is one of the cells isolated from series of this squamous cell carcinoma or an undifferentiated type. Squamous epithelial cells are a flat type of cell found in the skin and the membranes that line some body cavities. Differentiation means how different the cancer cells are from normal cells. Undifferentiated is a word used to describe cells that do not have their mature features or functions [250-252] (Figure 8.22).

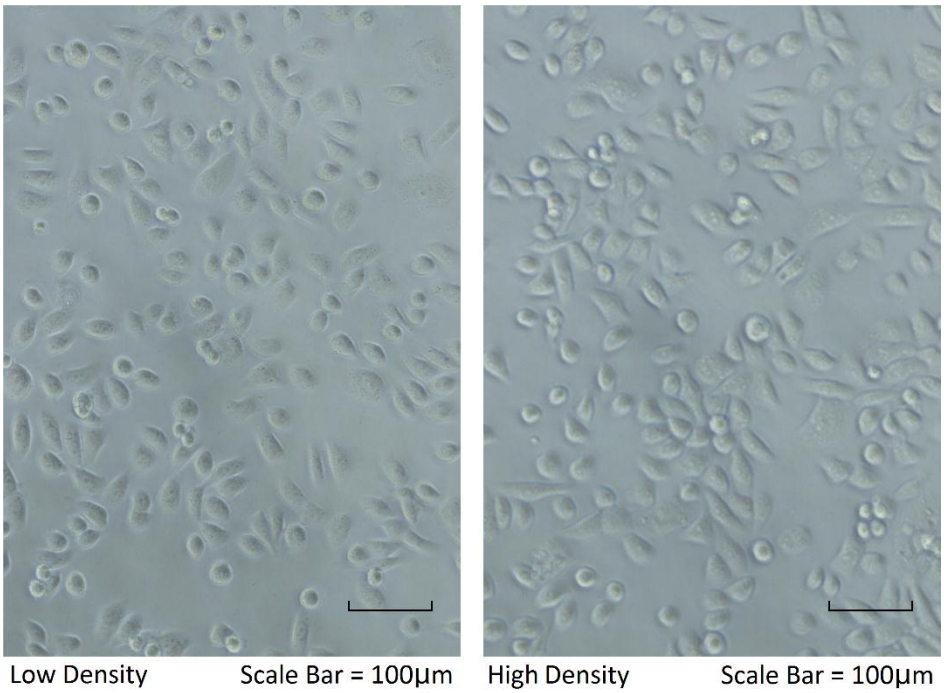


Figure 8.22: HK-1 human nasopharyngeal carcinoma cells.

In the present study, only the bark chloroform extract of *Melodinus eugeniifolius* revealed significant anticancer activity against the HK-1 human nasopharyngeal cancer cell line with a GI_{50} value of 66.7 µg/mL. Moreover, leaf chloroform and ethanol extracts also shown certain antitumor capacity with GI_{50} values of 170.7 µg/mL and 159.2 µg/mL respectively.

The carcinogenetic mechanism may help to conjecture the inhibition mechanism of the crude extracts of the plant *Melodinus eugeniifolius* on HK-1 cells. The squamous cell carcinoma caused by the gene over-

expression, DNA and RNA transform signal. The inhibition mechanism may present in the induction of apoptosis of squamous epithelial cells and gene regulation.

The MRC-5 human fetal lung fibroblast cell line is commonly utilized in vaccine development, as a transfection host in virology research, and for in vitro cytotoxicity testing. Initiated in 1966 by J. P. Jacobs, the cell line was derived from the human lung tissue of a 14-week-old male fetus aborted from a 27-year-old woman [253-255]. MRC-5 cells, which grow adherently in culture and exhibit fibroblast morphology, may double in population size 42 to 46 times before the onset of senescence. They are susceptible to poliovirus 1, herpes simplex, and vesicular stomatitis (Indiana strain). The line is, however, negative for reverse transcriptase, indicating the lack of integral retrovirus genomes [256, 257] (Figure 8.23).

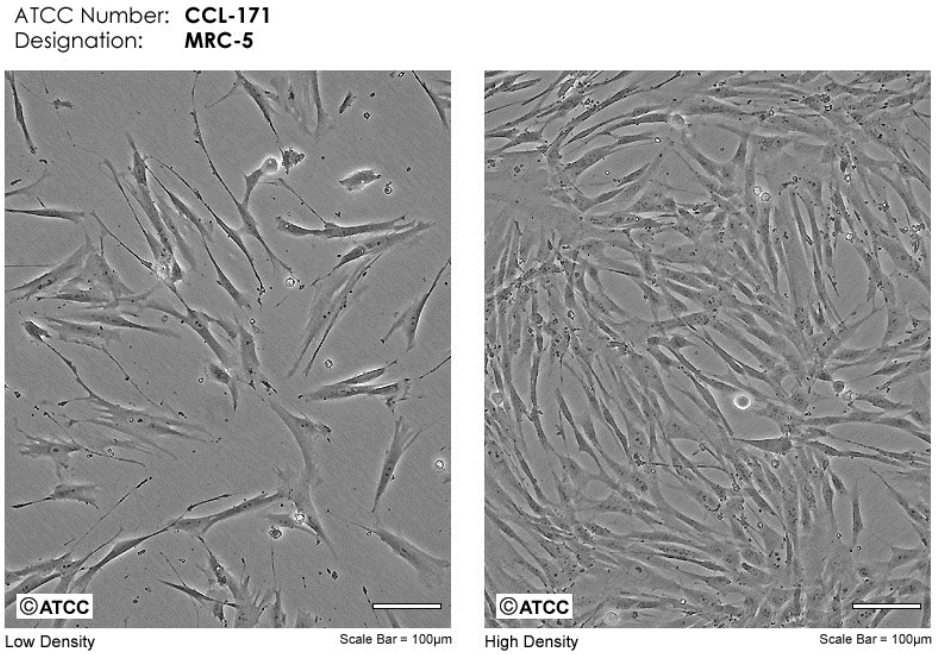


Figure 8.23: MRC-5 human lung fibroblasts cells.

In this study, six crude extracts of *Melodinus eugeniifolius* displayed obvious protective activity against the MRC-5 human lung fibroblast cell line. None of the fractions from extracts revealed inhibition activity as all of the GI_{50} values were higher than 1000 µg/mL.

It is known that nature is able to produce a wide variety of chemical entities of novel structure. Many of the new and novel compounds isolated from natural sources might otherwise have never been discovered, especially those of considerable complexity requiring the development of methods for the creation of new ring systems. Natural products appear to be a promising source for new types of compounds with antitumor activity [258].

8.5 Conclusion

Plant substances continue to serve as a wellspring of drugs for the world population and several plant-based drugs are in extensive clinical use [12]. Preliminary antitumor screening of six extracts of *Melodinus eugeniifolius* showed that the chloroform extract of barks and all the extracts of leaves had promising growth inhibition on the five human cancer cell lines, and not damage the normal cells. The possible anticancer mechanism of the crude extracts of the plant *Melodinus eugeniifolius* may include: ①: the induction of apoptosis; ②: cell-cycle arresting activity; ③: gene regulation; ④: the increase in oxidative stress and free redical scavenging activity; ⑤: enzyme inhibition. The results obtained suggest that further isolation studies can be performed on *Melodinus eugeniifolius* to identify the active constituents responsible for the antitumor activities.

CHAPTER Ⅸ

ISOLATION AND IDENTIFICATION OF COMPOUNDS FROM *MELODINUS EUGENIIFOLIUS*

9.1 Introduction

As one of the medicine sources, application of medicinal plant has formed the basis of health care since the earliest time of humanity and is still being used worldwide. The clinical, pharmaceutical and economic value continues to grow, varying between countries. Over the last century, natural products have provided considerable value to the pharmaceutical industry in the discovery of novel chemical structures and bioactive lead molecules for drug development [259]. Numerous natural products and their synthetically modified derivatives such as aspirin and morphine have been developed clinically to treat human diseases in all the therapeutic areas, particularly infectious disease and oncology [260].

Despite the availability of various approaches in drug discovery and development including synthetic and combinatorial chemistry, as well as computer-based molecular modelling design, none of them can substitute the central role of natural products as the majority of core structures or

scaffolds, for synthetic compounds are based upon natural products [261-263]. Most significantly, not all natural products can be prepared by total synthesis, and many of them possess highly complex structures that are too difficult and economically infeasible to synthesize on an industrial scale [264]. Consequently, isolation and characterisation of pharmacologically active compounds from natural products remain to be the only viable potion.

In this chapter, compounds were isolated from two of the crude extracts of the plant *Melodinus eugeniifolius* which had the best anticancer result in CHAPTER VIII to identify the active constituents responsible for the antitumor activities.

9.2 Methodology

9.2.1 Isolation of crude fractions

The chloroform of bark extract (BC – 10.00g) was subjected to silica gel 60 (0.063-0.200 nm, 70-230 mesh ASTM) (Merck, Germany) column chromatography eluted with hexane-chloroform (100:0 to 0:100, v/v) (Firendemann Schmidt, Australia) and chloroform-methanol (100:0 to 80:20, v/v) (Firendemann Schmidt, Australia) to afford 168 fractions. Each of collected fraction was monitored by analytical thin layer

chromatography (TLC) on silica gel 60 F_{254} aluminium sheet (0.2 mm thickness) (Merck, Germany) using chloroform-methanol (90:10 to 95:5, v/v) as eluent. The spots were visualised under ultraviolet (UV) light at 254nm and 365nm, followed by spraying with Dragendroff's reagent for alkaloid detection. Fractions with similar TLC profiles were combined to give 5 major fractions (BC-1 -- BC-5).

The chloroform of leaf extract (LC – 10.01g) was subjected to silica gel 60 (0.063-0.200 nm, 70-230 mesh ASTM) (Merck, Germany) column chromatography eluted with ethyl acetate-hexane-isopropanol (50:10:40) (Firendemann Schmidt, Australia), chloroform-methanol (100:0 to 10:90, v/v) (Firendemann Schmidt, Australia) and methanol-water (100:0 to 50:50) to afford 185 fractions. Each collected fraction was monitored by analytical thin layer chromatography (TLC) on silica gel 60 F_{254} aluminium sheet (0.2 mm thickness) (Merck, Germany) using chloroform-methanol (90:10 to 95:5, v/v) as eluent. The spots were visualised under ultraviolet (UV) light at 254nm and 365nm, followed by spraying with Dragendroff's reagent for alkaloid detection. Fractions with similar TLC profiles were combined to give 8 major fractions (LC-1 -- LC-8).

According to the TLC results of bark chloroform fractions, most of alkaloids were still stuck on the bottom part and were very difficult to

collect as their polarity was quite high. Therefore, acid-base extraction method was used to try to collect the polar alkaloids.

The bark of plant (500.00g) was soaked in 0.5% sulfuric acid (H_2SO_4) (R&M Chemical, UK) (pH 1.2), and the mixture was left in a water bath (Julabo, Germany) at 50 ^{o}C for 24 hrs. After that, the mixture was filtered and the filtrate was collected, then, a few drops of ammonia (NH_3) (R&M Chemical, UK) was added to the filtrate until the pH value was 8 (pH 8-8.5). Next, the solvent was mixed with chloroform ($CHCl_3$, Merck, Germany) in a separatory funnel and shaked gently, the chloroform layer was collected and filtered with magnesium sulfate auhydrous ($MgSO_4$, Merck-k40896267, Germany), and the collected filtrate was concentrated to dryness under reduced pressure at 40 ^{o}C with using a rotary evaporator (Buchi Labortechnik AG, R-200, Switzerland). The procedure was repeated 3 times. The dried extracts obtained (labeled: BAB) were weighed (1.2202 g) with an analytical balance (Sartorius AG, Germany) and stored in glass scintillation vials (Kimble, USA) at -20 ^{o}C for further use.

The acid-base extraction of bark (BAB – 1.00g) was subjected to silica gel 60 (0.063-0.200 nm, 70-230 mesh ASTM) (Merck, Germany) column chromatography eluted with chloroform-methanol (80:20 to 90:10, v/v) (Firendemann Schmidt, Australia) and ethyl acetate -

methanol (80:20 to 90:10, v/v) (Firendemann Schmidt, Australia) to afford 19 fractions. Each of collected fraction was monitored by analytical thin layer chromatography (TLC) on silica gel 60 F_{254} aluminium sheet (0.2 mm thickness) (Merck, Germany) using ethyl acetate-methanol-cyclohexane-formic acid (60:10:10:2, v/v) as eluent. The spots were visualised under ultraviolet (UV) light at 254nm and 365nm, followed by spraying with Dragendroff's reagent for alkaloid detection. Fractions with similar TLC profiles were combined to give 2 major fractions (BAB-1, BAB-2).

9.2.2 Isolation of Compound 1-2

Fraction 4 of bark chloroform (BC-4) was purified by C-18 Reversed phased Flash (RP-18, Bunch, Japan) column chromatography, eluted with methanol-water (100:0 to 0:100, v/v) (Merck, Germany) to furnish 12 subfractions (BC-4-RP-a -- BC-4-RP-l). Subfractions 7-9 (BC-4-RP-g -- BC-4-RP-i) were combined and recrystalliesd from methanol to afford **Compound 1** (15.6 mg).

Fraction 5 of bark chloroform (BC-5) was purified by C-18 Reversed phased Flash (RP-18, Bunch, Japan) column chromatography eluted with methanol-water (100:0 to 0:100, v/v) (Merck, Germany) to furnish 7 subfractions (BC-5-RP-a -- BC-5-RP-g). Subfractions 2-5 (BC-5-RP-b --

BC-5-RP-e) were combined and recrystalliesd from methanol to afford **Compound 2** (4.2 mg).

9.2.3 Isolation of Compound 3

Fraction 7 of leaf chloroform (LC-7) was purified using silica gel 60 (0.063-0.200 nm, 70-230 mesh ASTM) (Merck, Germany) column chromatography eluted with ethyl acetate-diethylamine-toluene (80:10:10, v/v) (Merck, Germany) to furnish 10 subfractions (LC-7-1 -- LC-7-10). Subfractions 1-2 (LC-7-1 -- LC-7-2) were combined and recrystalliesd from methanol to afford **Compound 3** (2.1 mg).

9.2.4 Isolation of Compound 4-9 (HPLC)

Fractions 1, 2 from the acid-base extraction of bark (BAB-1, BAB-2), and fraction 7 from leaf chloroform extraction (LC-7) were separately chromatographed over High Performance Liquid Chromatograph (HPLC). Detailed methodology was presented in the following table (Table 9.1).

Table 9.1: HPLC methodology for **Compound 4-9**.

Fraction	Column	Mobile phase	Flow rate	Test wavelength	Compound
BAB-1	C-18	methanol - 0.2% acetic acid (30:70, v/v)	1 ml /min	316 nm	**4** (3.7 mg) **9** (1.3 mg)
BAB-2	NH_2	methanol - acetonitrile (50:50, v/v)	1 ml /min	201 nm	**5** (0.7 mg) **6** (1.7 mg)
LC-7	NH_2	methanol - acetonitrile (50:50, v/v)	1 ml /min	201 nm	**5** (1.4 mg) **6** (0.9 mg) **7** (1.6 mg) **8** (1.1 mg)

BAB-1: Fractions 1from the acid-base extraction of bark;

BAB-2: Fractions 2 from the acid-base extraction of bark;

LC-7: fraction 7 from leaf chloroform extraction;

The mobile phase was filtered through a Millipore 0.45 μm filter and degassed prior to use. The detection wavelength range was set among 201and 316nm.

9.2.5 Nuclear magnetic resonance (NMR)

Chemical characterization of the 9 isolated compounds was achieved by NMR. Nuclear magnetic resonance (NMR) spectroscopy was performed on Varian NMR spectrometer. Mps: uncorr. ^{1}H NMR: 400 MHz and ^{13}C NMR: 100 MHz. Compound samples were recorded at a room

temperature in deuterated chloroform ($CDCl_3$) or deuteromethanol (CD_3OD). Optical rotations were measured in methanol.

9.2.6 Chemicals and meterials

HPLC: Agilent 1100 High Performance Liquid Chromatograph (HPLC), includes: Alltech 2000 Evaporative Light Scattering Detector (ELSD), Agilent 1100 ChemStation, Alltech 1100 Full Wavelength Ultraviolet (UV) Detector, Agilent 1100 Online Vacuum Degasser, Agilent 1100 manual injector; Agilent Kro--masi NH_2 column (250mm×4.6mm，5μm，S/N： 22N25110); SB—C18 column (250mm × 4.6mm， 5 μm); Si mplicity Superpure Water System (Milipore); Analytical Balance (METTLER XS105); 50μl Microsyringe (Hamilton).

9.3 Results

Compound 1 (Yohimbine hydrochloride): It has an empirical formular of $C_{21}H_{27}ClN_2O_3$ ($C_{21}H_{26}N_2O_3 \cdot HCl$), and was identified as an alkaloid, yohimbine hydrochloride. This compound has been reported from other plant, thus, no more chemical structure and character introductions mentioned here. Its chemical structure, the ^{1}H-NMR and ^{13}C-NMR spectral analyses were presented in Figure 9.1-9.2.

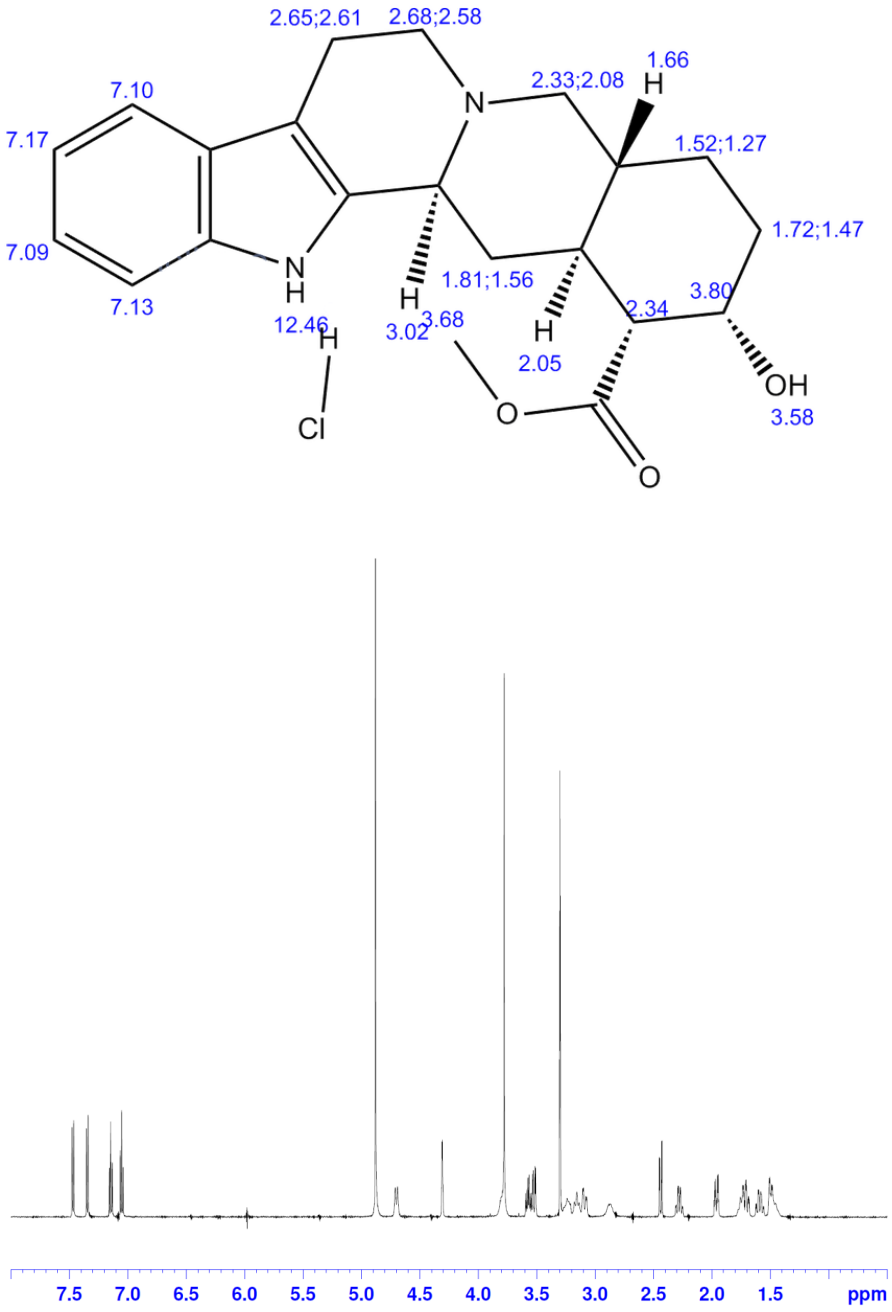


Figure 9.1: ^{1}H-NMR spectral analysis of Compound 1.

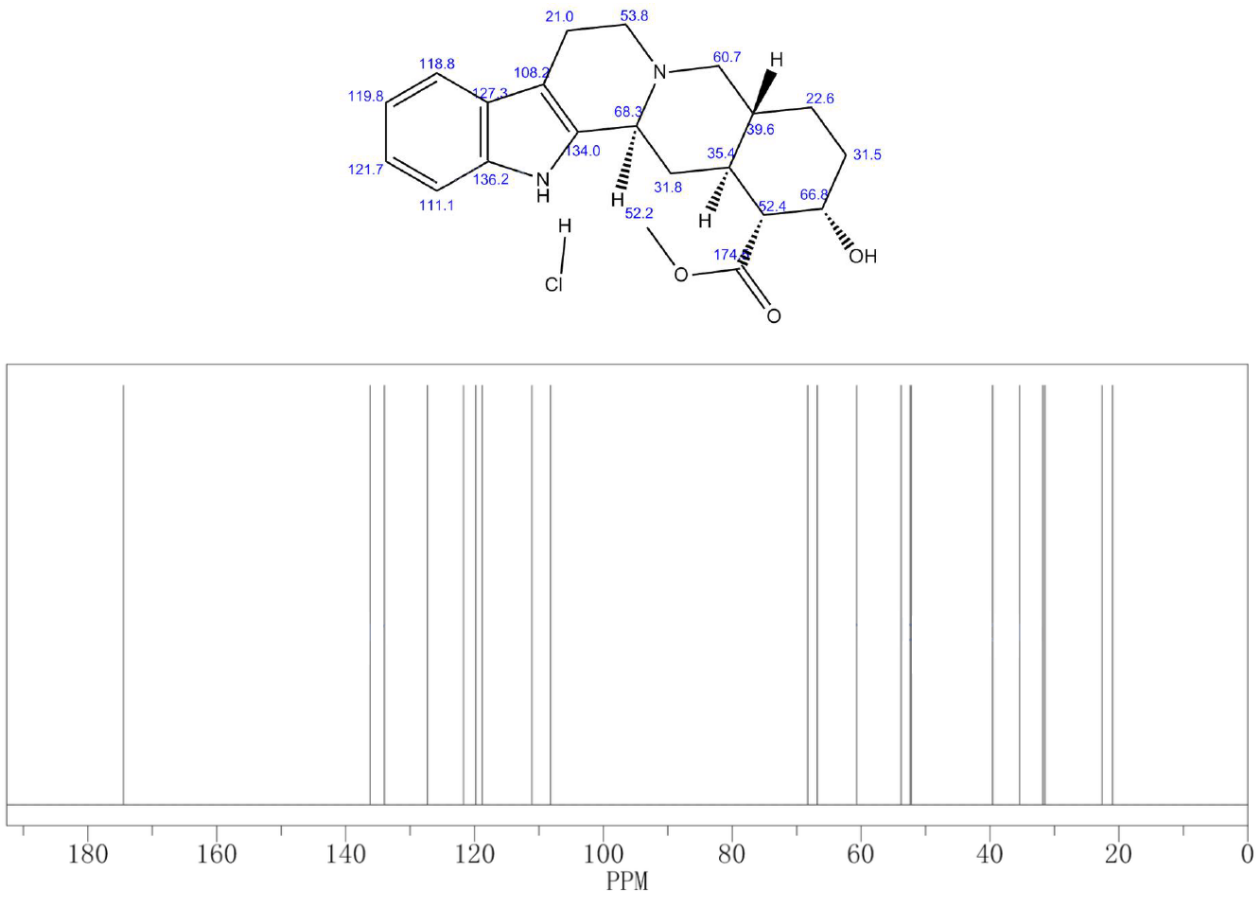


Figure 9.2: ^{13}C-NMR spectral analysis of Compound 1.

Compound 2 (Tabersonine): It has an empirical formular of $C_{21}H_{24}N_2O_2$, and was identified as an alkaloid, tabersonine. Molecular Weight: 336.42746 g/mol. This compound has been reported from other plant, thus, no more chemical structure and character introductions mentioned here. Its chemical structure, the ^{1}H-NMR and ^{13}C-NMR spectral analyses were presented in Figure 9.3-9.4.

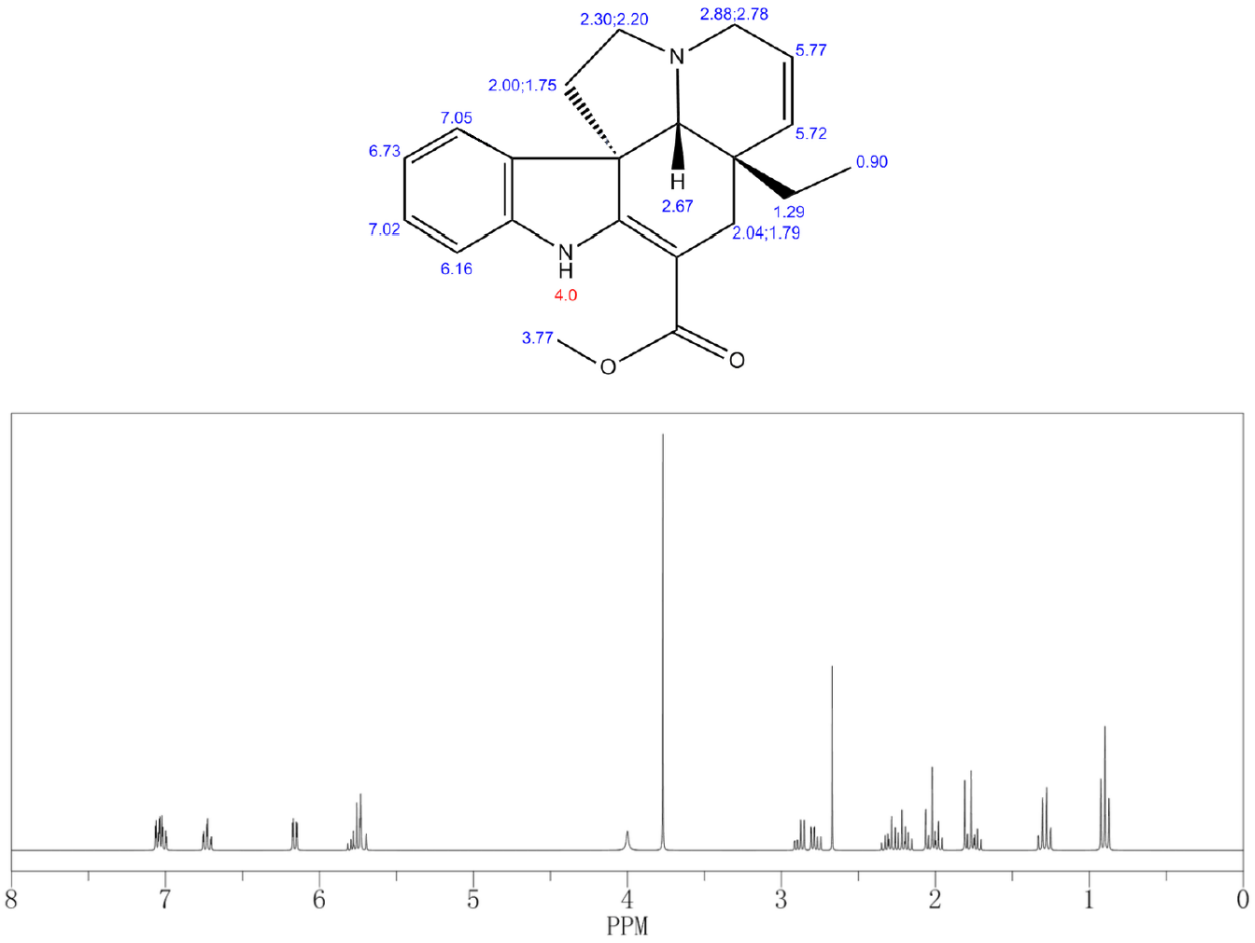


Figure 9.3: ^{1}H-NMR spectral analysis of Compound 2.

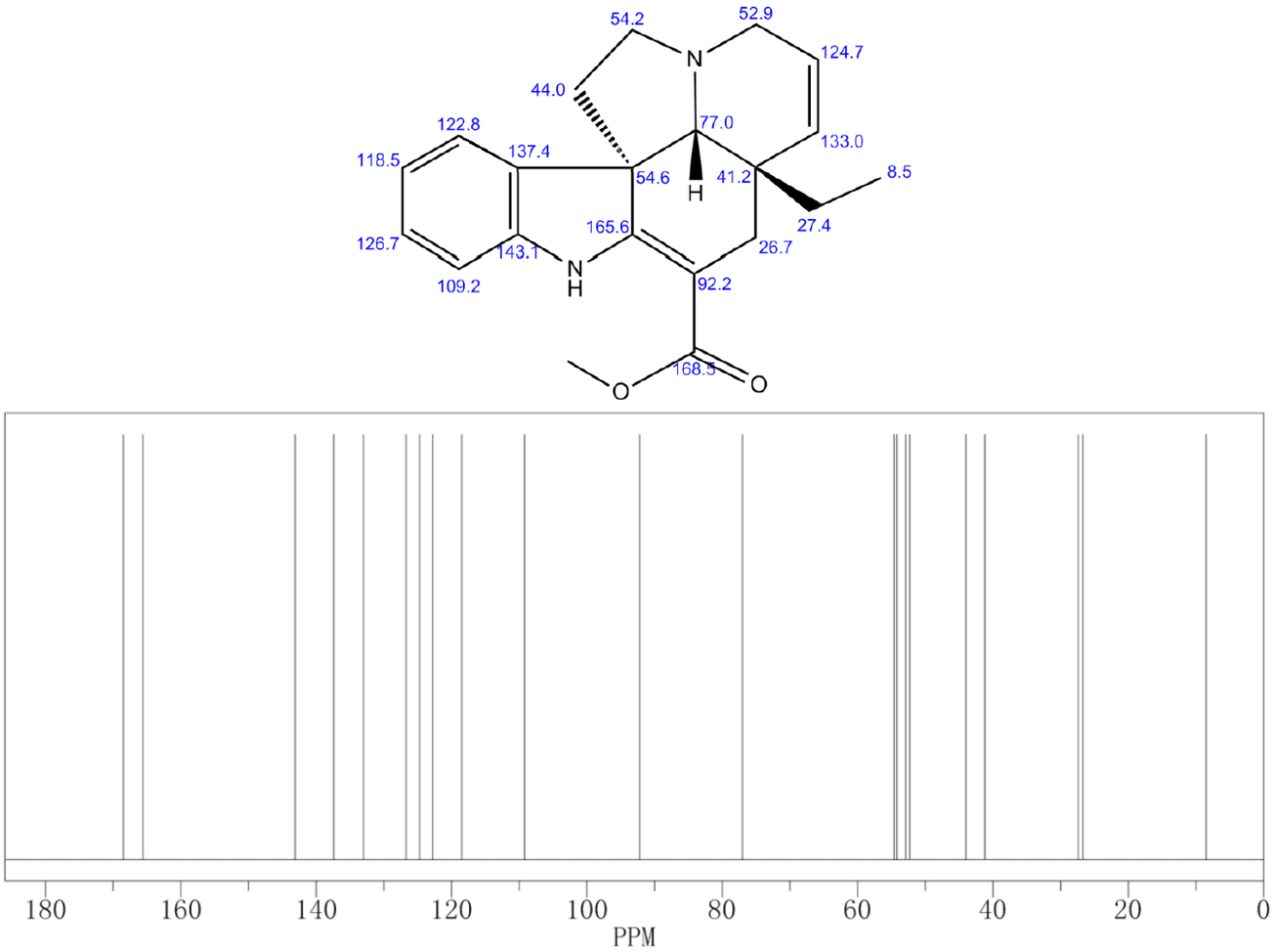


Figure 9.4: ^{13}C-NMR spectral analysis of Compound 2.

Compound 3 (Melodinoid): Prisms, mp 238-242 ° ($CHCl_3$-MeOH), $[\alpha]^{25}_D$ -88.0 ° (c 1.2), FAB-MS *m/z*: 311.1761, $C_{19}H_{22}N_2O_2$ +H requires 311.1759. 2D-NOESY cross-peaks: H-7/H-9, H-17β, H-19, H-9/H-6α, H-15α/H-3α; H-18/H-15β. COLOC cross-peaks: C-2/H-16, C-17, C-5/H-3, H-7, C-8/H-6, H-7, H-10, C-9/H-11, C-10/H-12, C-11/H-9, C-12/H-10 C-13/H-11, C-15/H-3, C-20/H-14, H-16, H-17, H-18, C-20/H-3β, H-6α, H-17.

Based on the presence of two carbonyl groups, **Compound 3** was considered to be an alkaloid. The FAB-mass spectrum, **Compound 3** afforded a $[M +H]^+$ peak at *m/z* 311.1761, suggesting a molecular formula of $C_{19}H_{22}N_2O_2$. Since the unsaturation number was 10 and no olefinic carbon signals were observed in the ^{13}C NMR spectrum except for those due to a benzene ring, **Compound 3** was determined to be a pentacyclic compound. Of the two quaternary carbons at δ 38.2 and 92.4, the former seemed to be C-20 by comparison with that of 3, and the latter was assigned to C-21 based on cross-peaks from H-6 and H-17 in the COLOC spectrum. Although no hydroxyl group was present, **Compound 3** showed a similar chemical shift for C-21, suggesting C-21 in **Compound 3** to be a diazaspirocarbon. Configuration of H-7 was determined to be β, based on the 2D NOESY between H-7/H- 17 and H-7/H-19. In an earlier paper by Goh et al. [265], a chlorinated pentacyclic

compound. In the ^{13}C NMR spectrum of **Compound 3**, signals were in good agreement with each other except for C-5, C-6, C-7, C-8 and C-19. **Compound 3** was then characterized as a 'diazaspiro-leuconolam' and named Melodinoid. (Table 9.2, Figure 9.5-9.7).

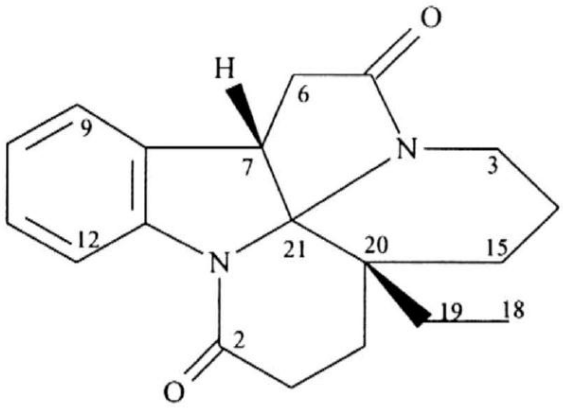


Figure 9.5: Chemical structure of Compound 3.

Table 9.2: ^{1}H-NMR and ^{13}C-NMR spectral data for Compound 3.

Position	δH	δC	Multiplicity (DEPT)
2	-	172.8	s
3	3.93	36.7	t
	2.76		
5	-	107.6	s
6	2.85	37.6	t
	2.66		
7	3.82	41.9	d
8	-	135.1	s
9	7.16	123.6	d
10	7.15	125.3	d
11	7.26	127.7	d
12	7.75	119.9	d
13	-	142.2	s
14	1.60	20.1	t
	1.62		
15	1.97	26.1	t
	1.76		
16	2.74	29.5	t
	2.47		
17	1.85	26.5	t
	1.64		
18	0.92	7.3	q
19	1.36	26.7	t
20	-	38.2	s
21	-	92.4	s

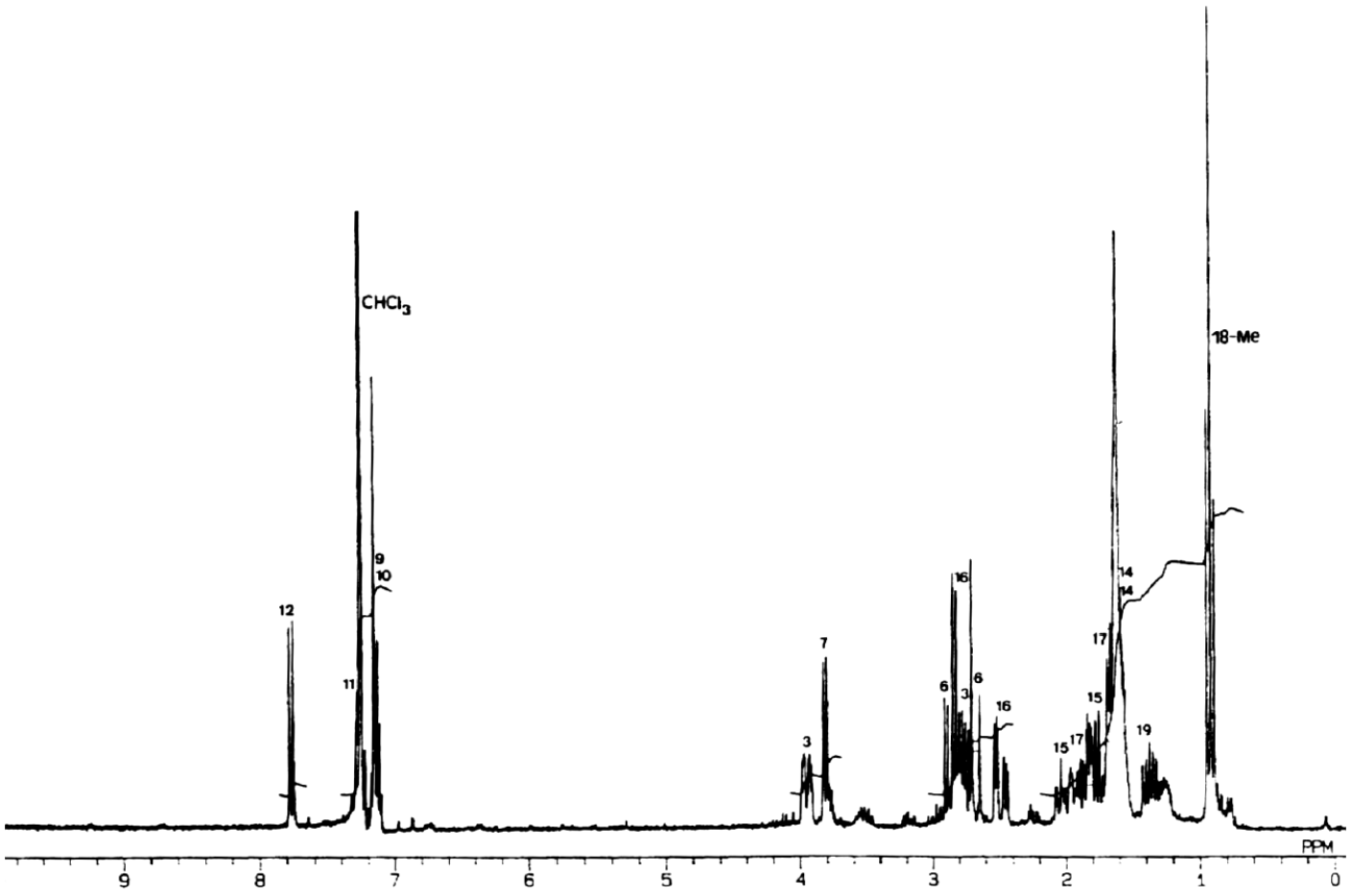


Figure 9.6: ^{1}H-NMR spectral analysis of Compound 3.

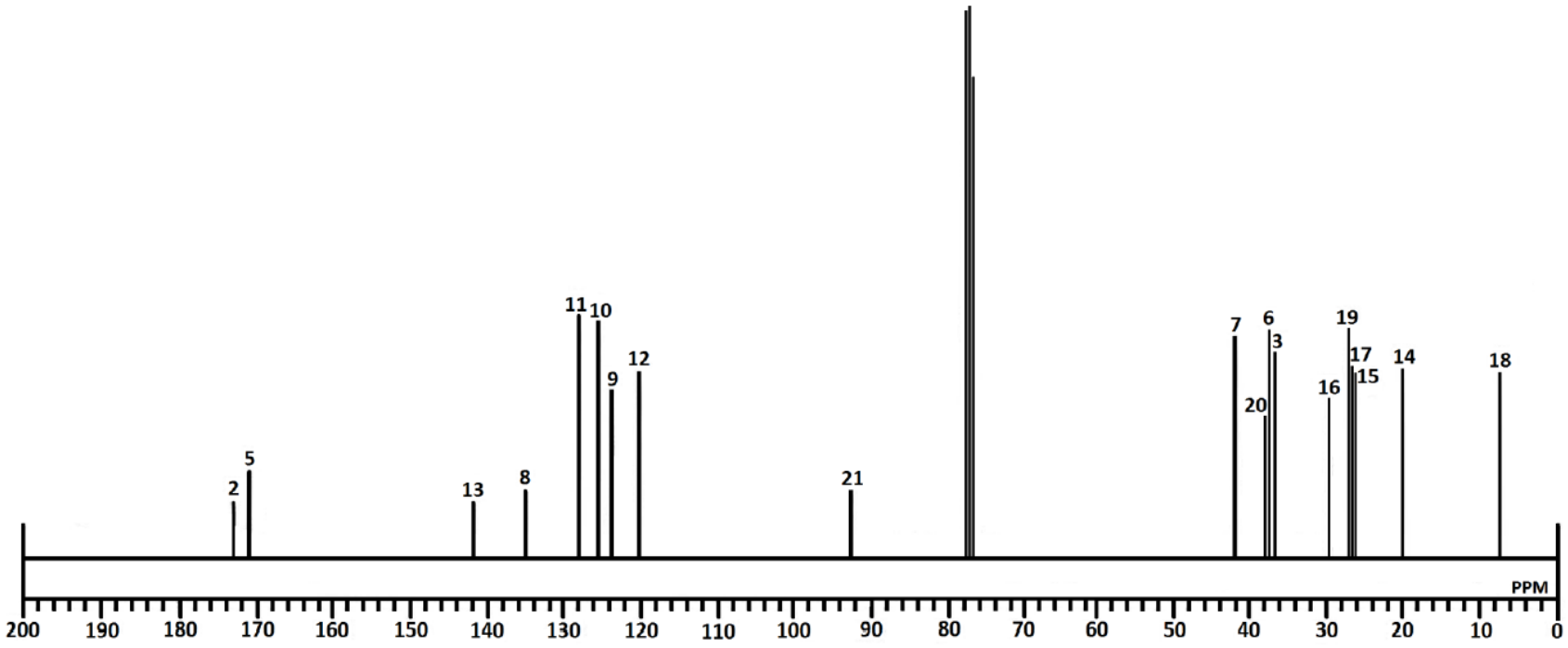


Figure 9.7: ^{13}C-NMR spectral analysis of Compound 3.

Compound 4 (Scandine): It has an empirical formular of $C_{21}H_{22}N_2O_3$, and was identified as an alkaloid, scandine. Its chemical structure, the ^{1}H-NMR and ^{13}C-NMR spectral analyses were presented in Figure 9.8-9.9.

Compound 5 (Yohimbine): mp 228-232° (dec), $[\alpha]^{26}_D$ +57.8 ° (c 0.90), negative FAB-MS *m/z*: 353.1868. It has an empirical formular of $C_{21}H_{26}N_2O_3$, based on the high resolution mass spectra. The presence of one hydroxyl, one carbomethoxyl and five methylene groups was suggested from the ^{1}H and ^{13}C NMR spectra, and the structures were confirmed as yohimbine (Figure 9.8, 9.9).

Compound 6 (β-yohimbine): mp 223-229° (dec), $[\alpha]^{27}_D$ -19.1 ° (c 0.35), negative FAB-MS *m/z* 353.1865. It afforded the same molecular formula as **Compound 5**, $C_{21}H_{26}N_2O_3$, and was identified as an alkaloid, β-yohimbine by comparison of their NMR data with those of authentic samples. Its chemical structure, the ^{1}H-NMR and ^{13}C-NMR spectral analyses were presented in Figure 9.10-9.11.

Compound 7 (Leuconolam): mp 263-267°, $[\alpha]^{28}_D$ -515.8 ° (c 0.25), *m/z*: 326.1637. It has an empirical formular of $C_{19}H_{22}N_2O_3$, and has two carbonyl groups, one of which is conjugated with an olefinic bond. Signals in the ^{1}H and ^{13}C NMR spectra were in good agreement with

those of leuconolam, reported previously from this species and also from Alstonia scholaris collected in Java [265, 266]. Its chemical structure, the ^{1}H-NMR and ^{13}C-NMR spectral analyses were presented in Figure 9.12-9.13.

Compound 8 (Epigallocatechin): It has an empirical formular of $C_{15}H_{14}O_{7}$, and was identified as epigallocatechin. Its chemical structure, the ^{1}H-NMR and ^{13}C-NMR spectral analyses were presented in Figure 9.14-9.15.

Compound 9 (Loganic acid): It has an empirical formular of $C_{16}H_{24}O_{10}$, and was identified as loganic acid. Its chemical structure, the ^{1}H-NMR and ^{13}C-NMR spectral analyses were presented in Figure 9.16-9.17.

Since compound 4-9 has been reported from other plants, thus, the ^{1}H-NMR and ^{13}C-NMR spectral analyses were combined together (page 199 to 208) and no more chemical structures and characters introductions mentioned here.

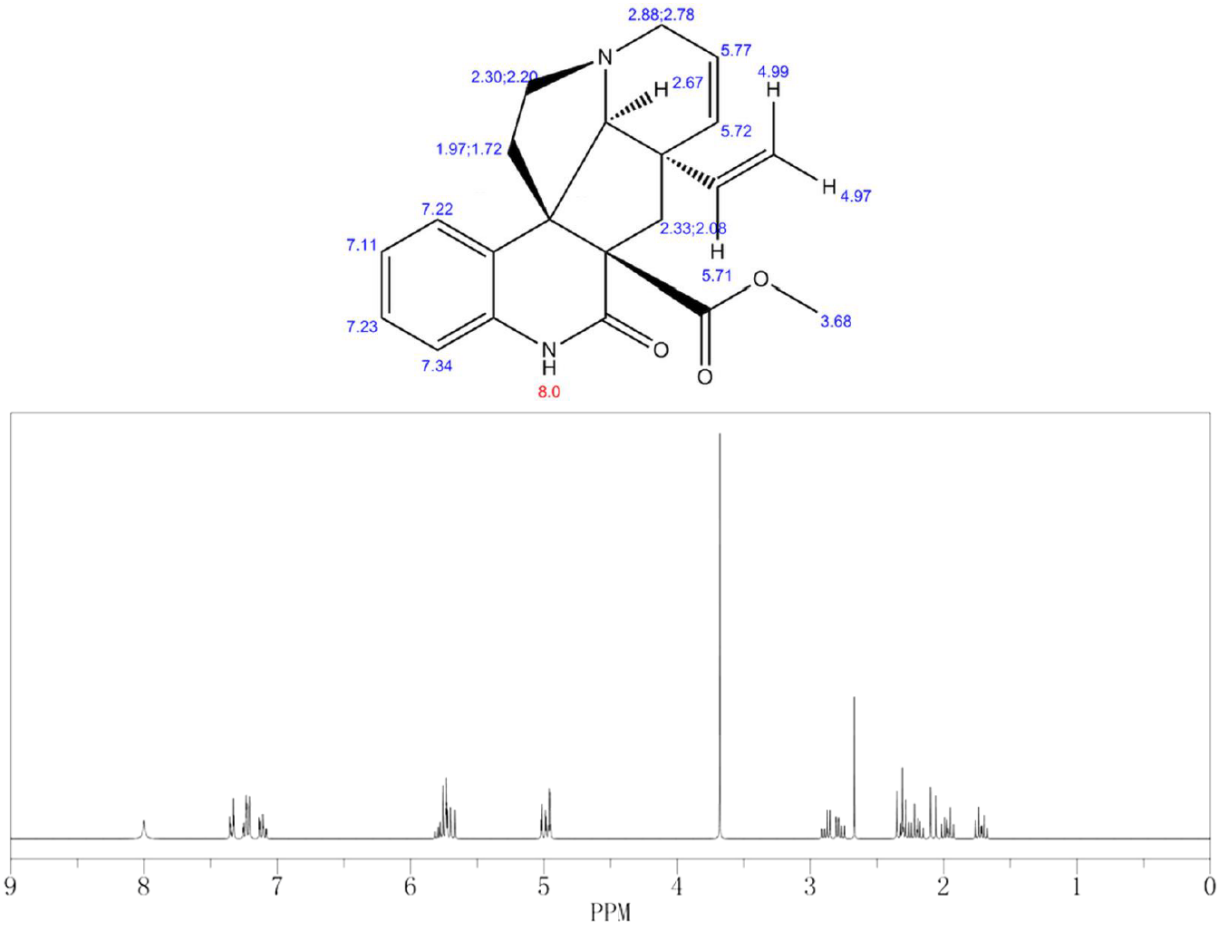


Figure 9.8: ^{1}H-NMR spectral analysis of Compound 4.

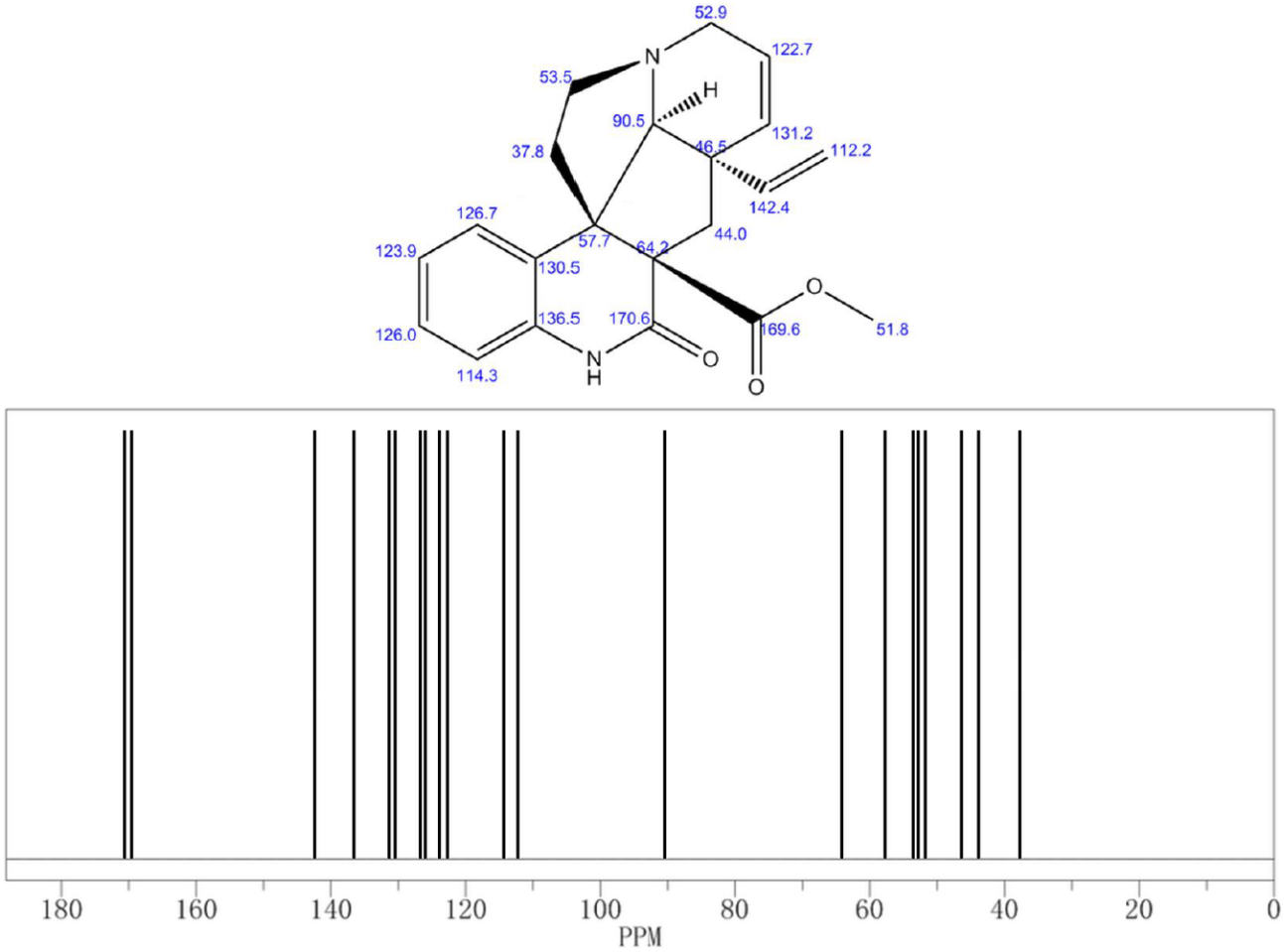


Figure 9.9: ^{13}C-NMR spectral analysis of Compound 4.

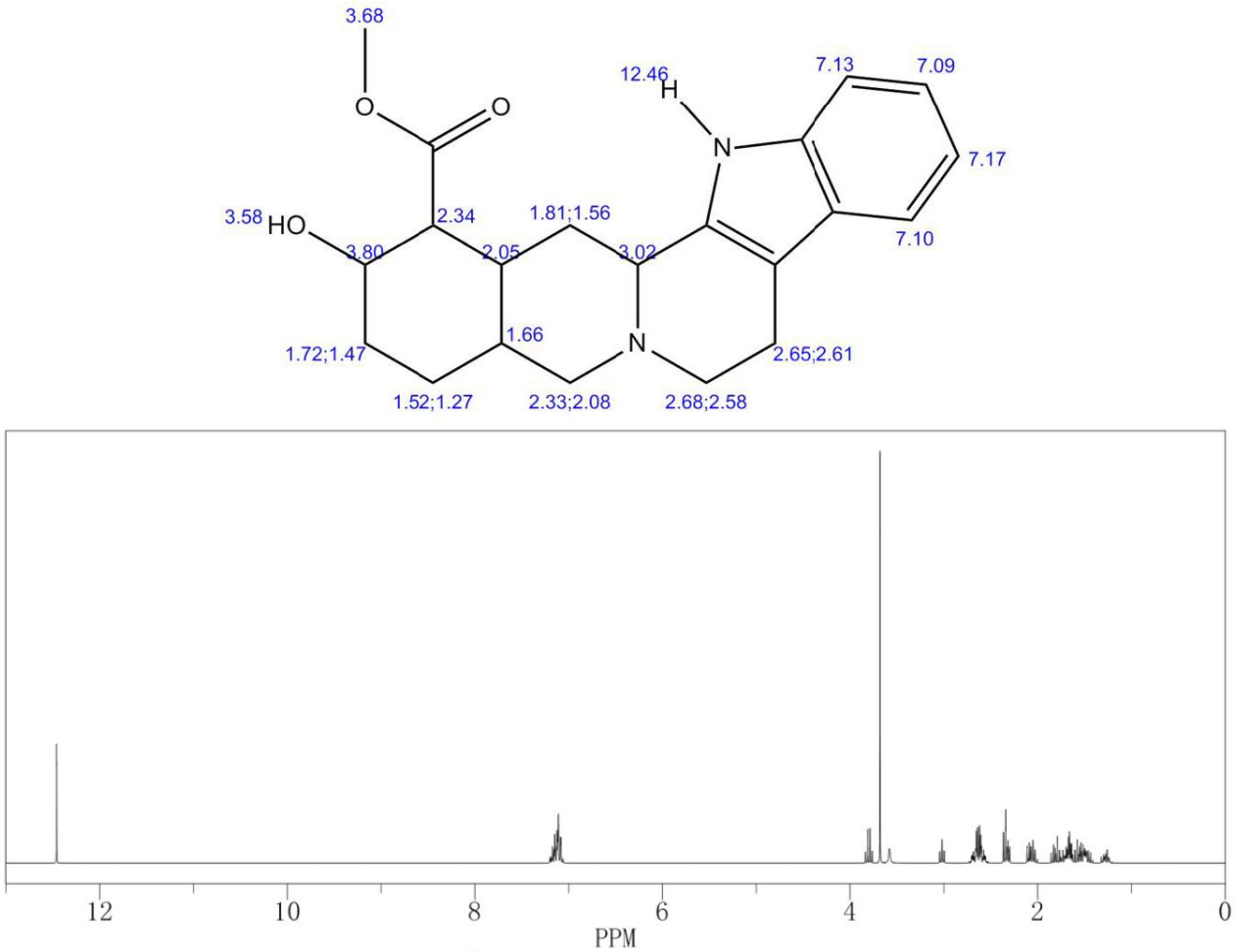


Figure 9.10: ^{1}H-NMR spectral analysis of Compound 6.

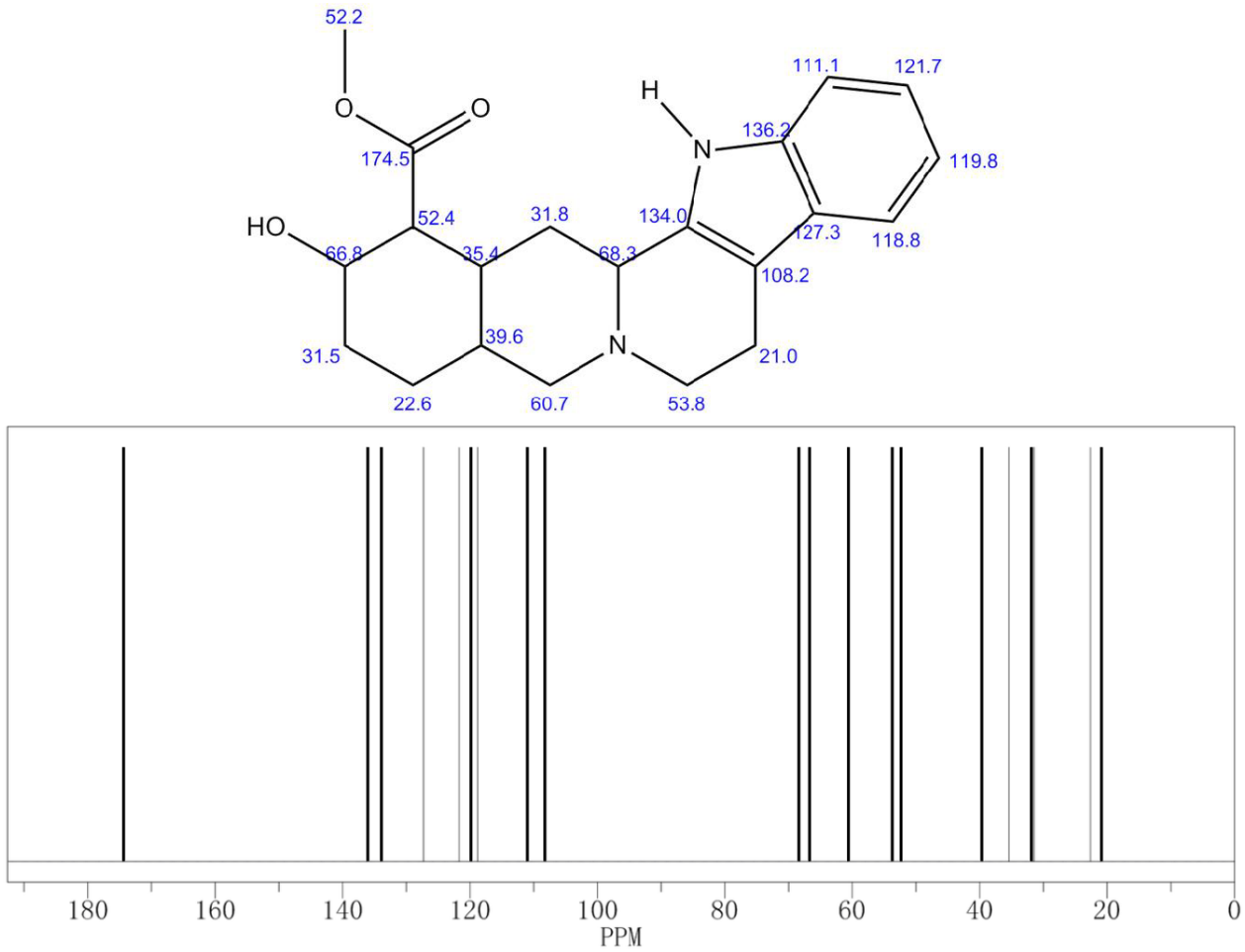


Figure 9.11: ^{13}C-NMR spectral analysis of Compound 6.

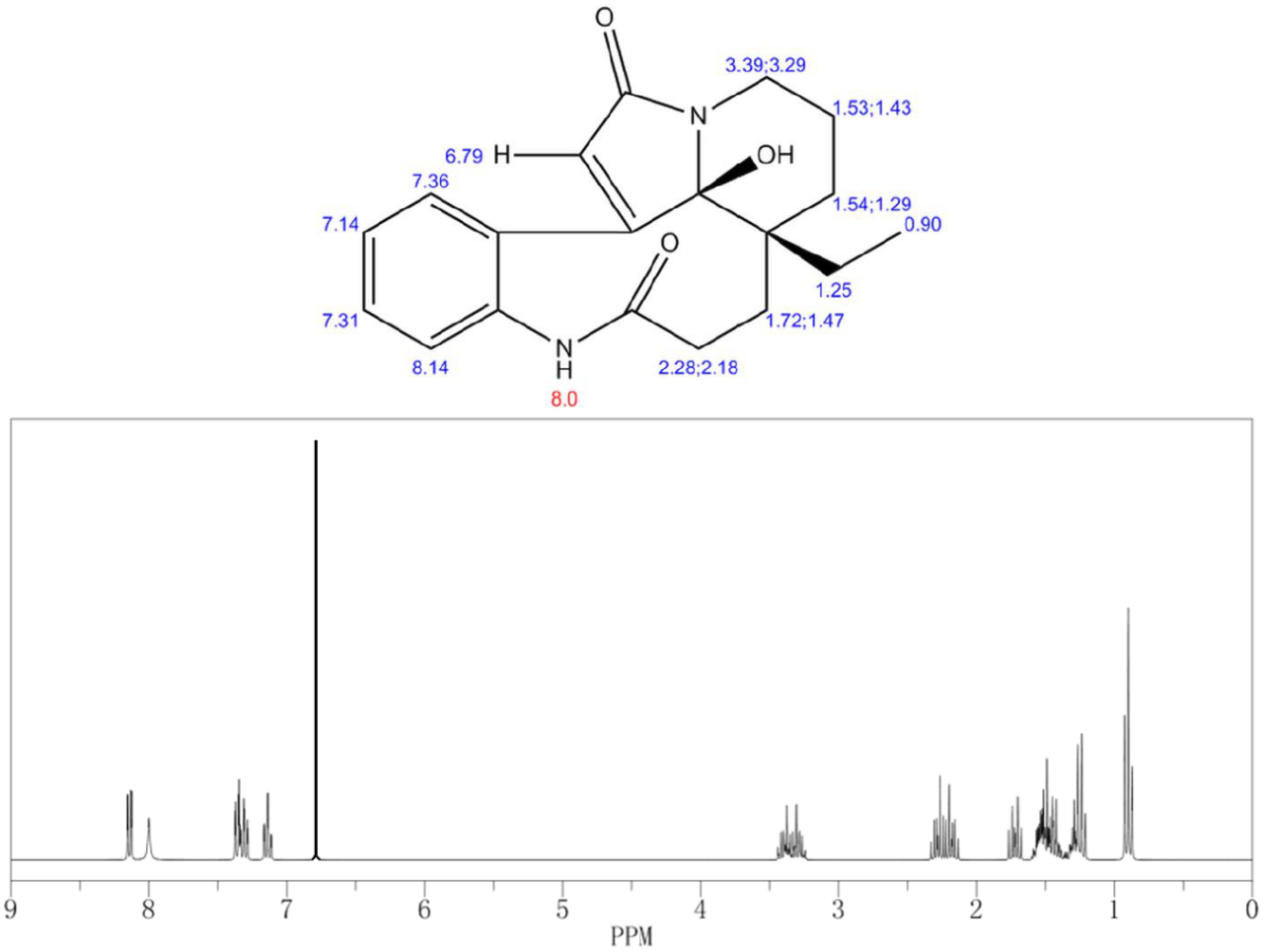


Figure 9.12: ^{1}H-NMR spectral analysis of Compound 7.

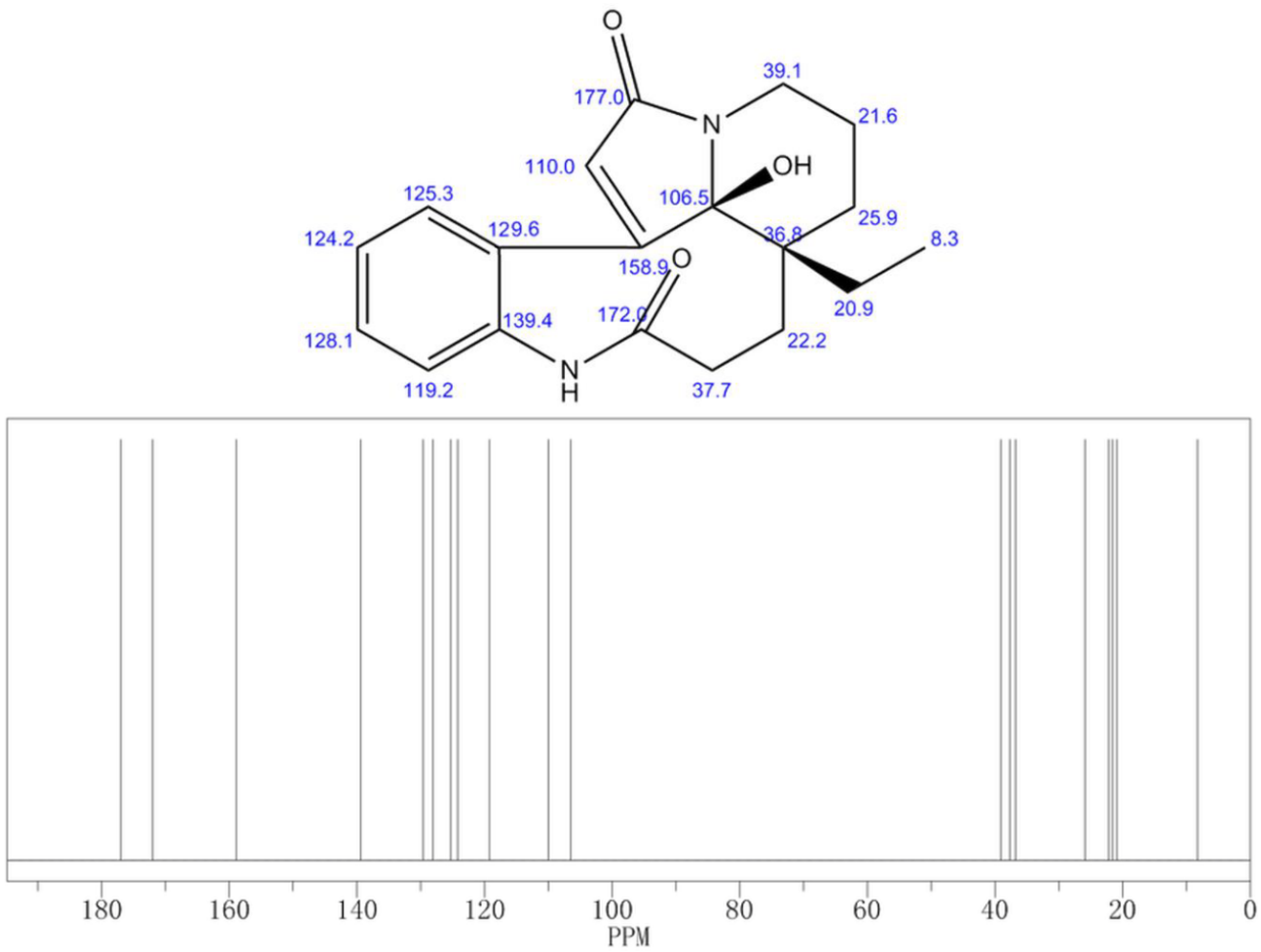


Figure 9.13: ^{13}C-NMR spectral analysis of Compound 7.

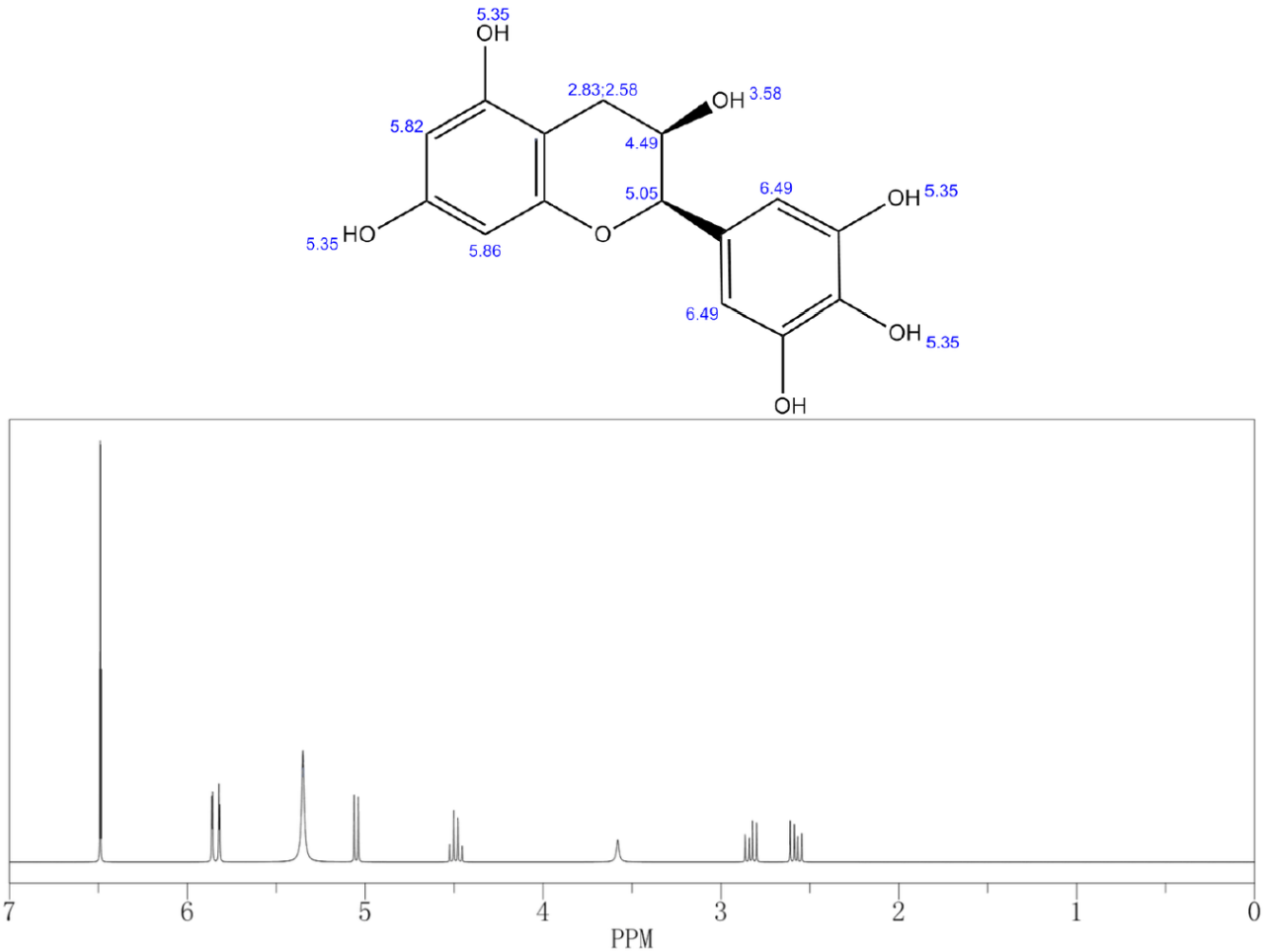


Figure 9.14: ^{1}H-NMR spectral analysis of Compound 8.

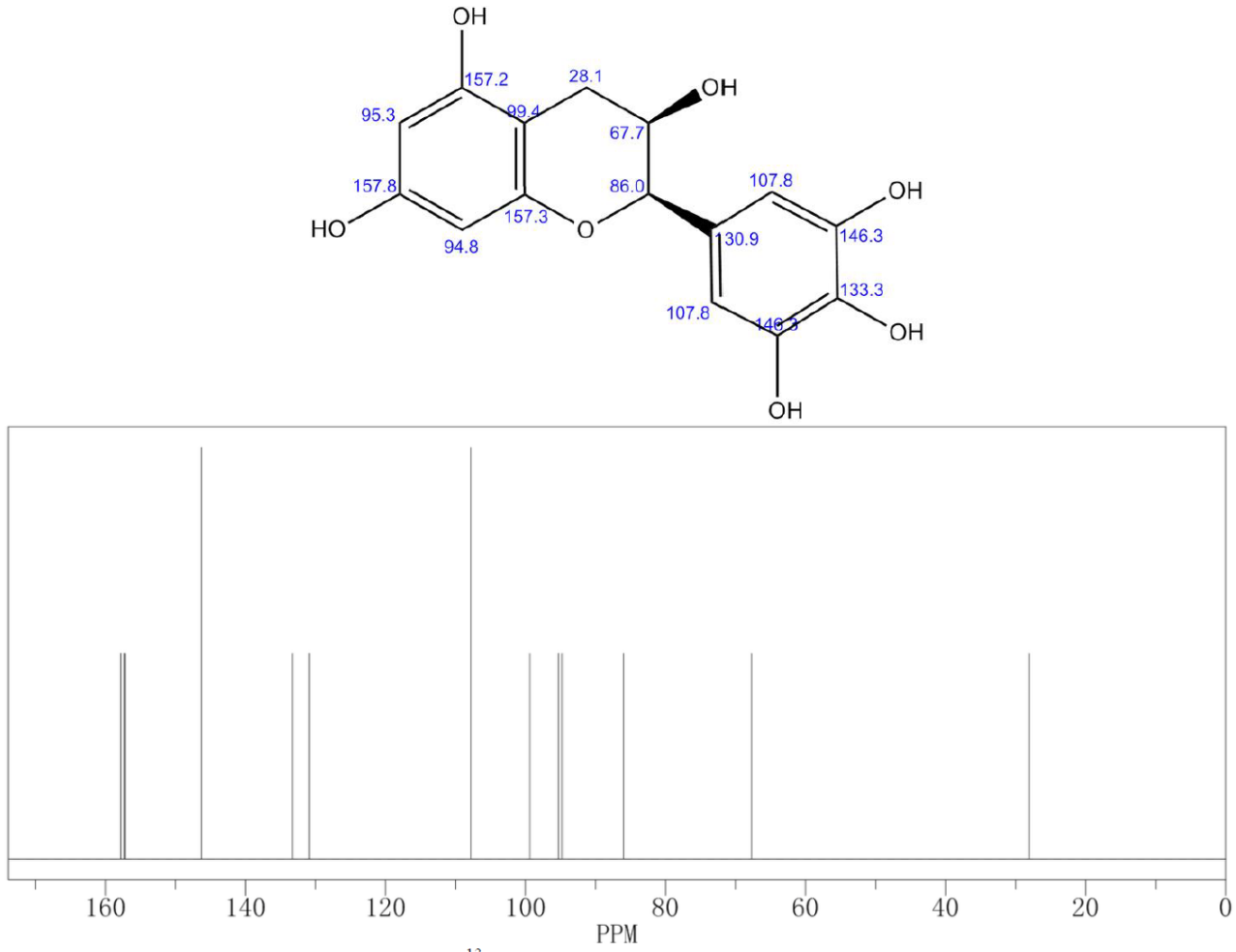


Figure 9.15: ^{13}C-NMR spectral analysis of Compound 8.

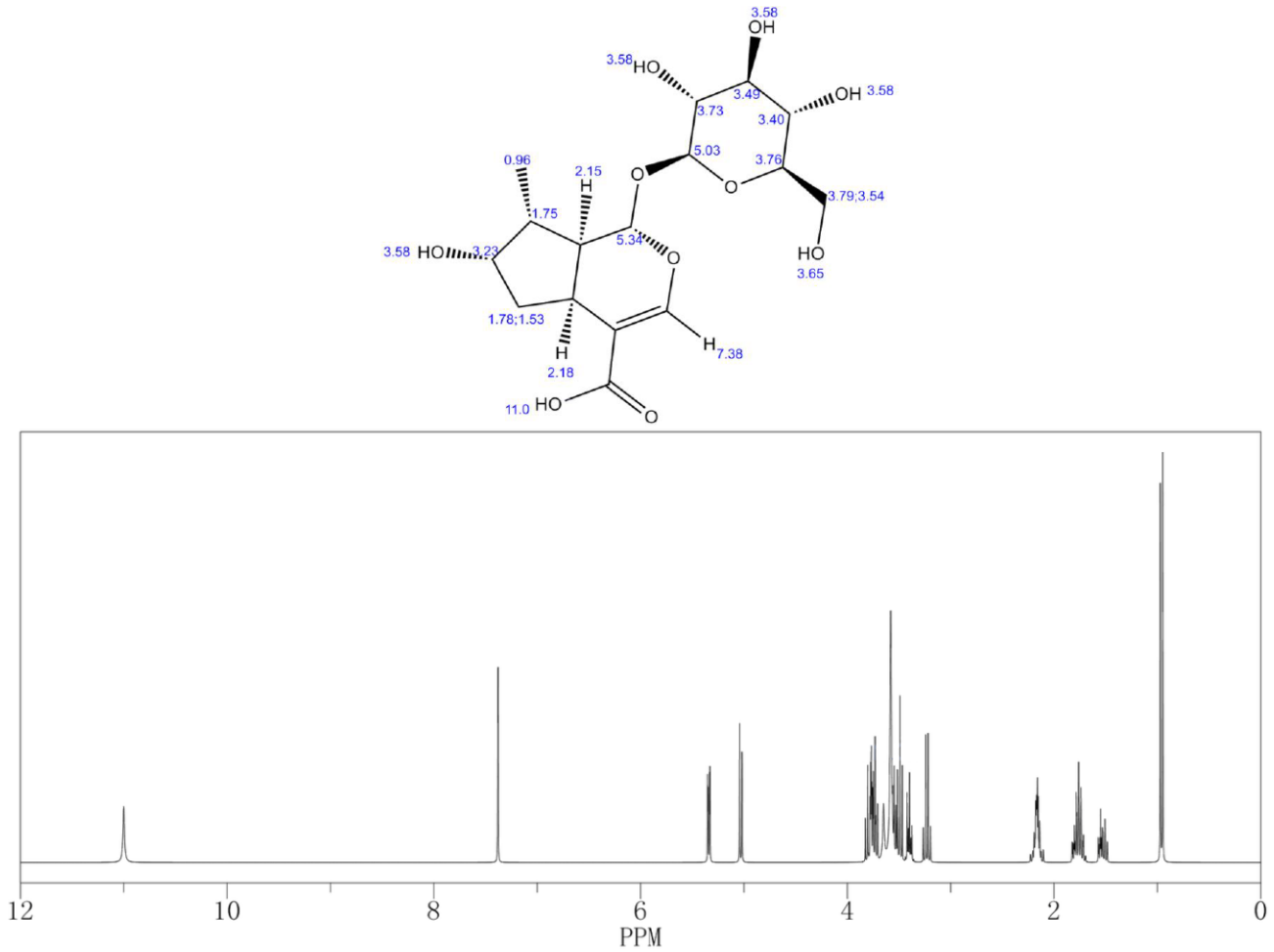


Figure 9.16: ^{1}H-NMR spectral analysis of Compound 9.

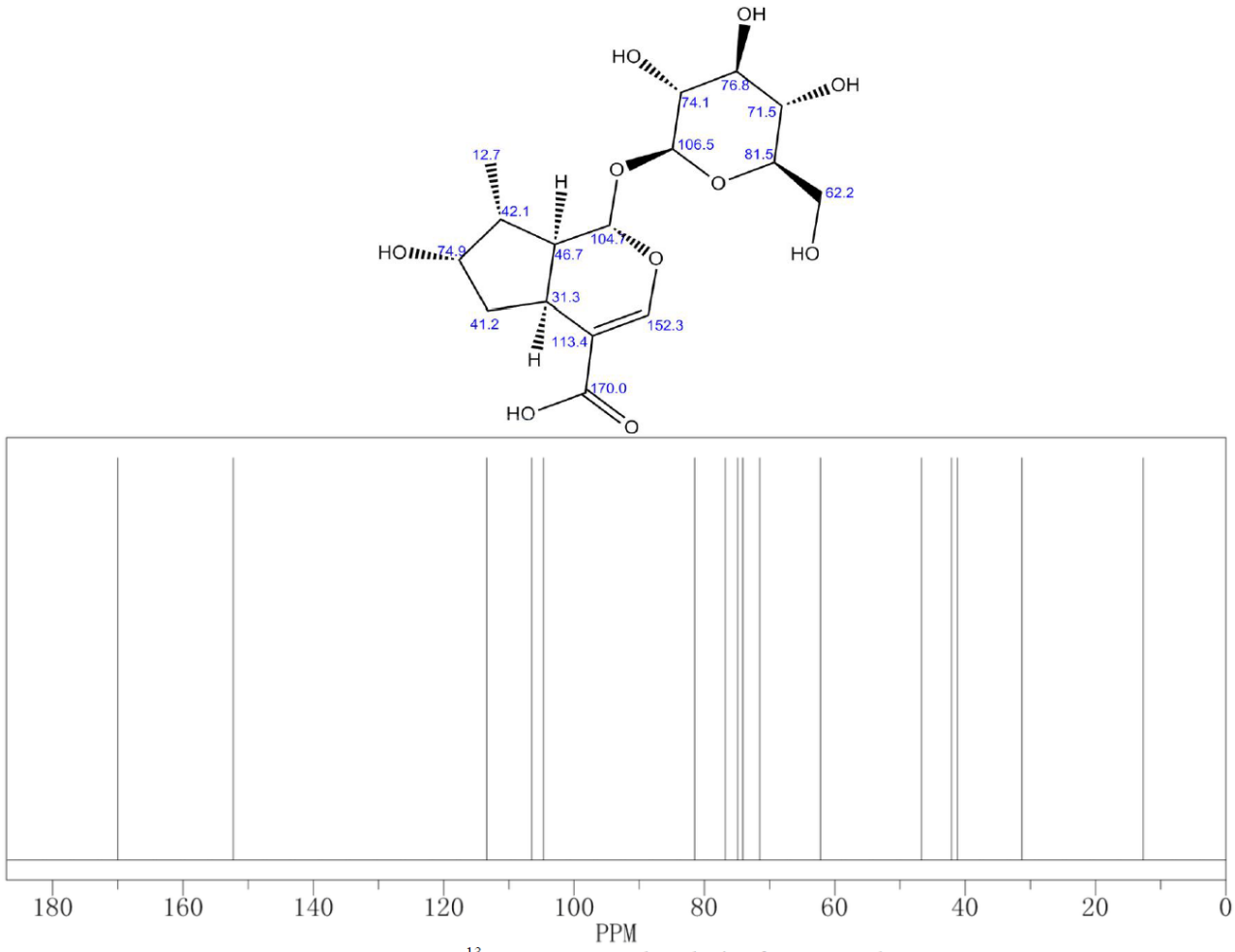


Figure 9.17: ^{13}C-NMR spectral analysis of Compound 9.

9.4 Discussion

The present study was focused on the isolation and identification of chemical compounds of *Melodinus eugeniifolius*. In this chapter, six indole alkaloids were isolated from leaves and barks extracts of *Melodinus eugeniifolius*. Six of them were identified as yohimbine hydrochloride, tabersonine, scandine, yohimbine, β-yohimbine, and leuconolam respectively. The new alkaloid, having a pentacyclic diazaspiro system, was named Melodinoid. Other constituents identified were epigallocatechin and loganic acid.

These compounds were isolated from two of the crude extracts of the plant *Melodinus eugeniifolius* which indicated the best anticancer results in CHAPTER VIII, and most of the compounds were alkaloids. The result was not just matched with the phytochemical analysis, but also explained why this plant indicate positive reactions on anticancer test. Among nine of the compounds isolated from the crude extracts of *Melodinus eugeniifolius*, the new alkaloid 'Melodinoid' and the rest of the alkaloids (yohimbine hydrochloride, tabersonine, scandine, yohimbine, β-yohimbine, and leuconolam) may possess the potential anticancer activities. Therefore, above-mentioned constituents should be

proceeded on the antitumor tests. Besides, since this plant is very rare, chemical modifications may also help to search and manufacture more potential active components.

9.5 Conclusion

Bioassay guided isolation and nine chemical compounds were isolated by column chromatography fractions and were identified by NMR techniques. Six indole alkaloids were isolated and were identified as yohimbine hydrochloride, tabersonine, scandine, yohimbine, β-yohimbine, and leuconolam respectively. The new alkaloid, having a pentacyclic diazaspiro system, was named Melodinoid. Other constituents identified were epigallocatechin and loganic acid. Some of the compounds isolated from the plant were subjected to anti-cancer tests.

CHAPTER X

IN VITRO ANTICANCER ACTIVITIES OF ISOLATED COMPOUNDS FROM *MELODINUS EUGENIIFOLIUS*

10.1 Introduction

Alkaloids are a group of naturally occurring chemical compounds that contain mostly basic nitrogen atoms. This group also includes some related compounds with neutral and even weakly acidic properties [267, 268]. Some synthetic compounds of similar structure are also termed alkaloids [269]. In addition to carbon, hydrogen and nitrogen, alkaloids may also contain oxygen, sulfur and more rarely other elements such as chlorine, bromine, and phosphorus [270]. Alkaloids are produced by a large variety of plants and which have proved to be an important natural source of anti-cancer therapy for several years. They can be purified from crude extracts of these organisms by series of extraction and isolation. Alkaloids have a wide range of pharmacological activities, and certain amount of them display extrodinary antitumor activity, which have been developed as efficient anticancer medicines. Table 10.1 list the representative alkaloids isolated from plants for anticancer use.

Table 10.1 List of alkaloids isolated from plants used in cancer therapy

Alkaloids	Original Plant	Anticancer Target	Reference
Vindesine Vinorelbine	*Catharanthus roseus*	Leukemias, lymphomas, advanced testicular cancer, breast cancer, lung cancer, Kaposi's sarcoma	[219]
Topotecan	*Camptotheca acuminate*	Epithelial ovarian cancer, small cell lung cancer	[271]
Irinotecan	*Camptotheca acuminate*	Metastatic, Colorectal cancer	[272]
Exatecan	*Camptotheca acuminate*	Potential anti-tumor activity both *in vitro* and *in vivo*	[273]
LE-SN-38	*Camptotheca acuminate*	Various cancer cell lines	[274]
Berbamine	*Berberis amarensis*	Chronic myeloid leukemia	[275, 276]
Berberine	*Hvdrastis canadensis L., Berberineeris sp., Arcungelisia flaw*	Osteosarcoma, lung, liver, prostate and breast cancer	[277, 278]
Ellipticine	*Ochrosia borbonica, Excavatia coccinea, Ochrosia elliptica*	Various cancer cell types	[279]

Table 10.1 List of alkaloids isolated from plants used in cancer therapy

(continued)

Alkaloids	Original Plant	Anticancer Target	Reference
Flavopiridol	*Amoora rohituka*, *Dysoxylum binectariferum*	colorectal, non-small cell lung cancer, renal cell carcinoma, non-Hodgkin's lymphoma, chronic lymphocytic leukemia, solid tumors	[280]
Harringtonine, Homoharringtonine	*Cephalotaxus harrintonia*, *Cephalotaxus hainanensis*, *Cephalotaxus qinensis*	Acute myeloid leukemia, chronic myeloid leukemia	[219, 281]
Schischkinnin	*Centaurea schischkinii*	Colon cancer lines in vitro	[282]
Montamine	*Centaurea Montana*	CaCo2 colon cancer cell line in vitro	[283]

Purpose in this chapter is to verify if the compounds isolated from the plant *Melodinus eugeniifolius* in CHAPTER IX contain antitumor capacity and may be applied on medical use in the future.

10.2 Methodology

According to the isolation work from *Melodinus eugeniifolius* in last chapter, three alkaloids with the highest amount collected were screened by MTT assays against two cancer cell lines and a normal cell line.

10.2.1 Principle of method

MTT assay is dependent on the reduction of the tetrazolium salt MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide) by the mitochondrial dehydrogenase of viable cells to form a blue formazan product. The assay measures cell respiration and the amount of formazan produced is proportional to the number of living cells present in culture which will result in lower optical density (OD) [226]: Biological in vitro assay was determined as follows [227].

10.2.2 Protocol

10.2.2.1 Cell lines and cell culture

Human derived cell lines were routinely cultivated at 37 ℃ in an atmosphere of 5% CO_2 in RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% fetal calf serum and subcultured twice weekly to maintain continuous logarithmic growth. Cells were seeded into 96-well microtiter plates at a density of 3-5 $\times 10^3$per well and allowed 24h to adhere before extracts were introduced (final concentration 200 μg/mL to 1 μg/mL).

10.2.2.2 Preparation of plant samples

Extracts were prepared as 500ug/mL top stock solutions, dissolved in DMSO, and stored at 40 ℃, protected from light for a maximum period of 4 weeks. Serial drug dilutions were prepared in medium immediately prior to each assay.

10.2.2.3 The MTT assay

The remainder of the cells were syringed through a 23G needle to attain a single cell suspension. The cells were counted and seeded at a density

of 3 x 10^3 cells per well in a 96-well plate. The cells were suspended in RPMI tissue culture medium per well. The outer columns of the plate were filled with medium to prevent evaporation from treatment wells in addition to providing blank readings for the plate reader. Cells were additionally seeded in a time zero (t0) plate in the same manner as the experimental treatment plate. The cells were incubated overnight at 37 ^oC in a 5% CO_2 environment to allow for attachment. Cells were then treated with serial dilutions of various concentrations the following day. A volume of 20 μL of each dilution was added to respective treatment wells (total volume 200 μL) to yield final concentrations of 0.1 μg/mL, 0.5 μg/mL, 1 μg/mL, 5 μg/mL, 10 μg/mL, 50 μg/mL, 100 μg/mL, 500μg/mL and 1mg/mL. A separate DMSO control trial was done to ensure that it did not affect any of the results obtained. A t_0 measurement was taken to obtain a measurement of cell viability at the time of treatment. Treated cells were incubated for 72 h at 37 ^oC. At the time of extract addition and following 72h exposure, MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide) was added to each well. Incubation at 37 ℃ for 4 h allowed reduction of MTT by viable cells to an insoluble formazan product. Well contents were aspirated and formazan solubilized by addition of DMSO: glycine buffer (pH 10.5) (4:1). The plate was then placed in an orbital plate shaker for 2-3 minutes to aid formazan dissolution before obtaining the absorbance

reading at 550-570 nm on microplate readers (Anthos Labtec , Perkin Elmer Envision, Biohit plc). The cell viability rate was calculated as follows:

$$Cell\ viability\ (\%) = \frac{(Absorbance\ of\ the\ treated\ wells)}{(Absorbance\ of\ the\ control\ wells)} \times 100$$

10.2.3 Statistical analysis

Concentration–response curves were calculated using the Prism software package 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com (GraphPad, San Diego, USA) and data were reported as mean and SD values obtained from a minimum of three determinations. Non-linear best fit was plotted with SD and 95% confidence interval.

To calculate IC_{50}, a series of dose-response data are needed and growth inhibition or % cell viability. The values of y are in the range of 0-100.

Linear Regression: The simplest estimate of IC_{50} is to plot x-y and fit the data with a straight line (linear regression). IC_{50} value is then estimated using the fitted line, i.e., Y = a * X + b, IC_{50} = (50 - b)/a.

Log transformation: Frequently, linear regression is not a good fit to dose-response data. The response-curve fits better to a straight line if the x-axis is logarithm-transformed.

10.2.4 Reagents and materials

Dimethyl sulfoxide (DMSO, R&M), MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Life Technologies), FCS (fetal calf serum) (for cell culture, Sigma), RPMI-1640 medium (for cell culture, Sigma), 5-Fluorouracil (5-FU), defined keratinocyte-SFM (Life Technologies), PBS(dulbecco's phosphate buffered saline) (Sigma), trypan blue (for cell culture, Sigma), Trypsin EDTA (0.05% Trypsin, 0.53mM EDTA.4Na) (Gibco), Penicillin-Streptomycin (liquid, 100 mL/pk) (Gibco).

96-well TC Plate (for mammalian cell culture, Orange Scientific), centrifuge tube (Orange Scientific), TC flask (Orange Scientific), minisart syringe filter (SSI), kit, pipette and tips are all from Dragon Med.

10.3 Results

10.3.1 HT-29 human colon cancer cell line

Three alkaloids isolated from *Melodinus eugeniifolius* were evaluated in MTT assays against a panel of HT-29 human colon cancer cell lines. The best growth inhibition was observed on scandine, with the GI_{50} value of 9.88 μg/mL (Table 10.2). Cellular morphology of HT-29 human colon carcinoma cells in tested compounds and positive control were viewed in Figure 10.1-10.5.

Table 10.2: Growth inhibition of HT-29 human cancer cell lines by compounds

Compound	GI_{50} µg/mL
YHB-HCl	21.93
TBS	17.55
SCD	9.88
5-FU	3.02

YHB-HCl: Yohimbine Hydrochloride, TBS: Tabersonine, SCD: Scandine.

5-FU: 5-Fluorouracil

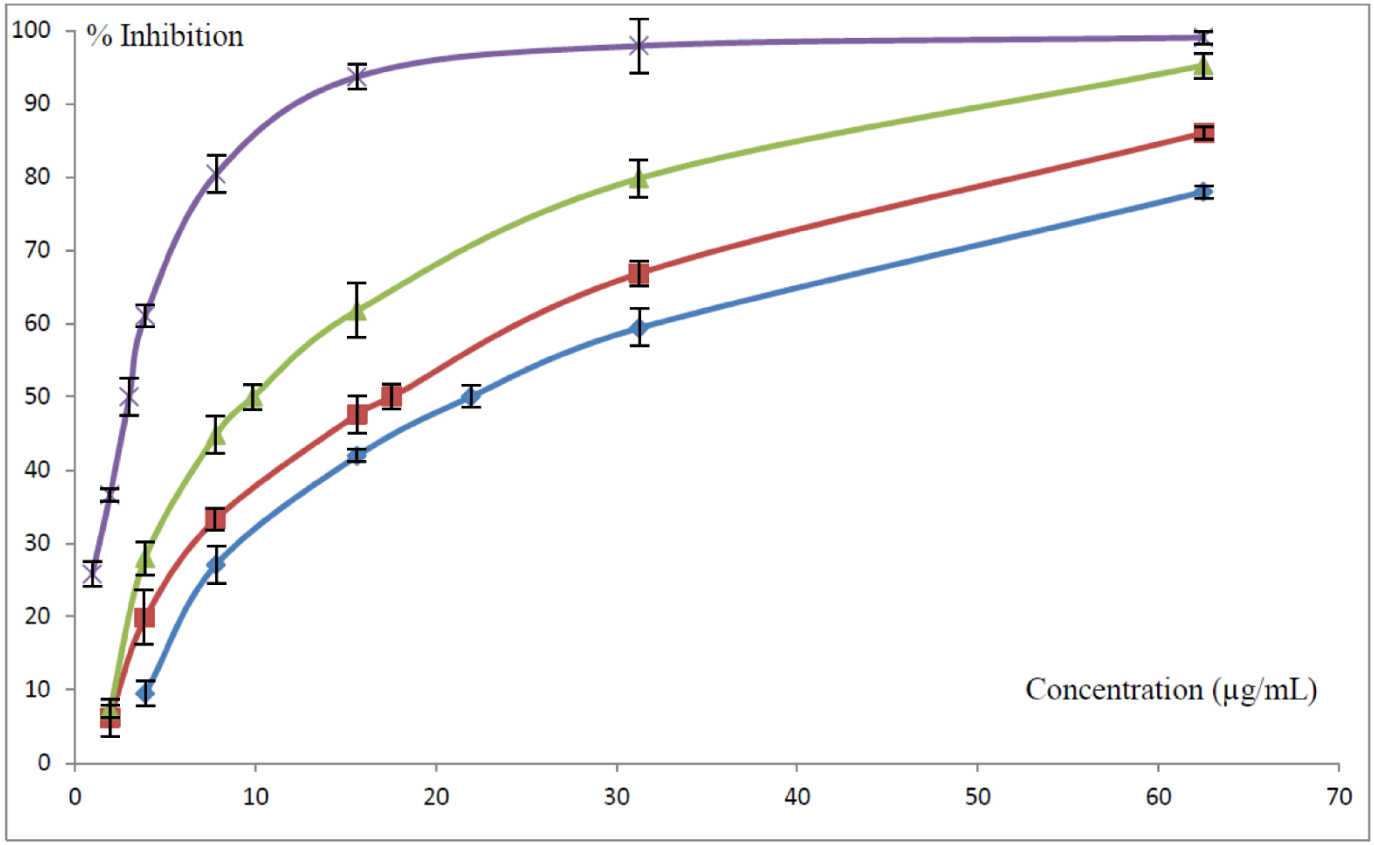


Figure 10.1: Effects of compounds on HT-29 cell line growth.

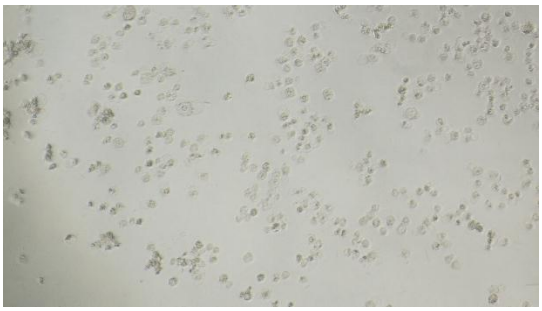


Figure 10.2: HT-29 human colon carcinoma cells (Yohimbine Hydrochloride).

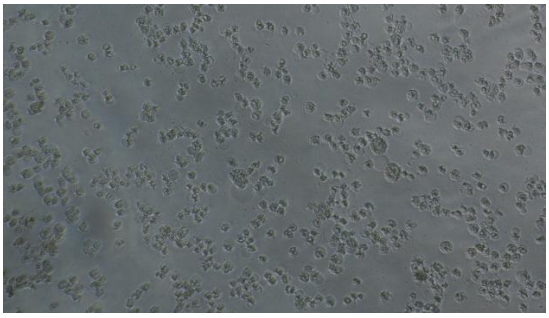


Figure 10.3: HT-29 human colon carcinoma cells (Tabersonine).

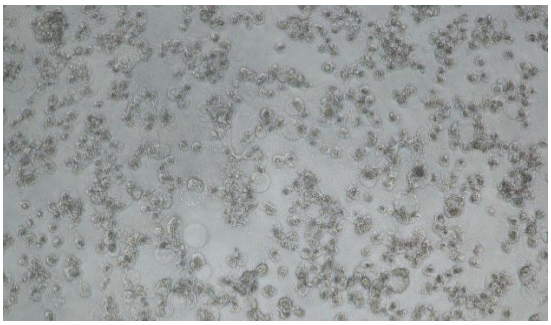


Figure 10.4: HT-29 human colon carcinoma cells (Scandine).

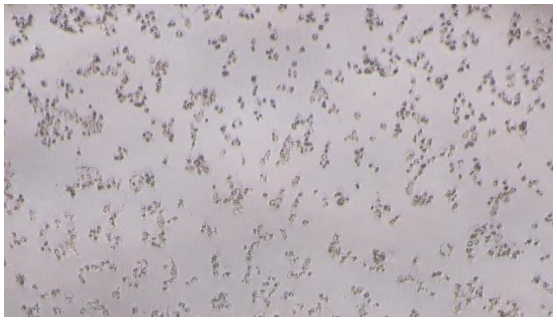


Figure 10.5: HT-29 human colon carcinoma cells (5- Fluorouracil).

10.3.2 HK-1 human nasopharyngeal carcinoma cell line

Three alkaloids isolated from *Melodinus eugeniifolius* were evaluated in MTT assays against a panel of HK-1 human nasopharyngeal cancer cell lines. The best growth inhibition was observed on tabersonine, with the GI_{50} value of 17.92 μg/mL (Table 10.3). Cellular morphology of HK-1 human nasopharyngeal carcinoma cells in tested compounds and positive control were viewed in Figure 10.6-10.10.

Table 10.3: Growth inhibition of HK-1 human nasopharyngeal carcinoma cell lines by compounds.

Compound	GI_{50} µg/mL
YHB-HCl	57.10
TBS	17.92
SCD	32.45
5-FU	10.50

YHB-HCl: Yohimbine Hydrochloride, TBS: Tabersonine, SCD: Scandine.

5-FU: 5-Fluorouracil

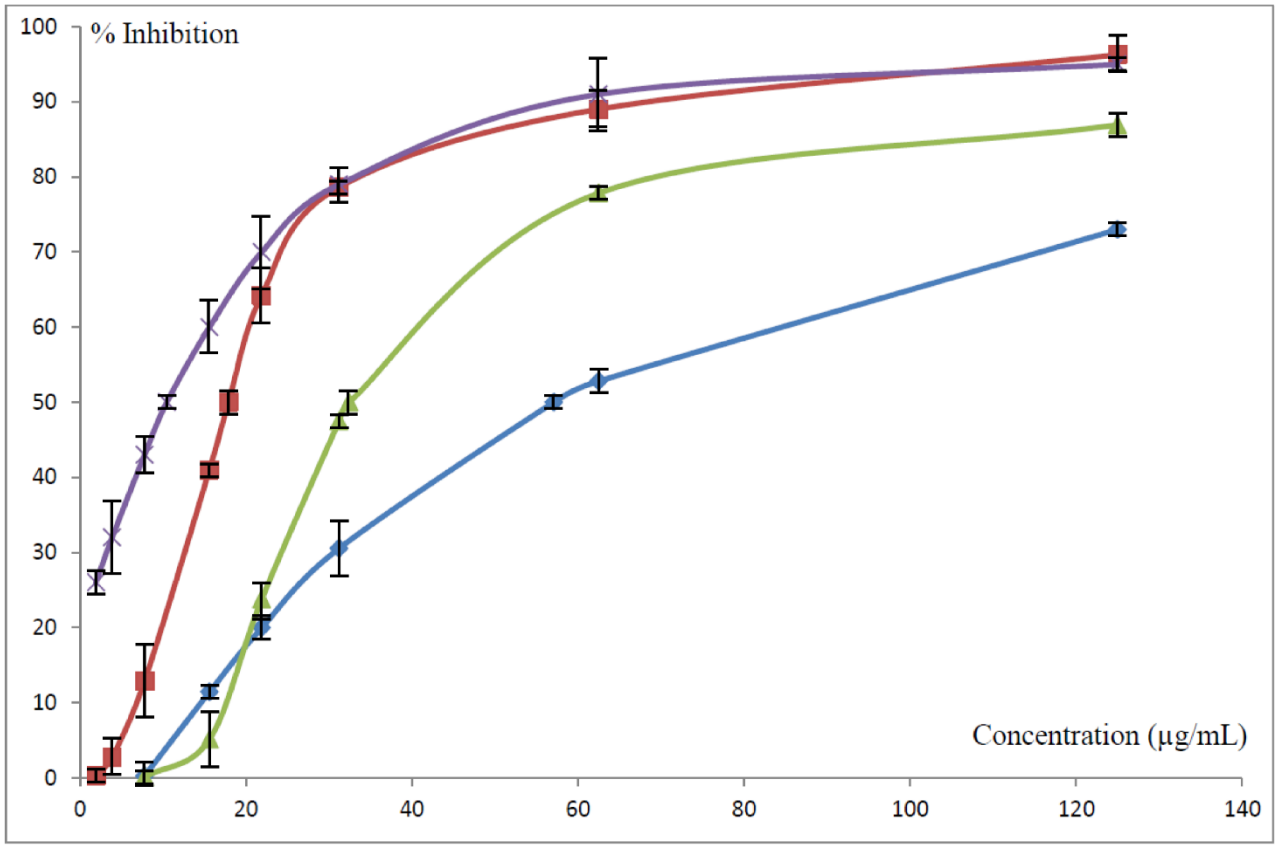


Figure 10.6: Effects of compounds on HK-1 cell line growth.

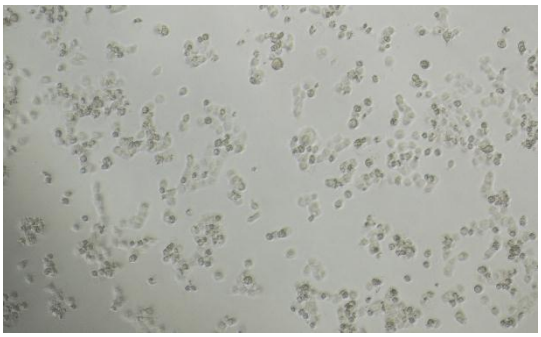


Figure 10.7: HK-1 human nasopharyngeal carcinoma cells (Yohimbine Hydrochloride).

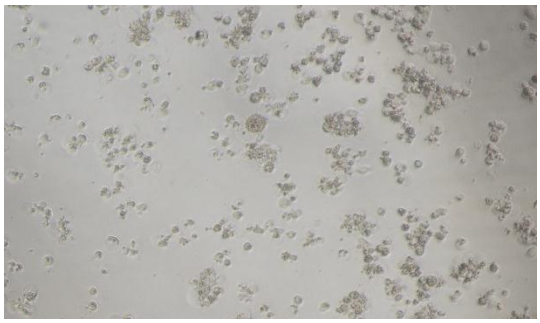


Figure 10.8: HK-1 human nasopharyngeal carcinoma cells (Tabersonine).

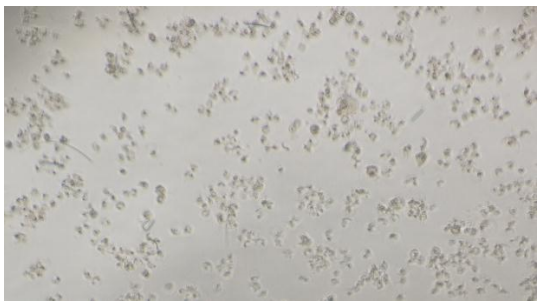


Figure 10.9: HK-1 human nasopharyngeal carcinoma cells (Scandine).

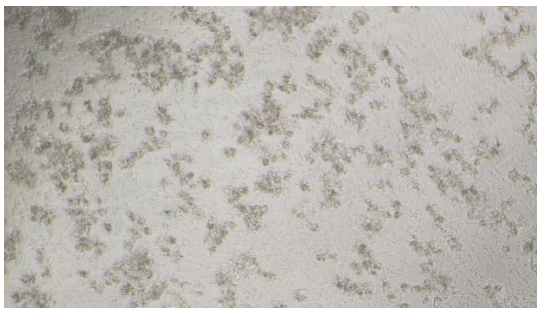


Figure 10.10: HK-1 human nasopharyngeal carcinoma cells (5-Fluorouracil).

10.3.3 MRC-5 human fetal lung fibroblast cell line

Three alkaloids isolated from *Melodinus eugeniifolius* were evaluated in MTT assays against a panel of MRC-5 human fetal lung fibroblast cell lines. None of the fractions from extracts revealed inhibition activity as all of the GI_{50} values of them are higher than 1000 μg/mL (Table 10.4).

Table 10.4: Growth inhibition of MRC-5 human fetal lung fibroblast cell lines by compounds.

Compound	GI_{50} µg/mL
YHB-HCl	>1000
TBS	>1000
SCD	>1000
5-FU	>500

YHB-HCl: Yohimbine Hydrochloride, TBS: Tabersonine, SCD: Scandine.

5-FU: 5-Fluorouracil

10.4 Discussion

Three of the alkaloids isolated from *Melodinus eugeniifolius* with the highest extraction yield rates have been tested for their *in vitro* anticancer activity screening. All the compounds revealed significant anticancer capacity against the two human cancer cell lines, and were not damaging to the normal cells. The best growth inhibition assay against HT-29 human colon cancer cell line was observed on scandine, with the GI_{50} value of 9.88 μg/mL (Table 10.1). And tabersonine revealed the best growth inhibition against HK-1 human nasopharyngeal cancer cell line with a GI_{50} value of 17.92 μg/mL (Table 10.2).

According to the analysis in CHAPTER VIII, the carcinogenesis of HT-29 cells is mainly focused on the production of Ig A and CEA which made by the intestinal epithelium. The antitumor mechanism of the compound isolated from the plant *Melodinus eugeniifolius* on HT-29 cells had been predicted the induction of apoptosis of intestinal epithelium. The carcinogenetic mechanism may help to conjecture the inhibition mechanism of the components isolated from the plant *Melodinus eugeniifolius* on HK-1 cells. The squamous cell carcinoma

caused by the gene over-expression, DNA and RNA transform signal. The inhibition mechanism may present in the induction of apoptosis of squamous epithelial cells and gene regulation.

Since all the alkaloid compounds tested in this chapter revealed comparative strong anticancer activities against different cancer cell lines. 5-Fluorouracil (5-FU) has been used as a positive control for all the anticancer assays. 5-Fluorouracil is a drug that is a pyrimidine analog which is used in the treatment of cancer [284]. It is a ‘suicide’ inhibitor and works through irreversible inhibition of thymidylate synthase, it means the 5-fluouracil is a killer for both the cancer cells and the normal cells. It belongs to the family of drugs called the antimetabolites [285]. It is on the World Health Organization's List of Essential Medicines, a list of the most important medications needed in a basic health system [286]. 5-FU acts in several ways, but principally as a thymidylate synthase (TS) inhibitor. Interrupting the action of this enzyme blocks synthesis of the pyrimidine thymidine, which is a nucleoside required for DNA replication. Thymidylate synthase methylates deoxyuridine monophosphate (dUMP) to form thymidine monophosphate (dTMP). Administration of 5-FU causes a scarcity in dTMP, therefore, rapidly dividing cancerous cells undergo cell death via thymineless death [287]. Calcium folinate provides an exogenous source of reduced folinates and

hence stabilises the 5-FU-TS complex, hence enhancing 5-FU's cytotoxicity [288].

However, comparing with 5-FU's cytotoxicity on normal cells, tabersonine, one of the alkaloids isolated from *Melodinus eugeniifolius*, revealed a similar level of anticancer activity towards the HK-1 human nasopharyngeal cancer cell line, but obviously has a higher security. Further research on the mechanism of tabersonine and relative in vivo anticancer tests should be proceeded for the following stage.

10.5 Conclusion

Alkaloids always present significant anticancer capacity [289]. Three alkaloids isolated from *Melodinus eugeniifolius* were screened by MTT assays against two cancer cell lines and a normal cell line. All the compounds revealed significant anticancer capacity on the two human cancer cell lines, and not damage the normal cells. The results obtained suggest that further *in-vivo* studies can be performed on isolated compounds from *Melodinus eugeniifolius*.

CHAPTER XI

GENERAL CONCLUSION

The following appropriate conclusions were down based on the experimental data obtained.

i. Extraction of samples

Extraction was done with maceration with solvents of increasing polarity. The yield for the hexane, chloroform and ethanol extract of barks were 1.02%, 0.67% and 0.56% respectively. The yield for the hexane, chloroform and ethanol extract of leaves for were 5.00%, 3.90% and 3.40%. Crude extracts were kept at -20^{o}C until further use.

ii. Phytochemical analysis

Phytochemical analysis of *Melodinus eugeniifolius* revealed that the chloroform extract of the leaves and barks accumulate substantial amounts of alkaloids and cardiac glycosides which could be well correlated with the activities measured. Flavonoids and steroids were detected in all extracts. Saponins were detected in the chloroform and ethanol

extracts. Tannins were not detected in any of the crude extracts.

iii. Antioxidant activities

The antioxidant activity reflected by the FRAP, DPPH, and β-carotene bleaching assay was clearly observed in the ethanol extracts of bark and leaves among the six crude extracts of *Melodinus eugeniifolius*. Results were followed by the chloroform extract of barks and leaves. The hexane extract of barks and leaves had the lowest result in the antioxidant activity tests.

iv. Antimicrobial activities

All crude extracts of *Melodinus eugeniifolius* under investigation exhibited exceptional antimicrobial activity against both Gram-positive and Gram-negative bacteria. The most promising activity was displayed against Gram-positive bacteria *Staphylococcus aureus* and Gram-negative bacteria *Escherichia coli*.

v. Antiparsitic activities

Six different extractions of *Melodinus eugeniifolius* leaves and barks were screened for their *in vitro* antileishmanial

activities, among the different extracts tested, the ethanol and hexane extract of barks showed significant antileishmanial activities with IC_{50} value of 159.9 µg/mL and 270.3 µg/mL.

vi. Anticancer activities

Six different extractions of *Melodinus eugeniifolius* leaves and barks were screened for their *in vitro* antitumor activities, among the different extracts tested, the chloroform extracts of barks and leaves showed significant antitumor activities. Among the study, different extracts showed significant antitumor activities on different cancer cell lines, and would not damage the normal cells. Leaf and bark chloroform extracts showed the best antitumor activities with GI_{50} values of 99.5 µg/mL and 124.2 µg/mL against the human colon cancer cell HT-29. Leaf hexane and chloroform extracts showed excellent antitumor activities with GI_{50} values of 50.22 µg/mL and 50.84 µg/mL against the human colon cancer cell HCT-116, and 53.93 µg/mL and 63.80 µg/mL against the human breast cancer cell MCF-7. Bark chloroform and leaf ethanol extracts showed significant antitumor activities with GI_{50} values of 193.7 µg/mL and 167.1 µg/mL against the human colon cancer cell CaCo-2. Among all the crude extracts of *Melodinus eugeniifolius*, only the bark chloroform

extract revealed the most significant anticancer activity against the HK-1 human nasopharyngeal cancer cell line with a GI_{50} value of 66.7 µg/mL, besides, the leaf chloroform and ethanol extracts also shown certain antitumor capacity with GI_{50} values of 170.7 µg/mL and 159.2 µg/mL respectively.

vii. Isolation and identification of the chemical compounds

Bioassay guided isolation and nine chemical compounds were isolated by column chromatography fractions and were identified by NMR techniques. Six alkaloids were isolated and were identified as yohimbine hydrochloride, tabersonine, scandine, yohimbine, β-yohimbine, and leuconolam respectively. The new alkaloid, having a pentacyclic diazaspiro system, was named Melodinoid. Other constituents identified were epigallocatechin and loganic acid.

viii. Anticancer activities on Isolated chemical compounds

Three alkaloids isolated from *Melodinus eugeniifolius* were screened by MTT assays against two cancer cell lines and a normal cell line. All the compounds revealed significant anticancer capacity on the two human cancer cell lines, and did not damage the normal cells. The best growth inhibition assays against HT-29 human colon cancer cell lines was

observed on scandine, with the GI_{50} value of 9.88 µg/mL. And tabersonine revealed the best growth inhibition against HK-1 human nasopharyngeal cancer cell lines with a GI_{50} value of 17.92 µg/mL. The results obtained suggest that further *in-vivo* studies can be performed on isolated compounds from *Melodinus eugeniifolius*.

To draw a conclusion, exploration of the pharmacycological properities of *Melodinus eugeniifolius* revealed that a series of matabolites with dual antioxidant and anticancer activities may represent a new generation of potential drug candidates for antioxidant products and the treatment of cancer. Hence, further research on the mechanism of some of the isolated compounds with target pharmacological activities, relative *in vivo* anticancer tests, and even clinical trials are recommended to ascertain the efficacy, safety and other mechanisms of action to application in the pharmaceutical industry as natural therapeutic agents.

CHAPTER XII

FUTURE PERSPECTIVES

This study intends to contribute towards the knowledge based of plant species with therapeutic potential. Material of this study was only collected from a single site. To account for possible geographical and chemotypic variation material should be studied from several populations.

12.1 Antioxidant activity

The crude extracts of the studied plant showed satisfactory antioxidant capacity, the isolated compounds should also be tested.

12.2 Anticancer

For those compounds that have already been isolated from *Melodinus eugeniifolius* fractions and extracts, other anticancer assays should be carried out, and for those compounds with excellent anticancer capability, they should be approved for further study in *in vivo* assays.

Besides, this study has undoubtedly proven the existence of other compounds with potential *in vitro* anticancer activity in different species or extracts of *Melodinus eugeniifolius*. Activity-guided fractionation, isolation and identification of these compounds is imperative and may lead to the development of novel treatment in the global struggle against cancer and cancer-related ailments.

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APPENDICES A: Extraction yields of crude extracts of *Melodinus eugeniifolius*

Extraction yields	Crude extracts	
	Extraction yields (g)	Extraction yields (%)
Bark Hexane (BH)	25.50	1.02
Bark Chloroform (BC)	16.75	0.67
Bark Ethanol (BE)	13.99	0.56
Leaf Hexane (LH)	15.01	5.00
Leaf Chloroform (LC)	11.70	3.90
Leaf Ethanol (LE)	10.21	3.40

APPENDICES B: CONFERENCES AND TRAININGS ATTENDED

APPENDICES B1

Post presentation in the 2013 Diabetic Foot Global Conference on the 21th-23th of March 2013 at Los Angeles, USA. – **"A Note on the Treatment of Diabetic Foot by the 'Heat - Clearing Method' (Qing Fa) of Traditional Chinese Medicine"**

"A NOTE ON THE TREATMENT OF DIABETIC FOOT BY THE 'HEAT - CLEARING METHOD' (QING FA) OF TRADITIONAL CHINESE MEDICINE"

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ABSTRACT:

In pursuing the study of different treatments of diabetic foot in Traditional Chinese Medicine, we became interested in the 'Heat - Clearing Method' (Qing Fa). The 'Heat - Clearing Method' is one of the most important current standards of care for diabetic foot in China. Diabetic foot involves the formation of an ulcer that induces gangrene, disability and eventually death. In Traditional Chinese Medicine, diabetic foot is incorporated in the 'gangrene' (Tuo Ju) type of pathology and oddly enough, little is currently known about this method. Indeed, treatises about the diabetic foot treatment by the 'Heat-Clearing Method' are rare or part of other treatment descriptions. With this premise in mind we launched an ethnopharmacological study about the 'Heat-Clearing Method' providing a comprehensive theoretical basis for the Traditional Chinese Medicine treatment of diabetic foot. To the best of our knowledge, this is the first report dedicated to the usage of the 'Heat-Clearing Method' on diabetic foot treatment.

Keywords: Diabetic foot, traditional Chinese medicine, medical treatment, heat-clearing method.

A Note on the Treatment of Diabetic Foot by the 'Heat - Clearing Method' (Qing Fa) of Traditional Chinese Medicine

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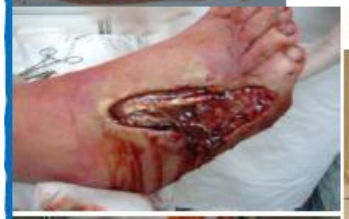
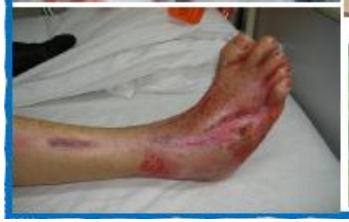
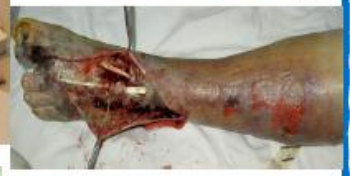
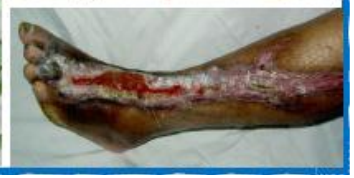




In pursuing the study of different treatments of diabetic foot in Traditional Chinese Medicine, we became interested in the 'Heat - Clearing Method' (Qing Fa). The 'Heat - Clearing Method' is one of the most important current standards of care for diabetic foot in China. Diabetic foot involves the formation of an ulcer that induces gangrene, disability and eventually death. In Traditional Chinese Medicine, diabetic foot is incorporated in the 'gangrene' (Tuo Ju) type of pathology and oddly enough, little is currently known about this method. Indeed, treatises about the diabetic foot treatment by the 'Heat-Clearing Method' are rare or part of other treatment descriptions. With this premise in mind we launched an ethnopharmacological study about the 'Heat-Clearing Method' providing a comprehensive theoretical basis for the Traditional Chinese Medicine treatment of diabetic foot. To the best of our knowledge, this is the first report dedicated to the usage of the 'Heat-Clearing Method' on diabetic foot treatment.





APPENDICES B2

Oral presentation in the 2014 7th annual World Cancer Congress on the 16th-18th of May 2014 at Nanjing, CHINA. – **"Anti-cancer Research on *Melodinus sp.* (Apocynaceae Juss.)"**

"ANTI-CANCER RESEARCH ON *MELODINUS sp.* (APOCYNACEAE JUSS.)"

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ABSTRACT:

Melodinus eugeniifolius. a very rare medicinal plant belongs to the family of Apocynaceae. Almost no pharmacological and phytochemical studies in genera *Melodinus*. This study was undertaken to screen the phytochemical composition and anticancer activity of different extracts of *Melodinus eugeniifolius.*

Qualitative phytochemical analysis of the crude extract was determined for the presence of tannins, flavonoids, alkaloids, saponins or sterols. Phytochemical analysis of *Melodinus eugeniifolius.* revealed presence of alkaloids, cardiac glycosides, sterols, steroids and flavonoids in crude extracts of leaves and barks.

Cancers remain the leading cause of death worldwide and the search for novel anti-cancer agents from medicinal plants has become crucial. Infections and free radical generation are recognized in the pathogenesis of cancer. Six different extractions of *Melodinus eugeniifolius.* leaves and barks were screened for their *in vitro* antitumor activities, among the

different extracts tested, the chloroform extracts of barks and leaves showed significant antitumor activities. According to the current result, the most susceptible cell lines were found to be the human breast cancer cell line, MCF-7. Besides, HCT-116 colorectal carcinoma cells lines and HT-29 human colon cancer line also have excellent results.

Results from our phytochemical analysis revealed that the chloroform extract of leaves and barks of *Melodinus eugeniifolius.* accumulate substantial amounts of alkaloids and cardiac glycosides which could be well correlated with the activities measured. We shall conduct further work to isolate the antioxidant, antibacterial and anticancer constituents of the plant. *Melodinus eugeniifolius.* is a potential medicinal plant focus on anti-cancer field.

Keywords:
Melodinus eugeniifolius., anticancer activity, human breast cancer, MCF-7, medicinal plant.

APPENDICES B3

Oral presentation in the 2014 International Conference on Medicine Sciences And Bioengineering [ICMSB 2014] on the 16th-17th of August 2014 at Kunming, CHINA. – **"Anti-cancer Research on *Melodinus sp*. (Apocynaceae Juss.)"**

"Anti-cancer Research on *Melodinus sp*. (Apocynaceae Juss.)"

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Keywords:
Melodinus eugeniifolius., anticancer activity, human breast cancer, MCF-7, medicinal plant.

APPENDICES B4

ATTENDED TRAININGS

- Nature of PhD, Graduate School Nottingham University Malaysia Campus, 8th Oct 2012
- Planning and time management, Graduate School Nottingham University Malaysia Campus, 9th Oct 2012
- Getting into the habit of writing, Graduate School Nottingham University Malaysia Campus, 10th Oct 2012
- Creative thinking, Graduate School Nottingham University Malaysia Campus, 11th Oct 2012
- Working effectively in research, Graduate School, Nottingham University Malaysia Campus, 12th Oct 2012
- Project management for researchers, Graduate School, Nottingham University Malaysia Campus, 17th Oct 2012
- Funding workshop, Graduate School, Nottingham University Malaysia Campus, 19th Oct 2012
- What do I want to get out of a conference, Graduate School, Nottingham University Malaysia Campus, 23th Oct 2012
- Presentation skills, Graduate School, Nottingham University Malaysia Campus, 19th Nov 2012
- Communicating your research, Graduate School, Nottingham University Malaysia Campus, 23th Nov 2012.
- Demonstrating in Laboratory Practicals, Graduate School, Nottingham University Malaysia Campus. 5th May, 2015.
- Health and Safety, Graduate School, Nottingham University Malaysia Campus. 5th May, 2015.

APPENDICES C: PUBLICATIONS

APPENDICES C1

***Pharmacology & Pharmacy*, 2013, 4, 277-282**
doi:10.4236/pp.2013.43040 Published Online June 2013
(http://www.scirp.org/journal/pp) Copyright © 2013 SciRes. ***PP*** 277

Research of Quality Standards for Stachydrine Hydrochloride in Chinese Medicine TJF Granule

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ABSTRACT

A high-performance liquid chromatographic method was applied to the determination of stachydrine hydrochloride concentration in TJF granule (Chinese name: Tiao-Jing-Fang), using a mobile phase of methanol-acetonitrile (50:50, v/v) by the Agilent Kro-masi NH2 column (250 mm × 4.6 mm, 5 μm, S/N: 22N25110). Detection wavelength was 201 nm. The result revealed good linearity of stachydrine hydrochloride and was obtained within the range of 0.20 - 1.98 μg/mL (R = 0.9995). The average recovery was 97.01%; the relative standard deviation (RSD) was

0.19%. To the best of our knowledge, this is the first report dedicated to the determination of stachydrine hydrochloride by the evaporative light scattering detector-high-performance liquid chromatographic (ELSD-HPLC) method.

Keywords: TJF Granule; Quality Standards; Traditional Chinese Medicine; Evaporative Light Scattering Detector (ELSD); High-Performance Liquid Chromatographic (HPLC) Method; Stachydrine Hydrochloride; Pharmaceutical Analysis; Determination

APPENDICES C2

American Open Medical Sciences Journal Vol. 1, No. 2, December 2013, pp. 1-12
Available online at http://rekpub.com/Journals.php

A Note on the Treatment of Diabetic Foot by the 'Heat -Clearing Method' (Qing Fa) of Traditional Chinese Medicine

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ABSTRACT

In pursuing the study of different treatments of diabetic foot in Traditional Chinese Medicine, we became interested in the 'Heat -Clearing Method' (Qing Fa, 清法). The 'Heat -Clearing Method' is one of the most important current standards of care for diabetic foot in China. Diabetic foot involves the formation of an ulcer that induces gangrene, disability and eventually death. In Traditional Chinese Medicine, diabetic foot is incorporated in the 'gangrene' (Tuo Ju, 脱疽) type of pathology and oddly enough, little is currently known about this method. Indeed, treatises about the diabetic foot treatment by the 'Heat-Clearing Method' are rare or part of other treatment descriptions. With this premise in mind we launched an ethnopharmacological study about the 'Heat-Clearing Method' providing a comprehensive theoretical basis for the Traditional Chinese Medicine treatment of diabetic foot. To the best of our knowledge, this is the first report dedicated to the usage of the 'Heat-Clearing Method' on diabetic foot treatment.

Keywords: Diabetic Foot, Traditional Chinese Medicine, Medical Treatment, Heat-Clearing Method (Qing Fa, 清法).

APPENDICES C3

***Pharmacology & Pharmacy*, 2014, 5, 395-400**
Published Online April 2014 in SciRes. http://www.scirp.org/journal/pp
http://dx.doi.org/10.4236/pp.2014.54047

Antioxidant Activity Determination of Citronellal and Crude Extracts of *Cymbopogon citratus* by 3 Different Methods

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ABSTRACT

Cymbopogon citratus, better known as lemongrass, is a plant commonly used for culinary purposes. It is known to contain the compound citronellal, which is responsible for the lemon-scent of many of the plants of the genus *Cymbopogon*. A chloroform extract of *Cymbopogon citratus* was screened to determine its free radical scavenging activities. Three different methods were used to test the antioxidant activity of the extract, including FRAP assay (Ferric reducing antioxidant potential), DPPH radical scavenging assay (1,1-diphenyl-2-picryl hydrazyl radical reducing power methods), and *β*-carotene bleaching assay. *Cymbopogon citratus* showed low radical scavenging activities compared to ascorbic

acid, gallic acid and quercetin. The results obtained suggest that *Cymbopogon citratus* is best appreciated for its refreshing aroma and delicate taste, but has little to offer as a source of antioxidants.

Keywords: Antioxidant Activity; DPPH Assay; FRAP Assay; *β*-Carotene Bleaching Assay; Citronellal

APPENDICES C4

Pharmacology & Pharmacy, 2014, 5, 540-550
Published Online May 2014 in SciRes. http://www.scirp.org/journal/pp
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The Genus *Melodinus* (Apocynaceae): Chemical and Pharmacological Perspectives

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ABSTRACT

The plants of the genus *Melodinus* (Apocynaceae) are widely distributed, and have long been used in folk medicine for the treatment of various ailments such as meningitis in children and rheumatic heart diseases, hernia, infantile malnutrition, dyspepsia and testitis. Over 100 alkaloids together with flavonoids, lignans, steroids, terpenoids and coumarins have been identified in the genus, and many of these have been evaluated for biological activity. This review presents comprehensive information on the chemistry and pharmacology of the genus together with the traditional uses of many of its plants. In addition, this review discusses the structure-activity relationship of different compounds as well as recent developments and the scope for future research in this aspect.

Keywords: *Melodinus*, Apocynaceae, Ethnopharmacology, Anti-Cancer

APPENDICES C5

Pharmacology & Pharmacy, 2014, 5, 747-754
Published Online June 2014 in SciRes. http://www.scirp.org/journal/pp
http://dx.doi.org/10.4236/pp.2014.57084

Antileishmanial Assay and Antimicrobial Activity on Crude Extracts of *Melodinus eugeniifolius* Barks and Leaves from Malaysia

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Received 22 April 2014; revised 29 May 2014; accepted 23 June 2014

ABSTRACT

Melodinus eugeniifolius, a very rare medical plant which belongs to the family of Apocynaceae, has just been discovered in the Malaysia rain forest. Six different extracts of *Melodinus eugeniifolius* (a very rare medical plant belongs to Apocynaceae) leaves and barks were screened for the *in vitro* antileishmanial and antibacterial activities, among the different extracts tested, the ethanol and hexane extract of barks showed significant antileishmanial activities with IC50 value of 159.9 μg/mL and 270.3 μg/mL. All the extracts displayed remarkable antibacterial activity against *Staphylococcus aureus*, *Bacillus cereus* and *Escherichia coli*.

Keywords: Antileishmanial Activity, MTT Assay, Antimicrobial Activities, Apocynaceae.

APPENDICES C6

Pharmacology & Pharmacy, 2014, 5, 773-780
Published Online July 2014 in SciRes. http://www.scirp.org/journal/pp
http://dx.doi.org/10.4236/pp.2014.58087

Phytochemical Analysis and Antioxidant Activity Determination on Crude Extracts of *Melodinus eugeniifolius* Barks and Leaves from Malaysia

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Received 6 May 2014; revised 9 June 2014; accepted 26 June 2014

ABSTRACT

Six different extracts of *Melodinus eugeniifolius* (a very rare medical plant belonging to Apocynaceae) leaves and barks were screened for their phytochemical composition, and free radical scavenging activities. Three different methods were used to test the antioxidant activity for extracts which include FRAP assay (Ferric reducing antioxidant potential), DPPH radical scavenging assay (1,1-diphenyl-2-picryl hydrazyl radical reducing power methods), and β-carotene bleaching assay. Among the different extracts tested, the ethanol extract of barks showed significant radical scavenging activities. Phytochemical analysis of the extracts revealed that the radical scavenging activities are mainly due to the presence of alkaloids, cardiac glycosides, sterols, steroids and

flavonoids. The results obtained suggest that *Melodinus eugeniifolius* could be exploited in the management of various diseases like cancer, cardiovascular diseases and infection diseases. Highlights: *Melodinus eugeniifolius*, a very rare medical plant from South Asian rain forest which may be a potential drug for many diseases is first reported about its phytochemical characteristics and antioxidant activity by different methods.

Keywords: Antioxidant Activity, DPPH Assay, FRAP Assay, *β*-Carotene Bleaching Assay, Phytochemical Analysis, Apocynaceae, *Melodinus eugeniifolius*

APPENDICES C7

2014 International Conference on Medicine Sciences and Bioengineering [ICMSB 2014]
(In publishing)

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