CANARIUM PATENTINERVIUM MIQ. (BURSERACEAE KUNTH.): A PHYTOCHEMICAL AND PHARMACOLOGICAL STUDY

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DECLARATION

I, R.Mogana, 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, Malaysia. It has not been submitted before for any degree or examination at this or any other University.

……..

Signature

……..

Date

DEDICATION

To my beloved Sri Sri Radha Krsna, my beloved parents, G. Rajagopal and P. Muthuletchumy and my beloved husband P. Ramesh Kumar

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.

I offer my greatest thanks to my dear husband and beloved parents for their love, ongoing support and motivation.

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ABSTRACT

Canarium patentinervium Miq. belongs to the family of Burseraceae best known for producing resins of economic, medicinal, and cultural values such as frankincense, myrrh, and copal. This family consists of 18 genera and 700 species of trees. In the Asia-Pacific region, about 20 species of Burseraceae are used to treat haemorrhoids, heal wounds and to treat skin infections. This plant has been used to heal wounds amongst the indigenous people of Malaysia. Furthermore no pharmacological and phytochemical studies have been reported on this species. This study was undertaken to screen the phytochemical and pharmacological aspects of this plant. Qualitative phytochemical properties of the crude extract was determined for the presence of tannins, flavonoids, alkaloids, saponins or sterols. Phytochemical analysis of *Canarium patentinervium* Miq. revealed presence of tannins and flavonoids in the ethanol extract of leaves and barks. Bioassay guided fractionation led to isolation of eight secondary metabolites by chromatography which were identified by NMR techniques. Two coumarins (scopoletin and scoparone), five phenols (cynaroside, hyperin, (+)-catechin, lioxin and syringic acid) and a norsesquiterpene with cyclohexenone ring (vomifoliol). The latter three compounds were isolated for the first time from the genus *Canarium*. The plant and the isolated compounds were then subjected to six biological assays comprising of antibacterial, antioxidant, anticancer, anti-inflammatory, anti-acetylcholinesterase and anti-parasitic activities. Infectious diseases remain the leading cause of death worldwide and bacteria have become more resistant to conventional antibiotic and the search for novel antimicrobial agents from medicinal plants has become crucial. Antibacterial test was done using disc diffusion method, minimum inhibitory concentration (MIC), minimum bactericidal

concentration (MBC) and death kinetic assay with ampicillin as the positive control. The ethanol extract of leaves and the hexane extract of bark displayed remarkable antibacterial activity against both Gram-positive bacteria and Gram-negative bacteria. All isolated compounds tested against *S.aureus* ATCC 11632 showed bacterial growth inhibition. Scopoletin, scoparone, hyperin, cynaroside and syringic acid had bactericidal effect <100 µg/ml. Only scopoletin had bactericidal effect and complete kill at MBC 50.00 µg/ml. 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. 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. Five various antioxidant assays with different mode of action [2, 2-diphenyl-1-picrylhydrazyl (DPPH) and 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ferric reducing antioxidant power (FRAP), β-carotene bleaching assay and superoxide dismutase (SOD) assay] were used to test the antioxidant scavenging abilities of this plant. Vitamin C (L-ascorbic acid), quercetin and trolox were used as positive controls. The ethanol extract of leaves and barks displayed superior antioxidant capacities. The EC_{50} values of the samples were consistently low in SET methods (ABTS, DPPH and FRAP) superior to standard as opposed to HAT method (β-carotene bleaching assay). Hyperin and (+)-catechin exhibited the most consistent free radical scavenging capability across the five antioxidant assay. Hyperin and (+)-catechin have significantly lower IC_{50} (0.75±0.03 µg/ml and 0.94±0.27 µg/ml respectively) compared to SOD enzyme (IC50 1.59±04 µg/ml). Scopoletin exhibited potent antioxidant activity compared to scoparone with significantly lower EC_{50} values in ABTS (IC_{50} 1.08±0.03 µg/ml) compared to ascorbic acid (EC_{50} 1.54±0.03

µg/ml) and lower values in FRAP assay (FRAP value 49.00±0.64 µg/ml) than quercetin (FRAP value 86.00±0.24 µg/ml) and ascorbic acid (FRAP value 347.00±0.23 µg/ml). Vomifoliol had potent β-carotene bleaching activity with IC_{50} 6.85±0.37 µg/ml. Infections and free radical generation are recognized in the pathogenesis of cancer. The ethanol and chloroform extract of barks showed significant anticancer activities with GI_{50} values of 34.40µg/ml and 23.44µg/ml. The most susceptible cell lines were found to be the breast cancer cell line, MDA 468. Scopoletin displayed potent anticancer effect against breast cancer cell line MDA 468 (GI_{50} 0.09±0.25 µg/ml) and colorectal cancer cell line HT-29 (GI_{50} 0.17±0.05 µg/ml), the latter being more significant that positive control doxorubicin (GI_{50} 0.66±0.60 µg/ml). In recent years, roles have been identified for several inflammatory cells and for a large number of inflammatory mediators in important pathologies not previously linked to inflammation, such as Alzheimer's disease and cardiovascular disorders including atherosclerosis, as well as cancer. Recently, reports have appeared regarding so-called "dual inhibitors," agents that inhibit not only cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), but also 5-lipoxygenase (5-LOX). Chloroform extract of the barks had the lowest 5-LOX inhibition (IC_{50}=29.53±0.0 3µg/ml) when compared to NDGA (IC_{50}=29.19±0.02 µg/ml). Ethanol extract of leaves had superior COX-1 inhibition (IC_{50} = 0.60±0.01µg/ml) compared to COX-2 inhibition (IC_{50} = 1.07±0.01 µg/ml), whereas the barks had superior COX-2 inhibition (IC_{50} = 9.39±0.03 µg/ml) as opposed to COX-1 (IC_{50} = 11.41±0.03 µg/ml). All isolated compounds exhibited significantly lower 5-LOX inhibition than NDGA. Scopoletin and scoparone were potent inhibitors of 5-LOX recording lowest IC_{50} values (IC_{50} 0.34±0.01 µg/ml and 0.20±0.01 µg/ml respectively). However (+)-catechin had a more comprehensive anti-inflammatory activity with dual inhibition of

5-LOX (IC_{50} 16.10±0.03 µg/ml) and COX (COX-1; IC_{50} 12.08±0.02 µg/ml, COX-2; IC_{50} 83.89±0.03 µg/ml). Syringic acid exhibited potent 5-LOX inhibition (IC_{50} 1.38±0.03 µg/ml) and moderate COX-1 inhibition (IC_{50} 34.89±0.02 µg/ml). Furthermore, oxidative and inflammatory processes are among the pathological features associated with the central nervous system in Alzheimer's disease. There is evidence that acetyl cholinesterase (AChE) inhibitors have an anti-inflammatory role through action against free radicals and amyloid toxicity, as well as through decreasing release of cytokines from activated microglia in the brain and blood. Chloroform extract of the barks displayed the best activity (IC_{50} = 88.59±0.14 µg/ml) as opposed to positive control, galanthamine (IC_{50} = 0.74±0.06 µg/ml). The ethanol extract of barks and leaves follow through with IC_{50} = 186.00±0.15 µg/ml and IC_{50} = 201.24±0.15 µg/ml respectively. Only scopoletin, scoparone, vomifoliol and syringic acid showed AChE inhibition at IC_{50} <100 µg/ml. Syringic acid exhibited good AChE inhibition (IC_{50} 29.53±0.19 µg/ml), lowest of all compounds tested. Choline is the precursor of phosphatidylcholine (PC), a main component of *Leishmania* promastigote membranes. Therefore, inhibition of choline formation may decrease *Leishmania* survival. This hypothesis can be tested by using inhibitors of the acetylcholinesterase enzyme (AChE), which catalyzes the hydrolysis of acetylcholine to choline and acetic acid, as leishmanicidal compounds. The hexane extract of leaves showed moderate antileshmanial activity with IC_{50} values of 257.40±0.30 µg/ml. Only ethanol extracts showed activity against *Giardia intestinalis* and *Entamoeba histolytica* at concentration of 500 µg/ml. Scopoletin was tested against all three parasite amd it was more potent against *Leishmania donovani* (IC_{50} 163.30±0.32 µg/ml) and MIC of >200 µg/ml for both *Giardia intestinalis* and *Entamoeba histolytica*. Six kinds of major biological effects were evident in the crude and

compounds namely, antioxidant, antibacterial, anti-inflammatory, anti-AChE, anti-parasitic, and anticancer all of which were reported for the first time from this plant. Given the aforementioned evidence it is tempting to speculate that *Canarium patentinervium* Miq. represents an exciting scaffold from which to develop leads for treatment of inflammatory and oxidative stress related diseases.

PUBLICATIONS ARISING FROM THIS STUDY*

- **R. Mogana**, K. Teng-Jin, and C. Wiart, "In Vitro Antimicrobial, Antioxidant Activities and Phytochemical Analysis of *Canarium patentinervium* Miq. from Malaysia," Biotechnol. Res. Int., vol. 2011, p. 768673, 2011 **(abstract pg 377)**.
- **R. Mogana**, T. D. Bradshaw, T. J. Khoo, and C. Wiart, "In Vitro Antitumor potential of *Canarium patentinervium* Miq.," Acad. J. Cancer Res., vol. 4, no. 1, pp. 1–4, 2011 **(abstract pg 378)**.
- **R. Mogana** and C. Wiart, "*Canarium* L .: A Phytochemical and Pharmacological Review," J. Pharm. Res., vol. 4, no. 8, pp. 2482–2489, 2011 **(abstract pg 379)**.
- A Nematollahi , N Aminimoghadamfarouj , **M Rajagopal** , TJ Khoo, C.Wiart, "The first antibacterial activity report of three selected Malaysian rainforest medicinal plants," Planta Med, vol. 77–PL9, 2011 **(abstract pg 380)**.
- **R. Mogana** and C. Wiart, "Anti-Inflammatory, Anticholinesterase, and Antioxidant Potential of Scopoletin Isolated from *Canarium patentinervium* Miq. (Burseraceae Kunth)," Evidence-Based Complement. Altern. Med., vol. 2013, p. 734824, 7 pages, 2013 **(abstract pg 381)**.
- **R. Mogana**, K. Teng-Jin, and C. Wiart, "The Medicinal Timber *Canarium patentinervium* Miq. (Burseraceae Kunth.) Is an Anti-Inflammatory Bioresource of Dual Inhibitors of Cyclooxygenase (COX) and 5-Lipoxygenase (5-LOX)," ISRN Biotechnol., vol. 2013, pp. 1–8, 2013 **(abstract pg 382)**.

*Appendix C

CONFERENCE PROCEEDINGS AND TALKS RELATED TO THIS STUDY

- **Oral presentation** at the 25th Scientific Meeting of the Malaysian Society of Pharmacology & Physiology, 25th-26th of May 2011 at Faculty of Medicine & Medical Sciences, Universiti Putra Malaysia. – **"*Canarium*- a source of new antibiotics and antioxidants?"**
- **Poster presentation** at the 59th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research, 4 – 9th September 2011, Maritim Pine Beach Resort Hotel Antalya, Turkey. **"The first antibacterial activity report of three selected Malaysian rainforest medicinal plants"**
- **Poster presentation** at the International Conference on Natural Products, 13-16th November 2011, Palm Garden IOI Resort, Putrajaya, Malaysia. "***In vitro* antimicrobial, antioxidant, anticancer activities and phytochemical analysis of *Canarium patentinervium* Miq. From Malaysia "**
- **Oral presentation** at the International Conference on Natural Products, 4-6th March 2013, Shah Alam Convention Centre, Malaysia. "**Antiinflammatory, anticholinesterase and antioxidant activities of scopoletin isolated from *Canarium* sp. (Burseraceae Kunth.)"**
- **Oral presentation** at the Graduate School Talk, 27th June 2012, University of Nottingham (Malaysia Campus). **"*Canarium patentinervium* Miq.: The phytochemical and pharmacological study".**

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ABBREVIATIONS

^{1}H NMR- Proton nuclear magnetic resonance

^{13}C NMR- Carbon nuclear magnetic resonance

5-LOX- 5-lipoxygenase

5-HPETE- 5(*S*)- hydroxyperoxyeicosatetraenoic acid

5-*S*-HETE- 5-hydroxy- 6,8,11,1-eicosatetraenoic acid

µg- Microgram

µl- Microliter

µM- Micromolar

^{o}C- Celcius

AAE- Ascorbic acid equivalent

AChE- Acetylcholinesterase

ACh- Acetylcholine

AD- Alzheimer's disease

AIDS- Acquired immunodeficiency syndrome

amu- Atomic mass unit

AST- Antimicrobial susceptibility testing

ATCC- *American Type Culture Collection*

BChE- Butyrylcholinesterase

BDE- Bond dissociation energy

CAT- Choline acetyltransferase

CC- Column chromatrography

CCl_4- Carbon tetrachloride

cfu- Colony forming units

cm- Centimeter

CNS- central nervous system

COSY- correlation spectroscopy

COX-1- Cyclooxygenase-1

COX-2- Cyclooxygenase-2

COX-3- Cyclooxygenase-3

cPLA$_2$- Cytosolic phospholipase A_2

d- Doublet

dd- Doublet of doublet

D-GaIN- D-galactosamine

DMAPP- Dimethyl allyl diphosphate

DMSO- Dimethyl sulfoxide

DPPH- 1,1,-diphenyl-2-2-picrylhydrazyl

DTNB- 5,5´-Dithio-bis(2-nitrobenzoic) acid

EC$_{50}$- Effective concentration at 50 % activity

EDTA- Ethylene diamine tetra-acetic acid

FC- Folin-Ciocalteu

FLAP- 5-lipoxygenase activating protein

FRAP - Ferric reducing antioxidant power

g- Gram

GAE- Gallic acid equivalent

GC- Gas chromatography

GOT- Glutamic oxaloacetic transaminase

GPT- Glutamic pyruvic transaminase

HAT- Hydrogen atom transfer reaction

HMBC- heteronuclear multiple-bond correlation spectroscopy

HPLC- High performance liquid chromatography

hr- Hour

HO_2- Hydroperoxyl

IC_{50}- Inhibitory concentration at 50 % activity

iNOS- Inducible NO (nitric oxide) synthase

INT- *p*-iodonitrotetrazolium

IP- Ionization potential

IPP- Isopentyl diphosphate

IR- Infrared spectroscopy

i.p- Intra peritoneal

kg- Kilogram

L- Litre

lb- Pound

LOO•- Peroxy radical

LTA_4- Leukotriene A_4

LTB_4- Leukotriene B_4

M- Molar

m- Multiplet

mM- Milimolar

MBC- Minimum bactericidal assay

mg- Miligram

MHB- Mueller Hilton broth

MHA- Mueller Hilton agar

ml- Mililitre

MIC- Minimum inhibitory concentration

Min- Minutes

MS- Mass spectroscopy

MTT- Methylthiazol tetrazolium

NCE- New chemical entities

NDGA- Nordihydroguairetic acid

NCCLS- National Committee for Clinical Laboratory Standards

nm- Nanometer

NMR- Nuclear magnetic resonance

NSAID- Non-steroidal anti-inflammatory drugs

NO_2- Nitrogen dioxide

N_2O_3- Dinitrogen trioxide

O_2^- - Superoxide

ONOO- Peroxy nitrate

ORAC- Oxygen radical absorbance capacity

PC- Phospholipase C

PGs- Prostaglandins

PGG_2- Prostaglandin G_2

PGI_2- Prostacyclin

PLA_2- Phospholipase A_2

PNS- Peripheral nervous system

ppm- Parts per million

PTLC- Preparative thin layer chromatography

RA- Rheumatoid arthritis

ROS- Reactive oxygen species

RNS- Reactive nitrogen species

Rt- Retention time

ROO- Peroxyl

s- Singlet

SET- Single electron transfer

SD- Standard deviation

SOD- Superoxide dismutase

t- Triplet

TEAC- Trolox equivalent antioxidant capacity

TLC- Thin layer chromatography

TMPD- *N,N,N',N'*-tetramethyl-p-phenylenediamine

TPTZ- 2,4,6-tripyridyl-s-triazine

TSA- Tryptic soya agar

TSB- Tryptic soya broth

TxA_2- Thromboxane A_2

UV- Ultra-violet spectroscopy

WST-1- (2-(4-Iodophenyl)- 3-(4- nitrophenyl)-5-(2,4-disulfophenyl)- 2H-tetrazolium, monosodium salt)

XO- Xanthine oxidase

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Pharmacognosy is not a familiar term, even to many scientists which is defined as the study of crude drugs of plant and animal origin. The scope of pharmacognosy is also 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]. Natural products offer unmatched chemical diversity with structural complexity and biological potency. It is estimated about 100-fold higher hit rate for natural products over synthetic compounds. Natural products occupy different chemical space that is sometimes difficult to access compared with synthetic compounds. Not only do the natural product databases contain many more scaffolds, but an important proportion of the ring systems are not found at all in other drug databases. Such unexploited scaffolds represent promising new starting points in drug discovery.

Natural products compounds not only serve as drugs or templates for drugs but lead to a better understanding of targets and pathways involved in disease process. They also create opportunities for additional drug targets to be identified and exploited in these pathways. The elucidation of the anti-inflammatory mechanism of action of aspirin led to the discovery of the cyclooxygenase isozymes COX-1 and COX-2, which were used in the development of novel anti-inflammatory drugs [2]. Over half of the

world's top 25 best-selling pharmaceuticals drugs in 1991 owed their origin to natural products. 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].

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 [3]. Table 1.1 lists the drugs derived from plants, their clinical uses and sources as well as recent plant-derived drugs in the global market.

1.2 An 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 [4]. 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.

1.3 An introduction to the family Burseraceae Kunth. and genus *Canarium* L.

1.3.1 The family Burseraceae Kunth.

The Burseraceae Kunth is one of the nine flowering plant families belonging to the order Sapindales Juss. ex Bercht. & J. Pearl that comprise the monophyletic group (5700 species), whose first known fossils appear in Europe 65 million years ago (Ma). The Burseraceae Kunth. consists of approximately 18 genera and 700 species. This family is divided into 3 tribes namely Canariceae, Protieae which are determined to be monophyletic and Burserae which was shown to be polyphyletic. The list of genera in the Burseraceae Kunth., approximate number of species, tribal and subtribal in the Burseraceae Kunth., approximate number of species, tribal and subtribal placement, and geographic range of genera are shown in Table 1.2. The family is distributed pantropically across a broad range of low-elevation, frost-free habitats including rainforest, dry deciduous forest and deserts [5]. The Burseraceae Kunth. are best known for producing resins of economic, medicinal, and cultural values such as frankincense, myrrh and copal [6].

Table 1.1 : Drugs derived from plants, their clinical uses, sources and recent plant derived drugs in global market till 2004 [3][7].

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

1.3.2 The genus *Canarium* L.

The genus *Canarium* L. probably originated from the North American continent, not Gondwanaland [5]. This clade embraces 75 species of trees which are mainly found in tropical Asia and the Pacific, and a few species in tropical Africa [8], about 9 species were found in the Philippines [8]. The geographical centre of their genetic diversity is the Molucca Islands of eastern Indonesia, but their centre of cultivated diversity is undoubtedly western Melanesia. Twenty to 25 species are found in the South Pacific, of which 21 are in Papua New Guinea [9], eight in the Solomon Islands [10] and 3 or 4 in Vanuatu [11]. The known *Canarium* species and their distribution are listed in Table 1.3.

The word *Canarium L.* derives from the Malay name 'kanari'[8]. *Canarium* L. species often produce edible kernels, called canarium nut of commercial interest: *Canarium indicum* L.*, Canarium solomonense* B.L.Burtt, *Canarium harveyi* Seem, *Canarium odontophyllum* Miq. and *Canarium album* L. Another economical interest of *Canarium* L. species, is the production of resins used in foods (*Canarium luzonicum* Miq.), in the making of incense and varnishing. In spite of these commercial potentials of *Canarium* L.*,* little attention has been given to the collection and conservation of *Canarium* L. species [12]. The genetic diversity thus derogates at an alarming rate.

Table 1.2: List of genera in the Burseraceae Kunth., approximate number of species, tribal and subtribal placement, and geographic range [5].

Bursereae
- Burserinae
  - *Bursera* Jacq. ca. 100 spp. Caribbean, Mexico, Central, and S. America
  - *Commiphora* Jacq. ca. 190 spp. Africa, India, S. America
- Boswelliinae
  - *Aucoumea* Pierre 1 sp. W. Africa
  - *Beiselia* Forman 1sp. SW Mexico
  - *Boswellia* Roxb. ca. 30 spp. NE Africa, Arabia, India
  - *Triomma* Hook. f. 1sp. W Malesian region
  - Unnamed subtribe
  - *Garuga* Roxb. 4 spp. India, SE Asia

Canarieae
- *Canarium* L. ca. 105 spp. SE Asia, Malaysia, Africa
- *Dacryodes* Vahl 66 spp. Caribbean, Mexico, C. and S. America, SE Asia, Africa
- *Haplolobus* H.J. Lam 22 spp. E. Malaysia
- *Pseudodacryodes* R. Pierlot 1 sp. Central Africa
- *Rosselia* Forman 1 sp. Rossel Island, New Guinea
- *Santiria* Blume 24 spp. W. Malesian region, Philipines, Moluccas, New Guinea, Africa
- *Scutinanthe* Thwaites 2 spp. Sri Lanka, S. Myanmar, Celebes, Sumatra, Malay Peninsula,Borneo
- *Trattinnickia* Willd. 13 spp. C. and S. America

Protieae
- *Crepidospermum* Hook. f. 6 spp. S. America
- *Protium* Burm. f. 150 spp. Mexico, C. and S. America, Africa, SE Asia
- *Tetragastris* Gaertn. 9 spp. Central and S. America

Garuga was placed informally within the Bursereae by Harley and Daly (1995).

Table 1.3: *Canarium* L. species and their geographic distribution [13].

Species	Distribution
C. acutifolium (DC.) Merr.	Moluccas, New Guinea, Central Celebes
C. album (Lour.) Raeusch.	Annam, Tonkin, S. China, Hainan
C. apertum H.J. Lam	Sumatra, Malay Peninsula, Borneo
C. asperum Benth.	Solomon Is., Bawean and Kangean Is
subsp. *asperum* Lesser	Sunda Is., Borneo, Philippines,
subsp. *papuanum* (H.J. Lam)	Leenh. Celebes, Moluccas, New Guinea
C. australianum F.v.M	N. Australia, SE New Guinea
C. baileyanum Leenh.	Queensland, NS Wales
C. balansae Engl.	Loyalty Is.
C. balsamiferum Willd.	Celebes, Moluccas, New Guinea
C. luzonicum (Bl.) A. Gray	Philippines
C. macadamii Leenh.	New Guinea
C. madagascariense Engl.	Madagascar, Tanganyika,
subsp. *Madagascariense*	Mozambique
subsp. *Obtusifolium* (S.Elliot) Leenh.	
C. maluense Laut.	Central Celebes, Moluccas, New Guinea
subsp. maluense	
subsp. *borneense* Leenh.	
C. manii King	Middle and S. Andaman
C. megacarpum Leenh.	W. New Guinea
C. megalanthum Merr.	Sumatra, Malay Peninsula, Borneo
C. merrillii H. J. Lam	Borneo
C. muelleri F.M. Bailey	Australia (Queensland)
C. odontophyllum Miq.	Sumatra, Borneo, Philippines
C. oleiferum Baill.	New Caledonia
C. oleosum (Lamk) Engl.	New Britain, Lesser Sunda Island.,
	N. Celebes, Moluccas, New Guinea
C. ovatum Engl.	Philippines
C. paniculatum (Lamk) Benth. ex Engl.	Mauritius
C. parvum Leenh.	Tonkin, N. Annam
C. patentinervium Miq.	Sumatra, Malay Peninsula, Banka,
	Anambas Island., Borneo
C. perlisanum Leenh.	Malay Peninsula
C. piloso-sylvestre Leenh.	W. New Guinea
C. pilosum Benn.	Sumatra, Malay Peninsula, Borneo
subsp. *pilosum*	
subsp. *borneensis* Leenh.	
C. pimela Leenh.	S. China, Hainan, Tonkin, Laos,
	Annam, Cambodia
C. polyphyllum K. Sch.	New Guinea
C. pseudodecumanum Hochr.	Sumatra, Malay Peninsula, Borneo
C. pseudopatentinervium	S. Sumatra, Banka, Borneo
H.J. Lam	
C. pseudosumatranum Leenh.	Malay Peninsula
C. rigidum (Bl.) Zipp. ex Miq.	New Guinea
C. salomonense B.L. Burtt	Solomon Island., E. New Guinea
subsp. *salomonense*	
subsp. *papuanum*	
C. samoense Engl.	Samoa
C. schlecteri Laut.	New Britain, E. New Guinea

Table 1.3: *Canarium* L. species and their geographic distribution [13]. (continuation)

Species	Distribution
C. schweinfurthii Engl.	Trop. W and Central Africa
C. smithii Leenh.	Fiji
C. strictum Roxb.	SW Deccan, Sikkim, Assam, Upper Burm
C. subulatum Guill.	S. China, Hainan, Tonkin, Laos, Annam, Cambodia
C. sumatranum Boerl. and Koord.	Sumatra, Malay Peninsula
C. sylvestre Gaertn.	Moluccas, New Guinea
C. trifoliatum Engl.	New Caledonia
C. trigonum H.J. Lam	Central Celebes
C. vanikoroense Leenh.	New Hebrides, Fiji
C. vitiense A. Gray	Fiji
C. vrieseanum Engl.	Philippines, Central and N. Celebes
C. vulgare Leenh.	Kangean and Bawean Island., Lesser Sunda Island., Celebes, Moluccas
C. whitei Guill.	New Caledonia
C. zeylanicum (Retz.) Bl.	Sri Lanka

1.3.3. Botanical features of *Canarium* L.

The members of the genus *Canarium* L. consist of medium to large buttressed trees up to 40-50m tall, or rarely a shrub. The barks are greenish grey, fawn or light yellow brown that are usually smooth, scaly or dippled with many small lenticels. Outer bark are thin while the inner barks are pinkish brown or reddish brown, laminated, soft and aromatic with a clear sticky or rarely oily exudate. The stems are usually terete. The leaves are pinnate, spiral and stipulated. The rachis is terete flattened to channeled swollen at base, and bears 5-21 folioles. The folioles are oblique at base, entire, dentate or serrate at margin, often thick and acuminiate at apex. The secondary nerves are arching and joined near margin. The tertiary nerves are reticulate. The infloresence is an axillary or terminal panicle. The calyx is cupular. The corolla includes 3 creamy petals. The androecium comprises a whorl of 6 stamens. The disc within the stamens is 6 lobed. The gynaecium consist of 3 carpels united into a 3

lobular ovary. The drupes are seated on a persistent enlarged calyx and enclose a woody stone [14].

1.4 Traditional and medicinal uses of *Canarium* L.

Elemi (British Pharmaceutical Codex, 1934) is an oleoresin exuded through the bark of *Canarium luzonicum* Miq. or *Canarium commune* L. which has been used in the form of an ointment as a stomach stimulant and as an expectorant [15]. The barks of *Canarium indicum* L. has been used for chest pains where else the oil has been patented for treatment of arthritis pain and the oleoresin of the tree is applied as a poultice for ulcerated wounds. The resin of *Canarium tonkinense* Engl. has been used as a stimulant, rubefacient and anti-rheumatic when applied externally. The oleoresin has been applied as ointment for ulcers [16]. The dried fruit of chinese olive or *Canarium album* (Lour.) Raeusch. is used in China and used to treat bacterial and viral infections, inflammation, poisoning and for detoxification [17].

In Chinese folk medicine, the dried fruits of *Canarium album* (Lour.) Raeusch have been used for treatment of angina, dysentery, snake bites, cough-hematemesis, enteritis, diarrhoea, toxicosis from swellfish and alcohol [18]. *Canarium schweinfurthii* Engl. is used by traditional healers as a remedy for diabetes mellitus in southern Senegal [19] while in Congo and Central African Republic the plant is used in fever, as stimulant, emollient, in post-partum pain, constipation, malaria, diarrhoea, sexual infections and rheumatism [20]. In Indonesia, the bark of *Canarium littorale* Bl. is used to make a decoction taken to heal haemorrhoids [21].

1.4.1 *In vitro* and *in vivo* pharmacological activities of *Canarium* L.

Only 12 % of 75 species have been studied for their pharmacological activities. Extracts and pure compounds derived from *Canarium* L. were reported to have a variety of pharmacological activities of which antioxidant, antibacterial, antifungal, antitumor, anti-inflammatory, hepatoprotective, analgesic and anti-diabetic (Table 1.4).

i. Antioxidant

Antioxidant activities were reported in *Canarium album* (Lour.) Raeusch, *Canarium odontophyllum* Miq. and *Canarium schweinfurthii* Engl. A tonic soup made of *Canarium album* (Lour.) Raeusch used mainly in China displayed significant antioxidant activity by 1,1,-diphenyl-2-2-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assay [22]. Tannins extracted from the leaves, twigs and stem barks of *Canarium album* (Lour.) Raeusch showed potent antioxidant activity in the DPPH radical scavenging activity with IC_{50} values of 56.86 µg/ml, 62.31 µg/ml and 54.80 µg/ml respectively and ferric reducing power of 4.28, 3.74 and 4.49 mmol AAE/g equivalent of dried tannin [23]. Pure compounds from this species includes brevifolin, hyperin and ellagic acid which showed free radical scavenging activity in DPPH assay [24].

The essential oil of *Canarium schweinfurthii* Engl. was tested for the antioxidant activity with the DPPH assay and by β-carotene bleaching test. It exhibited highest antioxidant activity at 150 µg/ml activity in both assay [25]. The ethyl acetate fraction

of peel of *Canarium odontophyllum* Miq. exhibited 95±1.00% scavenging activity at the concentration of 40 µg/ml [26][27].

ii. Antibacterial and antifungal activities

Antibacterial activities were reported in *Canarium schweinfurthii* Engl. and *Canarium patentinervium* Miq. Dichloromethane extract of *Canarium schweinfurthii* Engl. had bactericidal activity against Gram-negative *Vibrio cholerae* with minimum inhibitory concentration (MIC) of 0.62 mg/ml while the ethylacetate extract was active against Gram-positive and Gram-negative bacteria namely *Staphylococcus aureus* and *Proteus vulgaris* with MIC values of 10 mg/ml and 5 mg/ml respectively. Ethanol extract was active against Gram-negative *Vibrio cholerae* and *Proteus vulgaris* with MIC values of 0.62 mg/ml and 10 mg/ml respectively [28]. In a separate disc diffusion assay, the essential oil of *Canarium schweinfurthii* Engl. abrogated the survival of Gram-negative *Salmonella enterica*, Gram-positive *Streptococcus pyogens* and *Staphylococcus aureus* with an inhibition zone of 27 mm, 25 mm and 18 mm respectively. However the author did not inform on the concentration of the extract per disc. The oil was also fungicidal against *Candida albicans* with an inhibition zone of 23 mm [25].

iii. Hepatoprotective activities

Hepatoprotective activity was exhibited in *Canarium manii* King. and *Canarium album* (Lour.) Raeusch. The biflavanoid agathisflavone from *Canarium manii* King. preserved the integrity of the liver cells membrane of rodents as evidenced by the

decrease in the CCl_4-induced rise of glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) levels. GPT which is predominantly found in the liver showed a dose-dependent and significant reduction [29]. The triterpenes urs-12-ene-3α,16β-diol, olean-12-ene-3α,16β-diol and urs-12-ene-3β,16β-diol from from *Canarium album* (Lour.) Raeusch markedly reduced the amount of alanine aminotransferase leakage from the primary cultured hepatocytes intoxicated with 0.2 mM of D-galactosamine (D-GaIN) [30]. The phenols brevifolin and ellagic acid protected rat hepatocytes against D-GaIN-induced insults [24].

iv. Other Biological Activities

The essential oil of *Canarium schweinfurthii* Engl. collected from the region of Cameroon inhibited the enzymatic activity of lipoxygenase with an IC_{50} value of 62.6 ppm [31]. However in a separate study of the same species collected from central African region did not show any activity in the cotton pellet induced granuloma formation experiment [20]. This may suggest possible evidence of variety between secondary metabolites constituents according to regions. The essential oil of *Canarium schweinfurthii* Engl. at a dose of 1, 2 and 3 mg/kg i.p. displayed potent analgesic effect in the acetic acid-induced writhing and hot plate experiments [20].

Anti-diabetic activity was reported in the methanol/methylene chloride extract of stem bark of *Canarium schweinfurthii* Engl. At a dose of 300 mg/kg there was 67.1 % reduction in blood glucose levels after a once daily subcutaneous injection on streptozotocin-induced diabetic male rats over 14 days, versus insulin that had 76.8 % reduction. Weight gain was only 6.6 % as opposed to untreated rats that had lost 14.1

% of body weight. There was also significant reduction in food and fluid consumption by 68.5 % and 79.7 %. These results showed the extract could reverse hyperglycemia, polyphagia and polydipsia provoked by streptozotocin, thus having anti-diabetic activity [19].

1.5 The phytochemistry of *Canarium* L.

Isolation and structure elucidations of secondary metabolites in *Canarium* L. has been carried out since the 50's [32]. Majority of investigations include the resin and the fruit of the species. To date about 96 compounds have been isolated from 9 species, which are *Canarium schweinfurthii* Engl., *Canarium boivinii* Engl., *Canarium odontophllum* Miq., *Canarium manii* King., *Canarium album* (Lour) Raeusch, *Canarium zeylanicum* (Retz.) Blume, *Canarium commune* L., *Canarium muelleri* F.M. Bailey and *Canarium bengalense* Roxb. The extensively researched species are *Canarium schweinfurthii* Engl. *and Canarium album* (Lour.) Raeusch [17][18][23][33][34].

Table 1.4: Biological and pharmacological activities (*in vitro*) of *Canarium* L. extracts and pure constituents

Extract/Compound	Species	Pharmacological activity	References
Polymeric procyanidins(tannins) from leaves, twigs and stem bark	*Canarium album* (Lour.) Raeusch	Significant DPPH radical scavenging activity, Ferric reducing antioxidant activity	[23]
Ethyl acetate fraction of the fruit peel	*Canarium odontophyllum* Miq.	Antioxidant activity with DPPH assay, FRAP assay and hemoglobin oxidation assay	[26]
Carotenoids from peel,pulp and seed extracts	*Canarium odontophyllum* Miq.	Significant antioxidant activity with beta-carotene bleaching assay, ABTS assay, DPPH assay and hemoglobin oxidation assay	[27]
Essential oil of resins	*Canarium schweinfurthii* Engl.	Bactericidal for *Enterococcus faecalis*, *Listeria innocua, Salmonella enterica, Staphylococcus aureus, Staphylococcus camorum* Fungicidal for *Candida albicans* Antioxidant for DPPH and β-carotene bleaching test	[25]
Essential oil of resins(monoterpenes hydrocarbon)	*Canarium schweinfurthii* Engl.	Significant anti-inflammatory activity via lipooxygenase method with IC_{50} of 62.6 ppm	[31]
Brevifolin, ellagic acid and hyperin	*Canarium album* (Lour.) Raeusch	Significant antioxidant activity and inhibitory effect on lipid peroxidation assay	[24]
Dichloromethane extract of barks	*Canarium schweinfurthii* Engl.	Antimicrobial activity against *V.cholerae*	[28]
Extract of whole plant	*Canarium album* (Lour.) Raeusch	Antioxidant activities in DPPH and FRAP assay	[22]

Table 1.5: Biological and pharmacological activities (*in vivo*) of *Canarium* L. extracts and pure constituents

Extract/Compound	Species	Pharmacological activity	References
Agathisflavone (biflavanoid)	*Canarium manii* King.	Hepatoprotective activity against experimentally-induced carbon tetrachloride-hepatotoxicity in rats and mice	[29]
urs-12-ene-3α, 16β-diol, olean-12-ene-3α, 16β diol (triterpene)	*Canarium album* (Lour.) Raeusch	Hepatoprotective activity in primary cultured rat hepatocytes intoxicated with D-galactosamine	[30]
brevifolin, ellagic acid	*Canarium album* (Lour.) Raeusch	Reduction of carbon tetrachloride induced liver damage in mice. Reduction in elevated GPT and GOT levels after intraperitoneal administration	[24]
Essential oil of resins(composed mainly of nerolidol and octylacetate)	*Canarium schweinfurthii* Engl.	Significant analgesic effect using acetic acid-induced writhing and hot plate methods with swiss mice	[20]
Methanol/methylene chloride extract of stem barks	*Canarium schweinfurthii* Engl.	Anti-diabetic activity that reverses hyperglycemia, polyphagia and polydipsia in streptozotocin-induced diabetic rats. Significant reduction 69.9 % reduction in blood glucose level after 14 days at 300 mg/kg	[19]

The isolation and separation technique is very much dependent on the type of fractions. Essential oils are analysed with gas chromatography (GC) and mass spectroscopy (MS) [20][31][35]. Other substances are separated with liquid chromatography using different solvent mixtures with silica gel [29][36], charcoal [37], sephadex [18] and multiple column packing such as AB-8 adsorption resin, polyamide, and TSK Toyopearl HW-40(S). Other types of analytical techniques include thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) [17][27][34][38][39].

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 [29]. Among the secondary metabolites isolated from members of the genus *Canarium* L. are terpenes (monoterpenes, triterpenes, tetraterpenes like carotenoids, sesquiterpenes, cyclohexane and sterols), carboxylic acids, coumarins, furans, lipids and phenols (flavonoids, tannins, phenolic acids). The main secondary .metabolites isolated so far from the genus *Canarium* L. consists of terpenes with 58 compounds and flavonoids with 11 compounds. The profile of all known secondary metabolites of *Canarium* L. as found in literature are included in Table 1.6 and their structures are included in Figure 1.1.

1.6 The commercial uses of *Canarium* L. produces

Elemi derived from the Arabic 'Al-lami' is a collective term applied to several oleoresins obtained from different plants of the family Burseraceae Kunth. The most important and widely known of these oleoresins is Manila Gum Elemi which exudates from the trunk of *Canarium luzonicum* Miq. or *Canarium commune* L. The natural constituent of elemi oil is elemicin. Variety of foodstuffs are flavoured with elemi oil and in Europe its used in spices and seasonings. In US elemi oil is also used in fragrances to approximately 1000 lb/year [40]. At present, at least 4 species of *Canarium* L. nuts are of economic importance. *Canarium ovatum* Engl. (known locally as 'pili' and 'pilaui') is the most important nut-producing species in the Philippines. *Canarium luzonicum* Miq. most commonly known in the Philippines as 'pisa' and 'basiad', is important, not as an edible nut but for its oily resin (known locally as 'sahing'), which is tapped from the trunk. When processed, it is called 'brea blanca'(white pitch) and is exported as Manila elemi *Canarium indicum* L. is an important nut-producing species in the Solomon Islands (locally called 'ngali'), Papua New Guinea (locally called 'galip') and Vanuatu (where it is known as 'nangai') [10]. *Canarium album* (Lour) Raeusch., known in English as Chinese olive, 'samo cheen' in Thailand and 'tram trang' in Vietnam, is important in these countries for its edible pulp and kernel [13].

Table 1.6 Secondary metabolites of *Canarium* L.

Compound name	Species	Chemical formula	Structure number	Plant part	References
CARBOXYLIC ACIDS					
Octyl acetate	*Canarium schweinfurthii* Engl.	$C_{10}H_{20}O_2$	1	resin,oil	[20]
2,5-Dimethoxytoluene	*Canarium schweinfurthii* Engl.	$C_9H_{12}O_2$	2	resin,oil	[20]
COUMARINS					
Scoparone	*Canarium album* (Lour.) Raeusch	$C_{11}H_{10}O_4$	3	fruit	[41]
Scopoletin	*Canarium album* (Lour.) Raeusch	$C_{10}H_8O_4$	4	fruit	[41]
HETEROCYCLIC COMPOUNDS-FURANS					
2-acetylfuran	*Canarium schweinfurthii* Engl.	$C_6H_6O_2$	5	resin,oil	[20]
n-octanol	*Canarium schweinfurthii* Engl.	$C_8H_{18}O$	6	resin,oil	[20]
LIPIDS					
Hexadecanoic acid	*Canarium schweinfurthii* Engl.	$C_{16}H_{32}O_2$	7	fruit,oil	[42]
9-octadecenoic acid	*Canarium schweinfurthii* Engl.	$C_{18}H_{34}O_2$	8	fruit,oil	[42]
6,9-octadecadienoic acid	*Canarium schweinfurthii* Engl.	$C_{18}H_{32}O_2$	9	fruit,oil	[42]
9,12,15-octadecatrienoic acid	*Canarium schweinfurthii* Engl.	$C_{18}H_{32}O_4$	10	fruit,oil	[42]
Oleic acid	*Canarium schweinfurthii* Engl.	$C_{18}H_{34}O_2$	11	fruit,oil	[43]
Linoleic acid	*Canarium schweinfurthii* Engl.	$C_{18}H_{32}O_2$	12	fruit,oil	[43]
n-decanol	*Canarium schweinfurthii* Engl.	$C_{10}H_{22}O$	13	resin, oil	[20]
n-dodecanol	*Canarium schweinfurthii* Engl.	$C_{12}H_{26}O$	14	resin,oil	[20]
PHENOLS-FLAVONOIDS					
Luteolin	*Canarium album* (Lour.) Raeusch	$C_{15}H_{10}O_6$	15	fruit	[18]
Luteolin-7-*O*-β-D-glucoside	*Canarium album* (Lour.) Raeusch	$C_{21}H_{20}O_{11}$	16	fruit	[18]
Quercetin	*Canarium album*	$C_{15}H_{10}O_7$	17	fruit	[18]

Table 1.6 Secondary metabolites of *Canarium* L. (continuation)

Compound name	Species	Chemical formula	Structure number	Plant part	References
Quercetin-3-*O*-β-D-glucoside	*Canarium album* (Lour.) Raeusch	$C_{21}H_{19}O_{12}$	18	fruit	[18]
Kaempferol	*Canarium album* (Lour.) Raeusch	$C_{15}H_{10}O_6$	19	fruit	[18]
7,8,3',4'-tetrahydroxyflavanone	*Canarium album* (Lour.) Raeusch	$C_{15}H_{10}O_6$	20	fruit	[18]
3,5,7,3'-tetrahydroxy-4'-methoxyflavanonol	*Canarium album* (Lour.) Raeusch	$C_{15}H_{10}O_6$	21	fruit	[18]
Hyperin/ Quercetin-3-galactoside	*Canarium album* (Lour.) Raeusch	$C_{21}H_{20}O_{12}$	22	dried stem, leaf,fruit	[24] [17]
Kaempferol-3-glucoside	*Canarium album* (Lour.) Raeusch	$C_{21}H_{20}O_{11}$	23	fruit	[17]
Amentoflavone	*Canarium album* (Lour.) Raeusch	$C_{30}H_{18}O_{10}$	24	fruit	[17]
Agathisflavone	*Canarium manii* King.	$C_{30}H_{18}O_{10}$	25	stem,bark	[29]
PHENOLIC ACIDS					
Sinapic acid	*Canarium album* (Lour.) Raeusch	$C_{11}H_{12}O_5$	26	fruit	[34]
Corilagin	*Canarium album* (Lour.) Raeusch	$C_{27}H_{22}O_{18}$	27	fruit	[41]
Gallic acid	*Canarium album* (Lour.) Raeusch	$C_7H_6O_5$	28	fruit	[17]
Ellagic acid	*Canarium album* (Lour.) Raeusch	$C_{14}H_6O_8$	29	dried stem,leaf	[24]
Brevifolin carboxylic acid	*Canarium album* (Lour.) Raeusch	$C_{13}H_8O_8$	30	fruit	[34]
3-O-galloyl quinic acid butyl ester	*Canarium album* (Lour.) Raeusch	$C_{18}H_{24}O_{10}$	31	fruit	[33]
3,4-dihydroxybenzoic acid ethyl ether	*Canarium album* (Lour.) Raeusch	$C_9H_{10}O_4$	32	fruit	[18]
2-hydroxybenzoic acid	*Canarium album* (Lour.) Raeusch	$C_7H_6O_3$	33	fruit	[18]
TANNINS					
Ethyl gallate	*Canarium album* (Lour.) Raeusch	$C_9H_{10}O_5$	34	fruit	[18] [17]
Methyl gallate	*Canarium album* (Lour.) Raeusch	$C_8H_8O_5$	35	fruit	[17]
Elemicin	*Canarium commune* L.	$C_{12}H_{16}O_3$	36	fruit	[40]

Table 1.6 Secondary metabolites of *Canarium* L. (continuation)

Compound name	Species	Chemical formula	Structure number	Plant part	References
SAPONINS-					
HYDROXY ACIDS					
Elemadienonic acid	*Canarium boivinii* Engl.	$C_{30}H_{46}O_3$	37	resin	[44]
	Canarium schweinfurthii Engl.	$C_{30}H_{46}O_3$	37	resin	[32]
TERPENES-					
CYCLOHEXANE					
Limonene	*Canarium schweinfurthii* Engl.	$C_9H_{12}O_3$	38	resin,oil	[20]
	Canarium boivinii Engl.			resin	[36]
	Canarium zeylanicum (Retz.) Blume .			oleoresin	[44]
Furfuryl butanoate	*Canarium schweinfurthii* Engl.	$C_9H_{12}O_3$	39	resin, oil	[20]
MONOTERPENES					
α-pinene	*Canarium album* (Lour.) Raeusch	$C_{10}H_{16}$	40	resin	[35]
β-Pinene	*Canarium album* (Lour.) Raeusch	$C_{10}H_{16}$	41	resin	[35]
Myrcene	*Canarium album* (Lour.) Raeusch	$C_{10}H_{16}$	42	resin	[35]
α-Fenchene	*Canarium album* (Lour.) Raeusch	$C_{10}H_{16}$	43	resin	[35]
p-1-Menthene	*Canarium album* (Lour.) Raeusch	$C_{10}H_{18}$	44	resin	[35]
Δ-3-Carene	*Canarium album* (Lour.) Raeusch	$C_{10}H_{16}$	45	resin	[35]
α-Terpinene	*Canarium album* (Lour.) Raeusch	$C_{10}H_{16}$	46	resin	[35]
cis-Sabinene hydrate	*Canarium album* (Lour.) Raeusch	$C_{10}H_{18}O$	47	resin	[35]
Terpinolene	*Canarium album* (Lour.) Raeusch	$C_{10}H_{16}$	48	resin	[35]
Linalool	*Canarium album* (Lour.) Raeusch	$C_{10}H_{18}O$	49	resin	[35]
	Canarium schweinfurthii Engl.			resin,oil	[20]

Table 1.6 Secondary metabolites of *Canarium* L. (continuation)

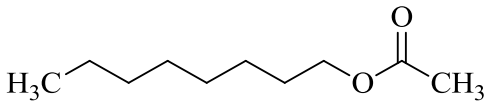
Compound name	Species	Chemical formula	Structure number	Plant part	References
cis-p-Menth-2-en-1-ol	*Canarium album* (Lour.) Raeusch	$C_{10}H_{18}O$	50	resin	[35]
trans-p-Menth-2-en-1-ol	*Canarium album* (Lour.) Raeusch	$C_{10}H_{18}O$	51	resin	[35]
Terpinen-4-ol	*Canarium album* (Lour.) Raeusch	$C_{10}H_{18}O$	52	resin	[35]
α-Terpineol	*Canarium album* (Lour.) Raeusch	$C_{10}H_{18}O$	53	resin	[35]
	Canarium schweinfurthii Engl.			resin,oil	[20]
cis-Piperitone	*Canarium album* (Lour.) Raeusch	$C_{10}H_{16}O_2$	54	resin	[35]
Isobornyl acetate	*Canarium album* (Lour.) Raeusch	$C_{12}H_{20}O_2$	55	resin	[35]
1,8-cineole	*Canarium schweinfurthii* Engl.	$C_{10}H_{18}O$	56	reisn,oil	[20]
Citronellyl acetate	*Canarium schweinfurthii* Engl.	$C_{12}H_{22}O_2$	57	resin,oil	[20]
Neryl acetate	*Canarium schweinfurthii* Engl.	$C_{12}H_{20}O_2$	58	resin,oil	[20]
Decyl Acetate	*Canarium schweinfurthii* Engl.	$C_{12}H_{24}O_2$	59	resin,oil	[20]
a-pinene/ α-pinene	*Canarium boivinii* Engl.	$C_{10}H_{16}$	40	Resin	[36]
	Canarium zeylanicum (Retz.) Blume			Oleoresin, timber	[44]
Carvone	*Canarium zeylanicum* (Retz.) Blume	$C_{10}H_{14}O$	60	oleoresin	[36]
α-phellandrene	*Canarium zeylanicum* (Retz.) Blume	$C_{10}H_{16}$	61	oleoresin, bark,timber	[36]
β-phellandrene	*Canarium zeylanicum* (Retz.) Blume	$C_{10}H_{16}$	62	oleoresin, bark,timber	[36]
Terpineol	*Canarium zeylanicum* (Retz.) Blume	$C_{10}H_{18}O$	63	oleoresin, bark	[36]
TRITERPENES					
α-amyrin/ a-amyrin/ (urs-12-en-3β-ol)	*Canarium album* (Lour.) Raeusch	$C_{30}H_{50}O$	64	dried stem, leaf	[30]
	Canarium boivinii Engl.			Resin, oleoresin,	[36]
	Canarium zeylanicum (Retz.) Blume			bark,timber	[44]

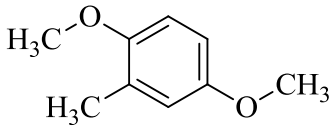
Table 1.6 Secondary metabolites of *Canarium* L. (continuation)

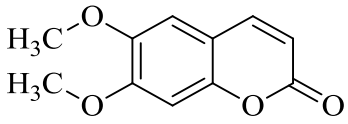
Compound name	Species	Chemical formula	Structure number	Plant part	References
β-amyrin(olean-12-en-3β-ol)	*Canarium album* (Lour.) Raeusch	$C_{30}H_{50}O$	65	dried stem, leaf	[30]
	Canarium boivinii Engl.			Resin	[36]
	Canarium zeylanicum (Retz.) Blume			oleoresin, bark,timber	[44]
3-epi-α-amyrin	*Canarium album* (Lour.) Raeusch	$C_{30}H_{50}O$	66	dried stem, leaf	[30]
3-epi-β-amyrin	*Canarium album* (Lour.) Raeusch	$C_{30}H_{50}O$	67	dried stem, leaf	[30]
α-Amyrenone (urs-12-en-3-one)	*Canarium zeylanicum* (Retz.) Blume	$C_{30}H_{48}O$	68	oleoresin	[36]
β-Amyrenone (olean-12-en-3-one)	*Canarium zeylanicum* (Retz.) Blume	$C_{30}H_{48}O$	69	oleoresin	[36]
Taraxerol	*Canarium zeylanicum* (Retz.) Blume	$C_{30}H_{50}O$	70	bark	[36]
urs-12-ene-3α, 16β-diol	*Canarium album* (Lour.) Raeusch	$C_{30}H_{50}O_2$	71	dried stem,leaf	[30]
olean-12-ene-3α, 16β diol	*Canarium album* (Lour.) Raeusch	$C_{30}H_{50}O_2$	72	dried stem, leaf	[30]
TETRATERPENES CAROTENOIDS					
All-trans-lutein	*Canarium odontophyllum* Miq.	$C_{40}H_{56}O_2$	73	peel,pulp, seed	[26]
9-cis-lutein	*Canarium odontophyllum* Miq.	$C_{40}H_{56}O_2$	74	peel,pulp, seed	[26]
13-cis-lutein	*Canarium odontophyllum* Miq.	$C_{40}H_{56}O_2$	75	peel,pulp, seed	[26]
15-cis-β-carotene	*Canarium odontophyllum* Miq.	$C_{40}H_{56}$	76	peel,pulp, seed	[26]
9-cis-β-carotene	*Canarium odontophyllum* Miq.	$C_{40}H_{56}$	77	peel,pulp, seed	[26]
All-trans-β-carotene	*Canarium odontophyllum* Miq.	$C_{40}H_{56}$	78	peel,pulp, seed	[26]
SESQUITERPENES					
α-Cubenene	*Canarium album* (Lour.) Raeusch	$C_{15}H_{24}$	79	resin	[35]
α-Copaene	*Canarium album* (Lour.) Raeusch	$C_{15}H_{24}$	80	resin	[35]

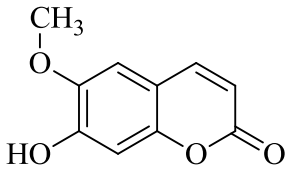
Table 1.6 Secondary metabolites of *Canarium* L. (continuation)

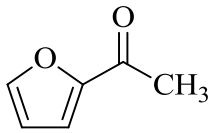
Compound name	Species	Chemical formula	Structure number	Plant part	References
β-Cubebene	*Canarium album* (Lour.) Raeusch	$C_{15}H_{24}$	81	resin	[35]
Canaric acid	*Canarium muelleri* F.M. Bailey	$C_{30}H_{48}O_2$	82	oleoresin	[36]
	Canarium zeylanicum (Retz.) Blume				[37]
(E)-β-Caryophyllene	*Canarium album* (Lour.) Raeusch	$C_{15}H_{24}$	83	resin	[35]
α-Humelene	*Canarium album* (Lour.) Raeusch	$C_{15}H_{24}$	84	resin	[35]
Germacrene D	*Canarium album* (Lour.) Raeusch	$C_{15}H_{24}$	85	resin	[35]
Spathulenol	*Canarium album* (Lour.) Raeusch	$C_{15}H_{24}O$	86	Resin	[20]
	Canarium schweinfurthii Engl.			resin,oil	[35]
Viridiflorol	*Canarium schweinfurthii* Engl.	$C_{15}H_{26}O$	87	resin, oil	[20]
Caryophyllene epoxide	*Canarium album* (Lour.) Raeusch	$C_{15}H_{24}O$	88	resin	[35]
(E)- Nerolidol	*Canarium schweinfurthii* Engl.	$C_{15}H_{26}O$	89	resin,oil	[20]
Elemene	*Canarium zeylanicum* (Retz.) Blume	$C_{15}H_{24}$	90	oleoresin, bark	[36]
Elemol	*Canarium zeylanicum* (Retz.) Blume	$C_{15}H_{26}O$	91	oleoresin, bark	[36]
Brevifolin	*Canarium album* (Lour.) Raeusch	$C_{10}H_{12}O_4$	92	dried stem, leaf	[24]
δ-Elemene	*Canarium album* (Lour.) Raeusch	$C_{15}H_{24}$	93	resin	[35]
STEROLS					
Sitosterol	*Canarium zeylanicum* (Retz.) Blume	$C_{29}H_{50}O$	94	oleoresin, bark,timber	[36]
β-sitosterol	*Canarium benglense* Roxb.	$C_{29}H_{50}O$	95	bark	[45]
Neoilexonol (3β-Hydroxyurs-12-en-11-one)	*Canarium zeylanicum* (Retz.) Blume	$C_{30}H_{48}O_2$	96	oleoresin, bark	[36]

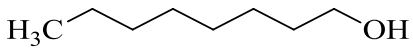
1. 

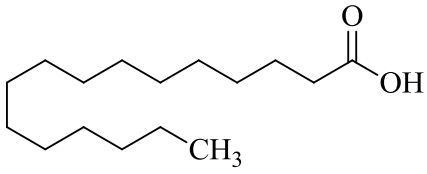
2. 

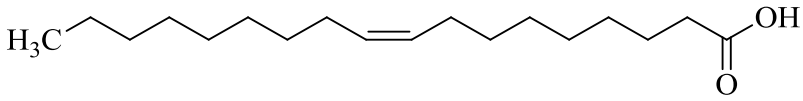
3. 

4. 

5. 

6. 

7. 

8. 

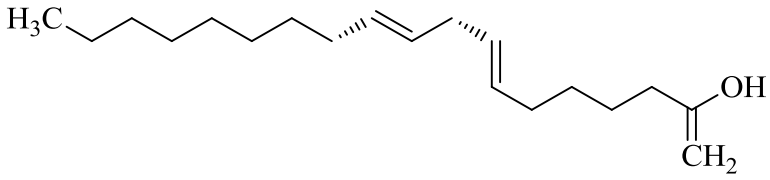
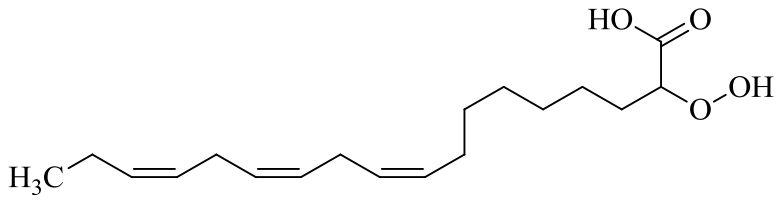
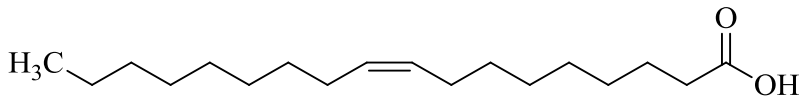
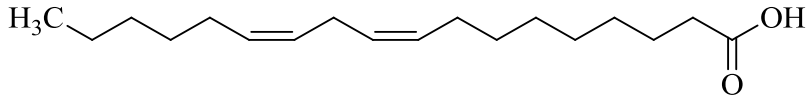
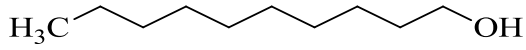
9. 

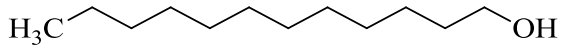
Figure 1.1: Chemical structures of secondary metabolites from *Canarium* L.

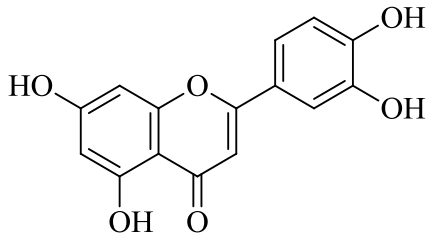
10. 

11. 

12. 

13. 

14. 

15. 

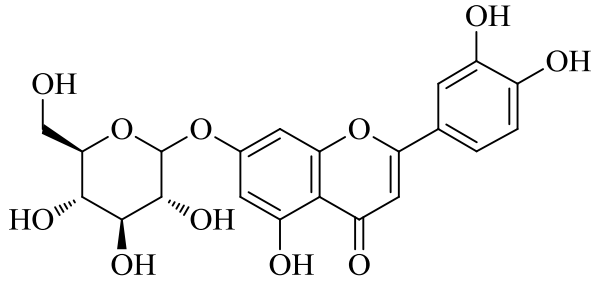
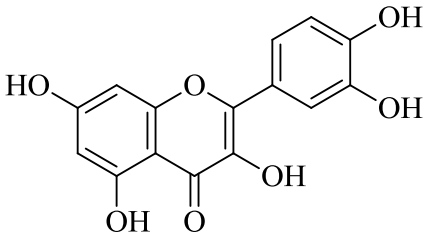
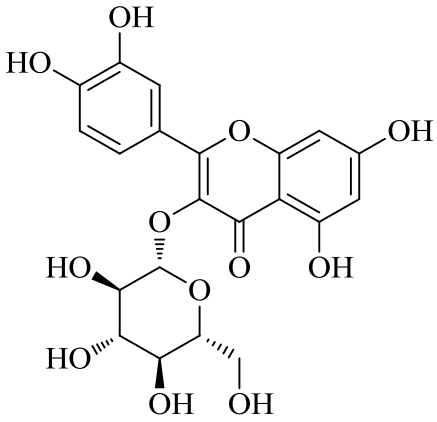
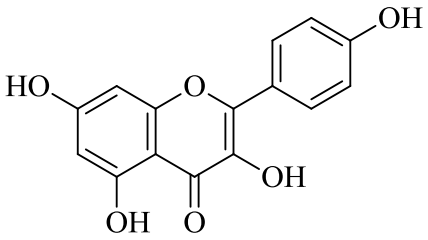
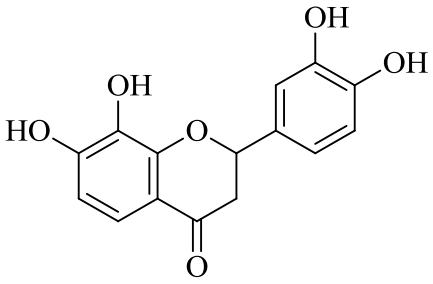
16. 

Figure 1.1: Chemical structures of secondary metabolites from *Canarium* L.

17. 

18. 

19. 

20. 

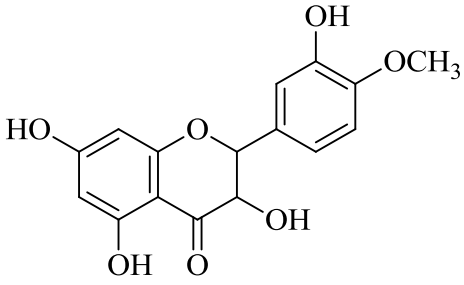
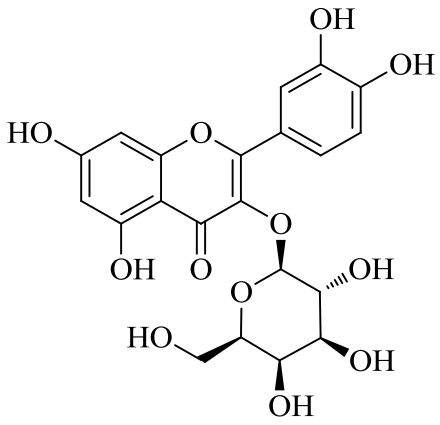
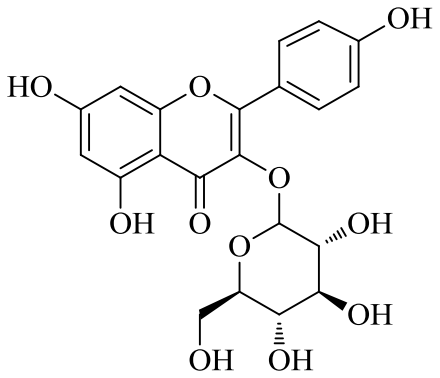
21. 

Figure 1.1: Chemical structures of secondary metabolites from *Canarium* L.

22. 

23. 

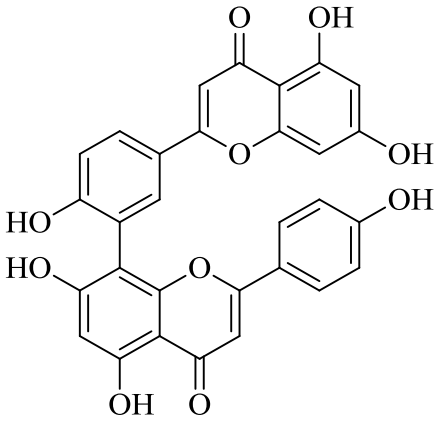
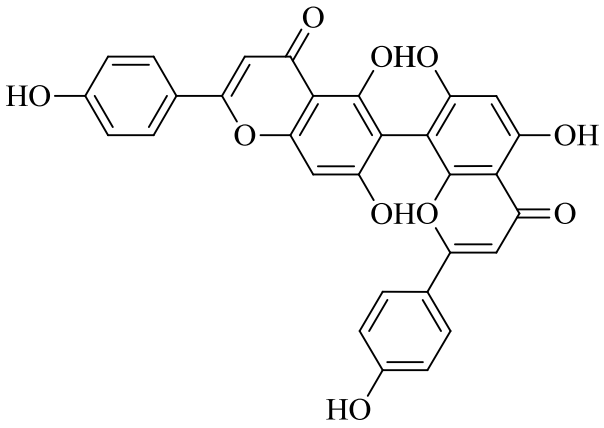
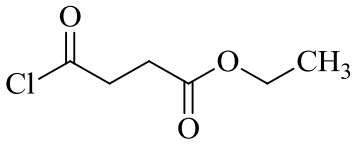
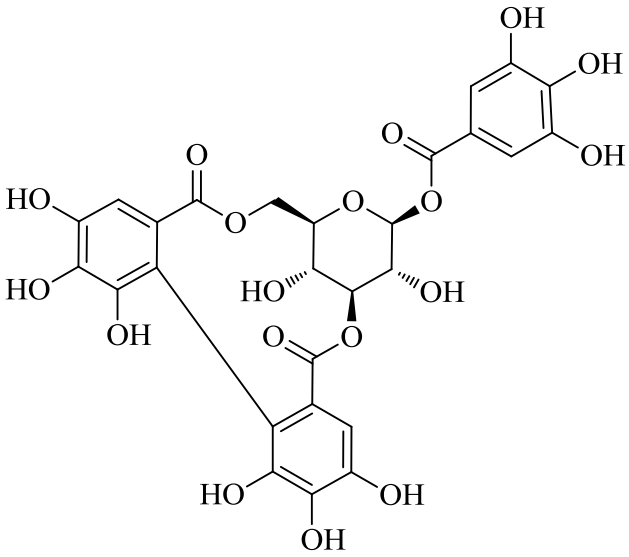
24. 

Figure 1.1: Chemical structures of secondary metabolites from *Canarium* L.

25. 

26. 

27. 

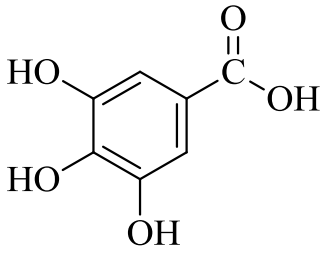
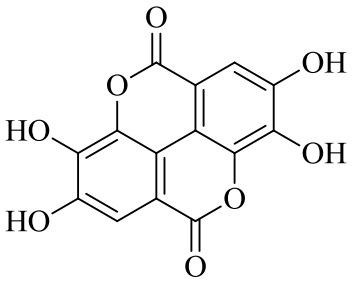
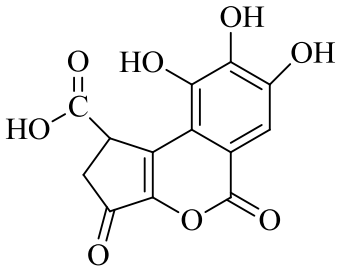
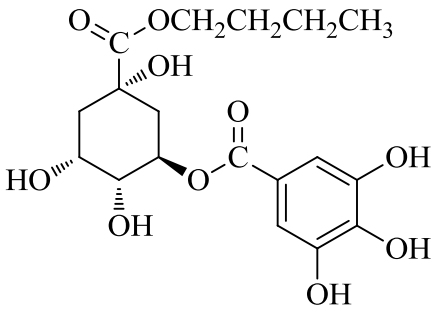
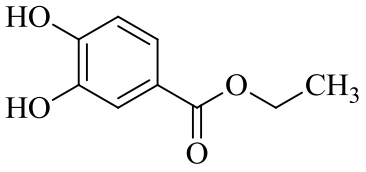
28. 

Figure 1.1: Chemical structures of secondary metabolites from *Canarium* L.

29. 

30. 

31. 

32. 

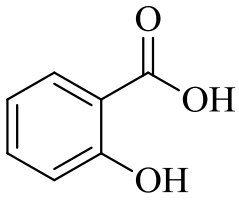
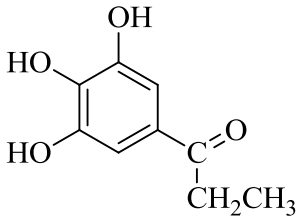
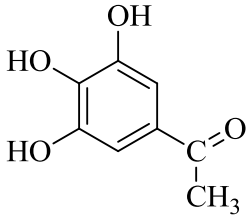
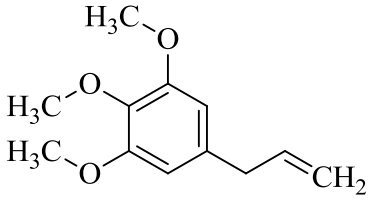
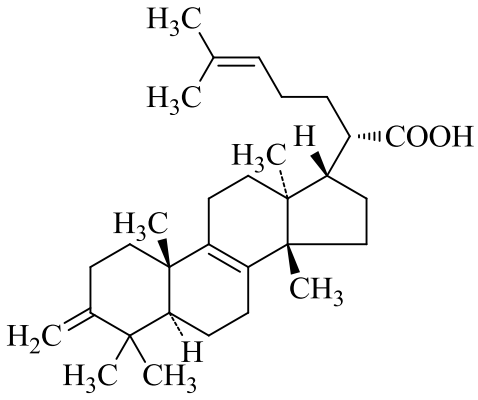
33. 

Figure 1.1: Chemical structures of secondary metabolites from *Canarium* L.

34. 

35. 

36. 

37. 

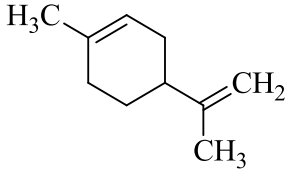
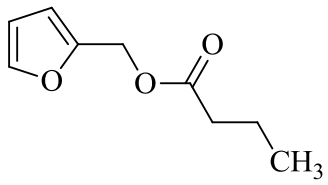
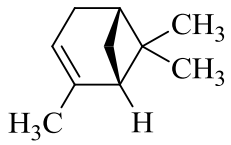
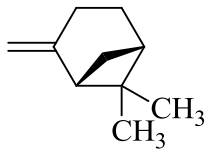
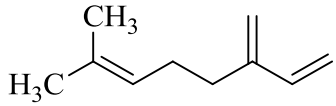
38. 

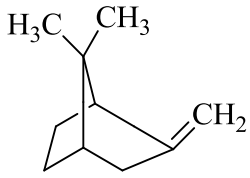
Figure 1.1: Chemical structures of secondary metabolites from *Canarium* L.

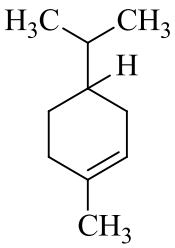
39. 

40. 

41. 

42. 

43. 

44. 

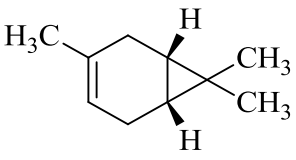
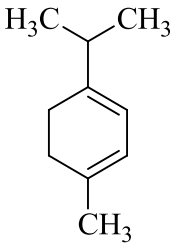
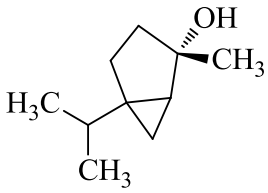
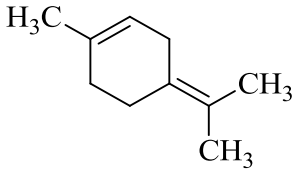
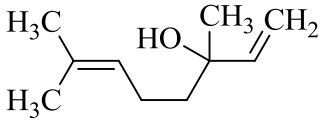
45. 

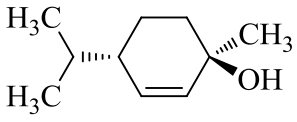
Figure 1.1: Chemical structures of secondary metabolites from *Canarium* L.

46. 

47. 

48. 

49. 

50. 

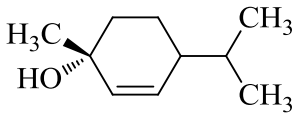
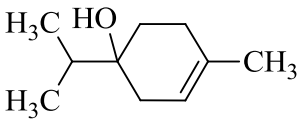
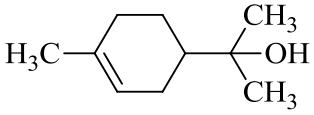
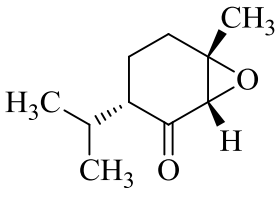
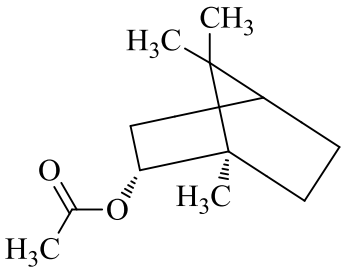
51. 

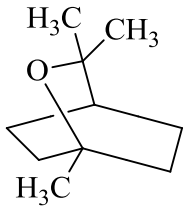
Figure 1.1: Chemical structures of secondary metabolites from *Canarium* L.

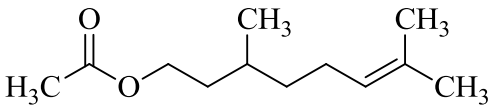
52. 

53. 

54. 

55. 

56. 

57. 

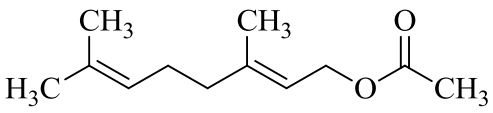
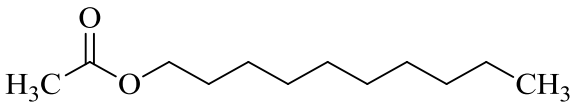
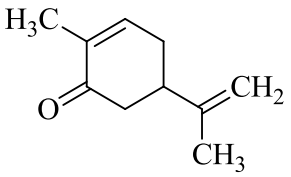
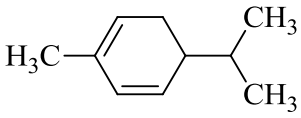
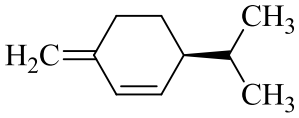
58. 

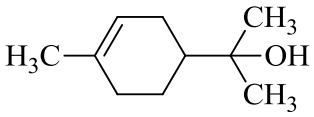
Figure 1.1: Chemical structures of secondary metabolites from *Canarium* L.

59. 

60. 

61. 

62. 

63. 

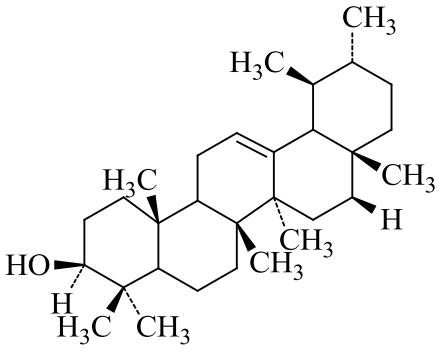
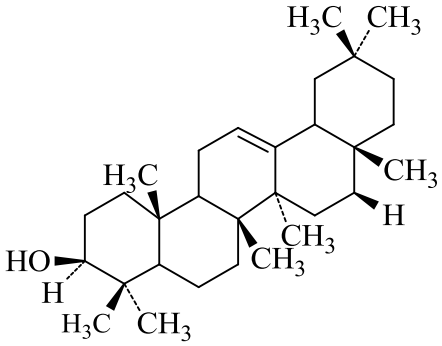
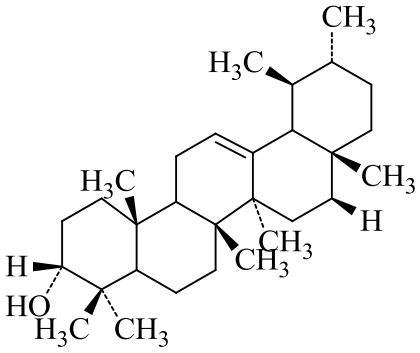
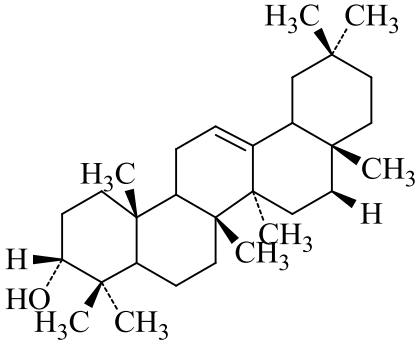
64. 

Figure 1.1: Chemical structures of secondary metabolites from *Canarium* L.

65. 

66. 

67. 

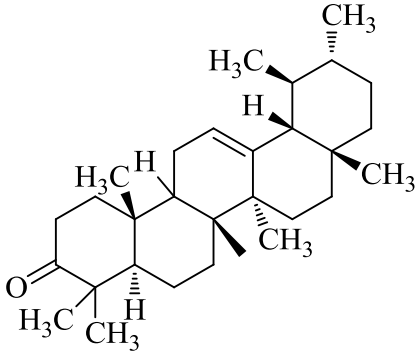
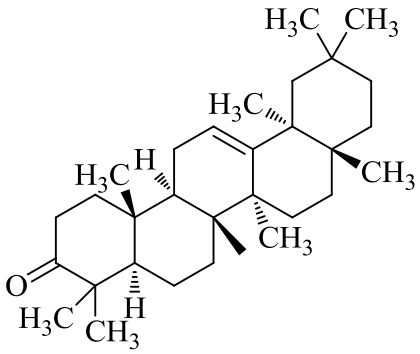
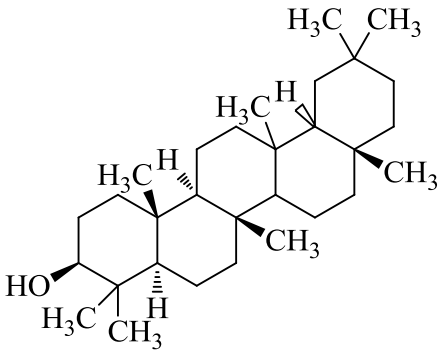
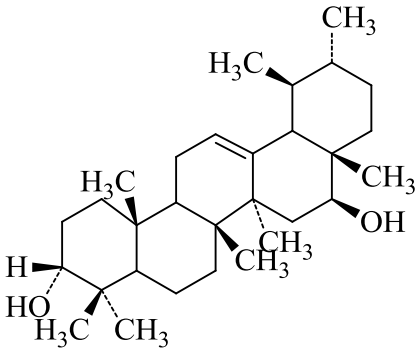
68. 

Figure 1.1: Chemical structures of secondary metabolites from *Canarium* L.

69. 

70. 

71. 

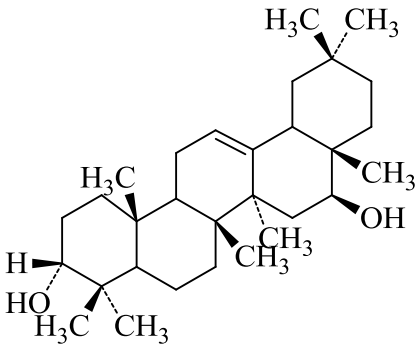
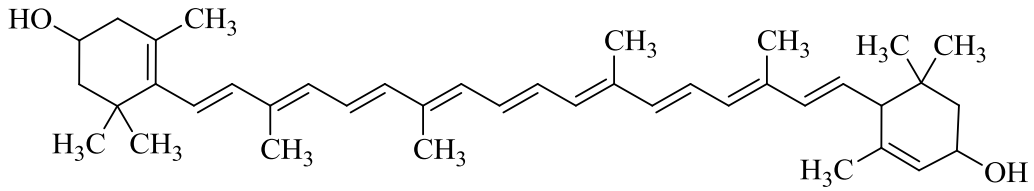
72. 

Figure 1.1: Chemical structures of secondary metabolites from *Canarium* L.



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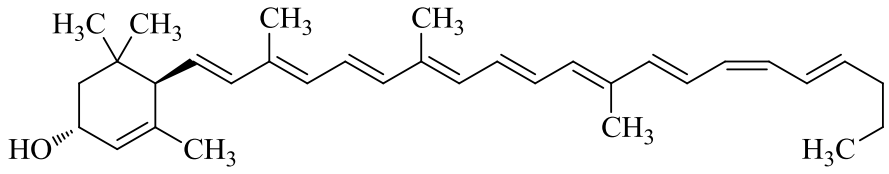

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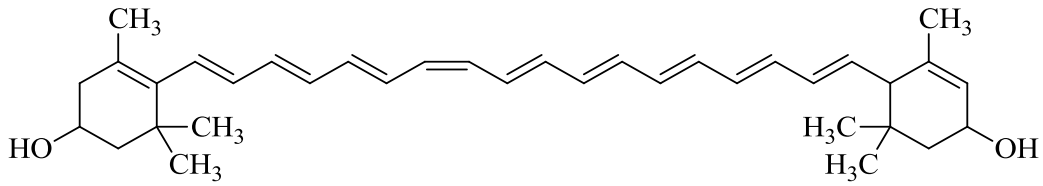

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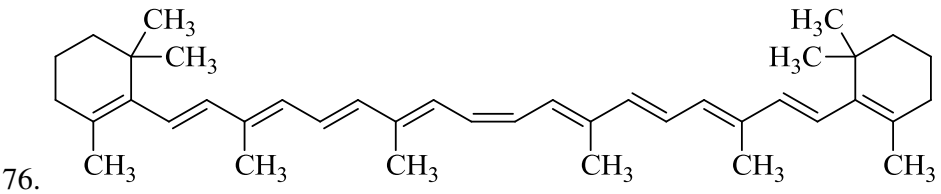

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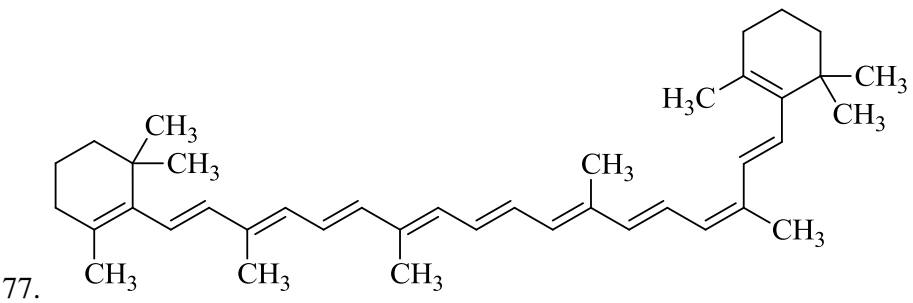

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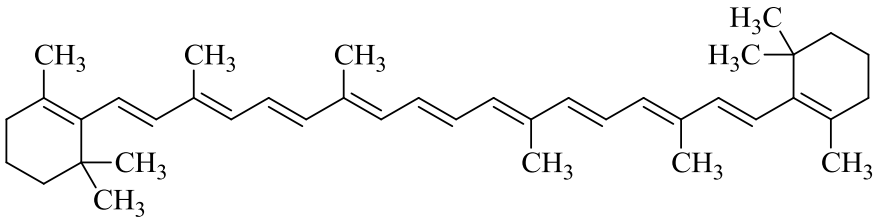
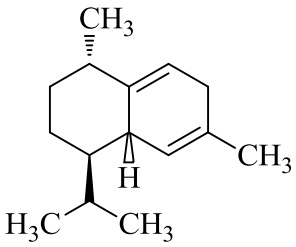
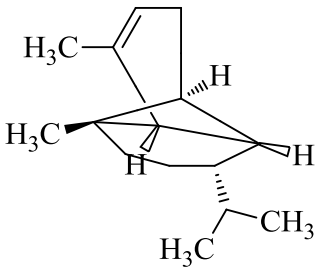
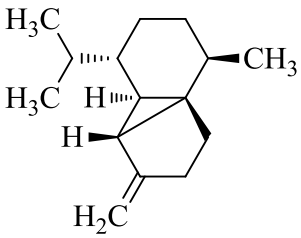
Figure 1.1: Chemical structures of secondary metabolites from *Canarium* L.

78. 

79. 

80. 

81. 

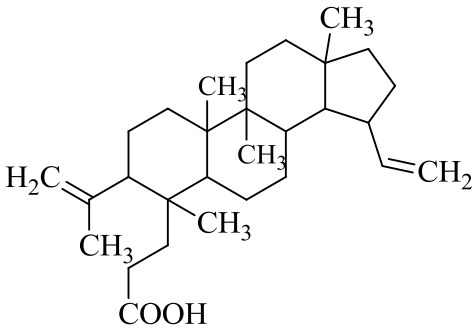
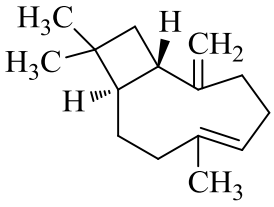
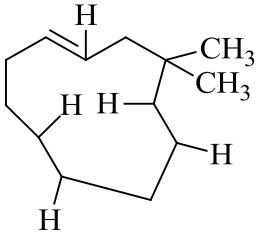
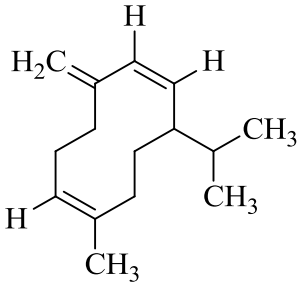
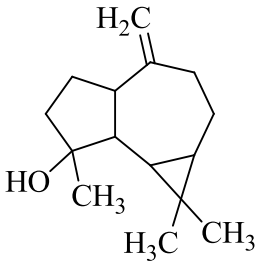
82. 

Figure 1.1: Chemical structures of secondary metabolites from *Canarium* L.

83. 

84. 

85. 

86. 

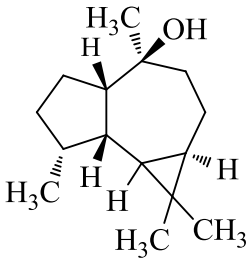
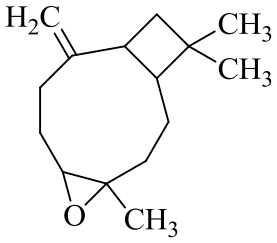
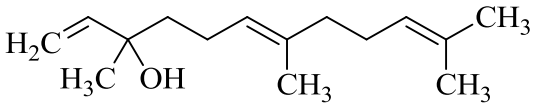
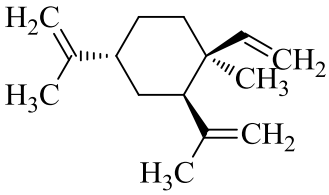
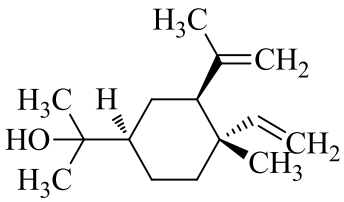
87. 

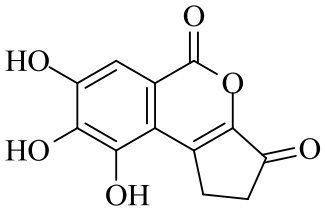
Figure 1.1: Chemical structures of secondary metabolites from *Canarium* L.

88. 

89. 

90. 

91. 

92. 

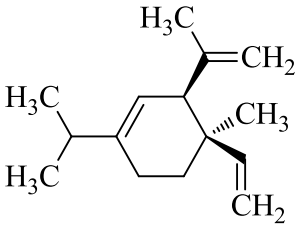
93. 

Figure 1.1: Chemical structures of secondary metabolites from *Canarium* L.

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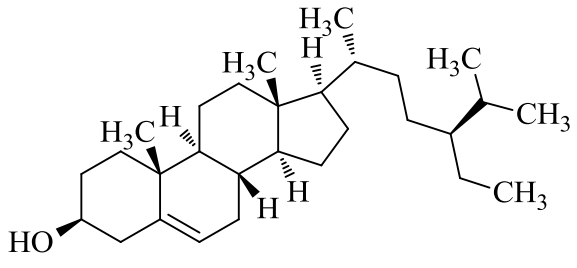

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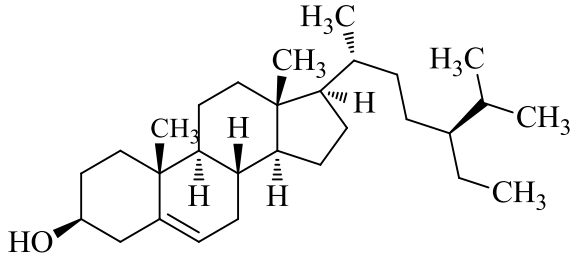


96.

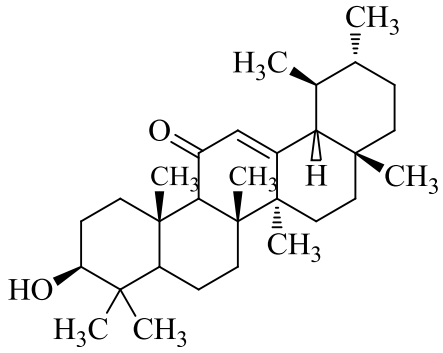


Figure 1.1: Chemical structures of secondary metabolites from *Canarium* L.

In Melanesia, marketing operations for *Canarium indicum* L. and *Canarium harveyi* Seem. range from private and community based production, processing, and marketing of kernel oil for cosmetic and medicinal use, to government-backed nationwide purchasing of kernels for sale as confections and oil. *Canarium solomonense* B.L. Burtt in the Solomons islands are used as general-purpose timber [46]. Because of the potential of *Canarium* L. as a high-value export crop for nuts (for confections) and/or oil extraction, some research is being done on its taxonomy, production and marketing. Additional research needs include selection, evaluation, and improvement of promising varieties for timber production, investigation of cultural aspects, phenological studies, and vegetative propagation [47].

1.7 Selection of plant material

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.

1.7.1. The selection of *Canarium patentinervium* Miq.

Despite reasonable traditional use of *Canarium* L. species, as well as extreme botanical diversity, the *Canarium* species in the Malaysian region have neither been the subject of pharmacological nor extensive phytochemical studies. Claims of the traditional usage to heal wounds [12] therefore requires validation and accurate documentation. For this purpose, this study was initiated as a basis for scientific verification regarding the traditional use of *Canarium patentinervium* Miq. . The leaves and barks of *Canarium patentinervium* Miq. were collected from one individual tree in April 2010 from Bukit Putih, Selangor, Malaysia (3°5'24 "N 101°46'0"E). The plant was identified by Mr. Kamaruddin (Forest Research Institute of Malaysia). A herbarium sample (PID 251210-12) has been deposited in the Forest Research Institute of Malaysia.

1.7.2 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, anti-inflammatory, anti-acetylcholinesterase, antimicrobial, antiparasitic and anticancer activities.

1.8 Aims of study

This study is undertaken to extract and to isolate and purify active constituents from plants by phytochemical techniques and to characterize, elucidate and identify the structure of the pure compound and to assay isolated plant active constituents for antibacterial, antiparasitic, antioxidant, anti-inflammatory, anti-acetylcholinesterase and anticancer activities (Figure 1.2).

1.9 Objectives of the study

i. Extraction of leaves and barks of plant with solvents of different polarity to obtain crude extracts.

ii. Investigation of the secondary metabolites present in the crude extracts of leaves and barks by qualitative phytochemical analysis.

iii. Investigation of the antioxidant activity of the crude extracts of both leaves and barks using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, ferric reducing ability of plasma (FRAP) assay, 2,2'-Azino-*bis* (3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS) assay, β-carotene bleaching assay and superoxide dismutase assay (SOD).

iv. Investigation of the anti-inflammatory potential of the crude extracts against 5-lipoxygenase (5-LOX) and cycloxygenase (COX) systems *in vitro.*

v. Investigation of the anti-acetylcholinesterase (AChE) potential of the crude extracts *in vitro.*

vi. Investigation of the *in vitro* antibacterial activity of the crude extracts of leaves and barks using the disc diffusion assay and determination of minimum inhibitory concentration, minimum bactericidal concentration and death kill rate against four ATCC bacterial strains namely Gram-positive bacteria *Staphylococcus aureus*, *Bacillus cereus*, methicillin-resistant *Staphylococcus aureus* and Gram-negative *Pseudomonas aeruginosa* and nine clinical strain bacteria.

vii. Investigation of the *in vitro* antifungal activity of the crude extracts of leaves and barks using the disc diffusion assay against three clinical strain fungi namely, *Candida parapsilosis, Candida glabrata* and *Candida albicans.*

viii. Investigation of the anticancer activity of the crude extracts of the leaves and barks against human derived cell lines [HCT 116,colon cancer cell line and MCF-7 (ER+), MDA 468 (ER-) breast carcinoma] using MTT assay.

ix. Investigation of the *in vitro* anti-parasitic activity of the crude extracts of leaves and barks against *Leishmania donovani* promastigotes, *Entamoeba histolytica* and *Giardia intestinalis.*

x. Isolation and identification of the chemical compounds responsible for the antibacterial and antioxidant activity in the most active extract through bioassay-guided fractionation using liquid-liquid extraction or solvent partioning, column chromatography, thin layer chromatography (TLC) and nuclear magnetic resonance (NMR).

xi. Bioactivity screening of all isolated compounds using the same bioassay as mentioned in iii-viii.

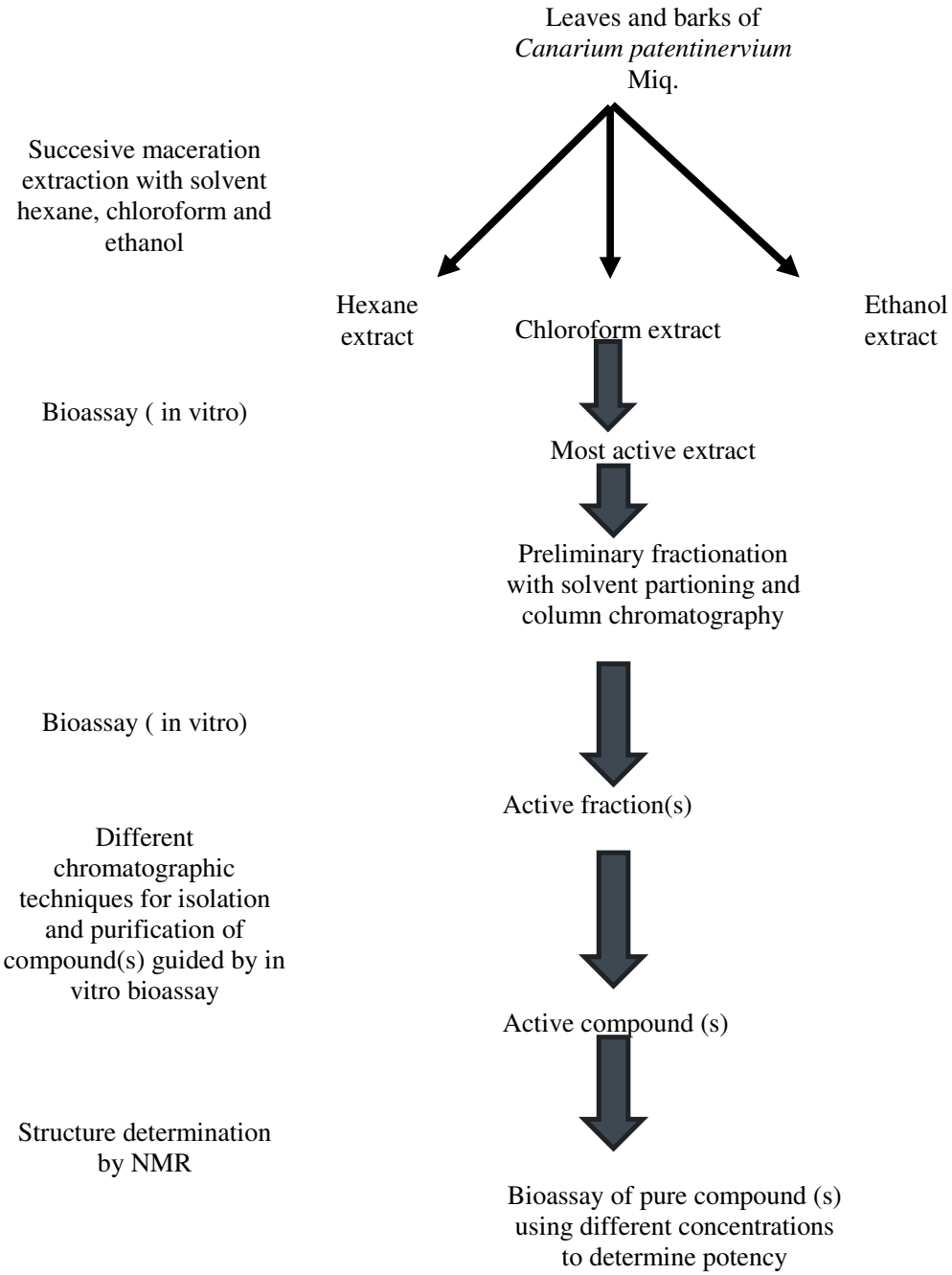


Figure 1.2: A diagrammatic summary of steps in the study of *Canarium patentinervium* Miq. evaluating the phytochemistry and pharmacological activities

CHAPTER 2

PLANT COLLECTION AND EXTRACTION

2.1 Brief introduction to *Canarium patentinervium* Miq.

Canarium patentinervium Miq. is fairly widespread species which occurs in lowland primary and secondary mixed dipterocarp forest and kerangas forest. It is recorded as a common understorey tree. It's a small to medium-sized tree reaching a height of 50-60 feet. The bark is grey brown that is smooth to cracking with many small lenticels and often inconspicuous hoop marks. The inner bark is reddish brown and loosely fibrous with clear resinous exudate. The sapwood is whitish with strong resinous smell from slash. Stipules are caduceus, inserted at the junction of twigs and rachis. Leaves are about 6-15 inches long and thickened at both ends, blade ovate to oblong lanceolate. Apex is gradually to rather shortly and bluntly acuminate. Fruits are ellipsoid to obovoid, rounded with a stone triangular in cross section. Its' strongly glaucous and strongly aromatic (Figure 2.1). Tree flowers mainly from November to May and fruits from September to April [14].

2.2 Plant collection and extraction

The leaves and barks of *Canarium patentinervium* Miq. were collected from one individual tree in April 2010 from Bukit Putih, Selangor, Malaysia (3°5'24 "N 101°46'0"E). The plant was identified by Mr. Kamaruddin (Forest Research Institute

Figure 2.1: Fresh and dried herbarium sample of *Canarium patentinervium* Miq.

of Malaysia). A herbarium sample (PID 251210-12) has been deposited in the Forest Research Institute of Malaysia. The leaves and barks were air dried at ambient temperature (25 ^{o}C) in the laboratory for about 15 days. Thereafter the leaves were separated from the barks and any fruit present were removed and stored separately. The plant material was crushed to a fine powder using an industrial grinder.

Extraction was performed with maceration that involves leaving the pulverized plant to soak in a suitable solvent in a closed container at room temperature [48]. Dried and grinded sample of leaves (2.8 kg) and barks (1.7 kg) were soaked in hexane with the ratio of 1:3 parts of sample to solvent for 2 hr in a 60 ^{o}C water bath, then filtered and concentrated with a rotary evaporator (Buchi, 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 and then ethanol respectively. The yield for the hexane, chloroform and ethanol extract of leaves were 1.25 %, 1.11 % and 6.45 % respectively. The yield for the hexane, chloroform and ethanol extract of barks were 1.04 %, 0.40 % and 2.61 %. Crude extracts were kept at -20 ^{o}C until further use.

CHAPTER 3

PHYTOCHEMICAL METHODS

3.1 Introduction

Phytochemical screening is 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 [48]. Qualitative phytochemical analysis of the crude extract was determined as outlined below.

3.2 Phytochemical assay protocol

Qualitative phytochemical analysis of the crude extract was determined as outlined below [48][49].

i. Alkaloids test

200 mg of the extract was dissolved in 10 ml of methanol and heated on a boiling water bath with 2M 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.

Mayer's reagent consists of 2 solutions described here as solution I and solution II. Solution I: 1.36 g $HgCl_2$ was dissolved in 60 ml water. Solution II: 5 g KI was dissolved in 10 ml water. Both solutions are combined and diluted with water to 100 ml. If alkaloids are present a white to yellowish precipitate will appear. Wagner reagent consists of 1.27 g I_2 (sublimed) and 2 g KI was dissolved in 20 ml water and water is made up to 100 ml. A brown precipitate indicates the presence of alkaloids.

ii. Flavonoids test

40 mg plant material was dissolved in 2 ml 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 color developed within 3 min.

iii. Saponins 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).

iv. Tannins test

About 10 mg of extract was dissolved in 6 ml of hot distilled water and filtered. The solution is divided in three test tubes. To the first 0.9 % sodium chloride solution was added, to the second 0.9 % sodium chloride and 1 % gelatine solution was added and to the third ferric chloride ($FeCl_3$) was added. Formation of a precipitate in the second treatment suggests the presence of tannins, and a positive response after addition of $FeCl_3$ to the third portion which will result in a characteristic blue, blue-black, green or blue-green color supports this inference.

v. Sterols (Salkowski reaction) test.

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

3.3 Results

Phytochemical analysis results exhibits the presence of flavonoid, tannin and sterols in the respective leaves and barks extracts as shown in Table 3.1. Presence of flavonoid and tannin was shown in the leaf and bark extract of ethanol (Figure 3.1 and 3.2). Steroids were present in all extracts except leaf extract of hexane and chloroform (Figure 3.3 and Figure 3.4).

3.4 Discussion

Phytochemical analysis of *Canarium patentinervium* Miq.(Table 3.1) revealed presence of tannins and flavonoids in the ethanol extract of leaves and barks. Triterpenoids can be divided into at least four groups of compounds namely true triterpenes, steroids, saponins and cardiac glycosides [50]. Steroids were detected in the ethanol extract of leaves and all tested bark extracts.

3.5 Conclusion

Results from our phytochemical analysis revealed that the ethanol extract of leaves and barks of *Canarium patentinervium* Miq. accumulate substantial amounts of flavonoids and tannins which could be possibly result in antioxidant or antibacterial activity [51].

Table 3.1: Preliminary phytochemical analysis of the crude extract of *Canarium patentinervium* Miq

Sample	Alkaloids	Flavonoids	Saponins	Tannins	Steroids
LH	-	-	-	-	-
LC	-	-	-	-	-
LE	-	+++	-	+++	+++
BH	-	-	-	-	+++
BC	-	-	-	-	++
BE	-	+++	-	+++	+++

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

Figure 3.1: Positive flavonoid result for leaf ethanol extract (left) against blank and and bark ethanol extract (right) against blank.

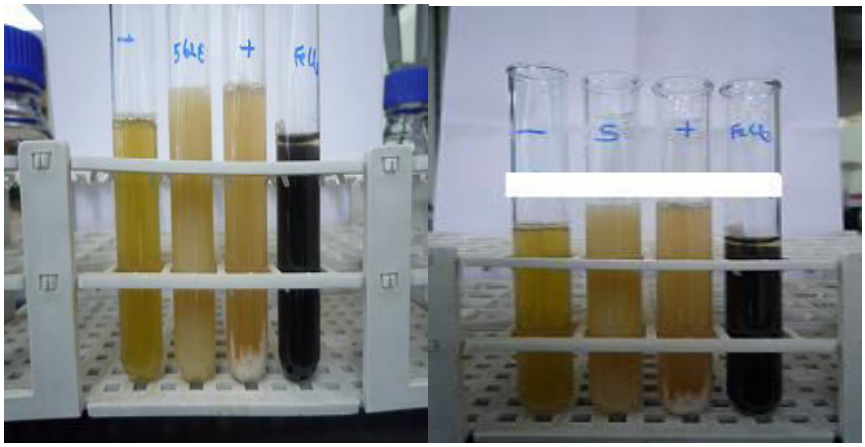


Figure 3.2: Positive tannin result for leaf ethanol extract marked as test tube '56LE' (left) and bark ethanol extract marked as test tube 'S' (right) against blank (-), positive standard tannic acid(+) and ferric chloride .

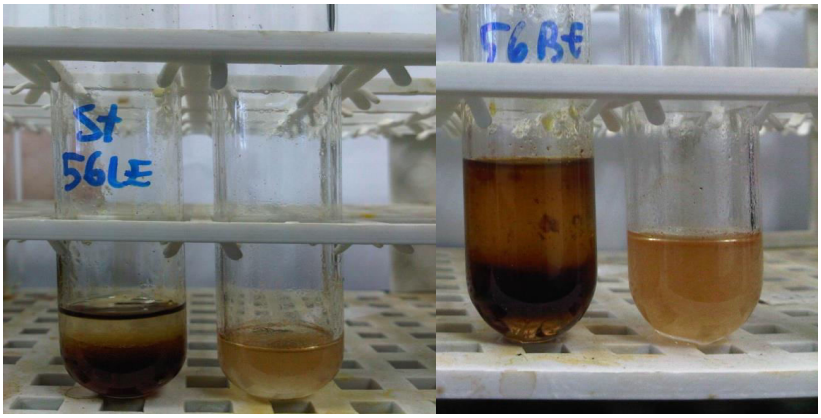


Figure 3.3: Positive sterol test for leaf ethanol extract (left) and bark ethanol extract (right) against blank.

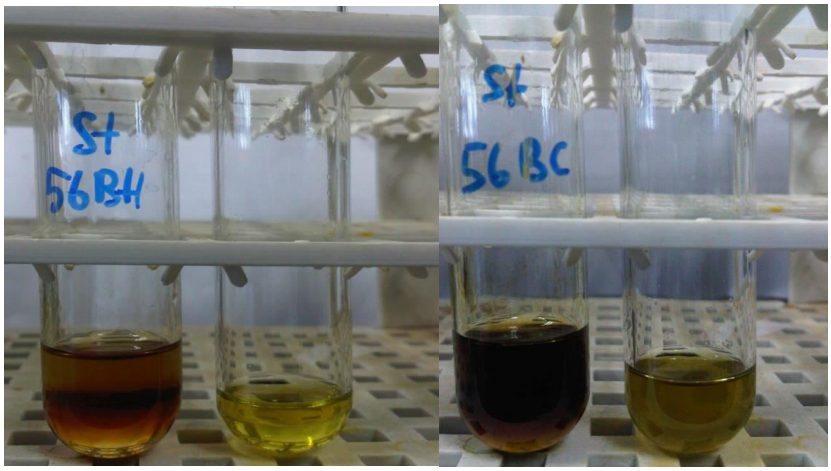


Figure 3.4: Positive sterol test for bark hexane extract (left) and bark chloroforml extract (right) against blank**.**

CHAPTER 4

THE ANTIOXIDANT ACTIVITY

4.1 Introduction

4.1.1 Free radicals and their mechanism of action

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 as natural by products of cell metabolism [52]. 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. Free radicals may either be oxygen derived (ROS, reactive oxygen species) or nitrogen derived (RNS, reactive nitrogen species) [53]. The oxygen derived molecules are O_2^- (superoxide), HO (hydroxyl), HO_2 (hydroperoxyl), ROO (peroxyl), RO (alkoxyl) as free radical and H_2O_2 oxygen as non-radical. Nitrogen derived oxidant species are mainly NO (nitric oxide), ONOO (peroxy nitrate), NO_2 (nitrogen dioxide) and N_2O_3 (dinitrogen trioxide) [54].

In normal cell, there are appropriate oxidant and antioxidant balance. However this balance can be shifted, when the production species is increased or when levels of antioxidants are diminished. This stage is called oxidative stress [53]. Oxidative stress results in the damage of biopolymers including nucleic acids, proteins, polyunsaturated fatty acids and carbohydrates. Oxidative stress causes serious cell

damage leading to a variety of human diseases like Alzheimer's disease, Parkinsons's disease, atherosclerosis, cancer, liver disease, inflammation, diabetes, AIDS, arthritis, immunological incompetence, neurodegenerative disorders and etc [55][56].

In aerobic organisms, the defence system against these free radicals is provided by free radical scavengers which act as anti-oxidants. Antioxidants are defined as a molecule capable of slowing or preventing the oxidation of other molecules, whereas a biological antioxidant has been defined as "any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate" [56] . There are at least four general sources of antioxidants: (1) enzymes, for example super oxide dismutase, gluthathione peroxidase and catalse; (2) large molecules (albumin, ceruloplasmin, ferritin, other proteins); (3) small molecules [ascorbic acid, glutathione, uric acid, tocopherol, carotenoids, (poly)phenols]; and (4) some hormones (estrogen, angiotensin, melatonin, etc) [54][56].

Mechanisms of antioxidant action include serving as (1) physical barriers to prevent ROS generation or ROS access to important biological sites, eg UV filters, cell membranes; (2) chemical traps/sinks that "absorb" energy and electrons, quenching ROS, eg carotenoids and anthocyanidins; (3) catalytic systems that neutralize or divert ROS, eg SOD (superoxide dismutase), catalse and gluthathione peroxidase; (4) binding/inactivation of metal ions to prevent generation of ROS, eg ferritin, ceruloplasmin, catechins; and (5) chain breaking antioxidants which scavenge and destroy ROS, eg ascorbic acid (vitamin C), tocopherols (vitamin E), uric acid, gluthathione, flavonoids [57].

4.1.2 Natural antioxidants

The term, antioxidant is used to describe a component that can function to decrease tissue damage by reactive oxygen. 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 [58]. 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 [51].

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 [59]. Other non-enzymatic anti-oxidants include antioxidant enzyme cofactors, oxidative enzyme inhibitors and transition metal chelators such as ethylene diamine tetra-acetic acid (EDTA) [58].

4.1.3 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 inflammation, age-related disorders, cancer and cardiovascular disease is derived from clinical trials and epidemiological studies [58][59]. 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 [58]. 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 [58].

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 one compound, due to synergism between different anti-oxidant compounds. Methods to measure the anti-oxidant 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 its consequent disappearance [60][61]. 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 [62].

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 [63][64]. 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 [65]. 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 [66].

4.1.4 Antioxidant potential of *Canarium* L.

Antioxidant activities were reported in *Canarium album* (Lour.) Raeusch, *Canarium odontophyllum* Miq. and *Canarium schweinfurthii* Engl. A tonic soup made of *Canarium album* (Lour.) Raeusch used mainly in China displayed significant antioxidant activity by 1,1,-diphenyl-2-2-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assay [22]. Tannins extracted from the leaves, twigs and stem barks of *Canarium album* (Lour.) Raeusch showed potent antioxidant activity in the DPPH radical scavenging activity with IC_{50} values of 56.86 µg/ml, 62.31 µg/ml and 54.80 µg/ml respectively and ferric reducing power of 4.28, 3.74 and 4.49 mmol AAE/g equivalent of dried tannin [67]. Pure compounds from this species includes

brevifolin, hyperin and ellagic acid which showed free radical scavenging activity in DPPH assay [24]. The essential oil of *Canarium schweinfurthii* Engl. was tested for the antioxidant activity with the DPPH assay and by β-carotene bleaching test. It exhibited highest antioxidant activity at 150 µg/ml activity in both assay [25]. The ethyl acetate fraction of peel of *Canarium odontophyllum* Miq. exhibited 95±1.00% scavenging activity at the concentration of 40 µg/ml [26][68].

4.2 Materials and methods

4.2.1 Antioxidant determination assays

Major antioxidant capacity methods have been generally divided into two categories: (1) hydrogen atom transfer (HAT) reaction and (2) single electron transfer (SET) reaction-based method. SET and HAT almost always occur together with the balance determined by antioxidant structure and pH. Bond dissociation energy (BDE) and ionization potential (IP) are two major factors that determine the mechanism and the efficacy of the antioxidants. HAT-based methods measure the classical ability of an antioxidant to quench free radicals by hydrogen donation. Relative reactivity in HAT methods are determined by the BDE of the H-donating group in the potential antioxidant and IP. Most HAT-based methods monitor competitive reaction kinetics and are rapid.

$$X\bullet + AH \longrightarrow XH + A\bullet$$

SET-based methods detect the ability of a potential antioxidant to transfer one electron to reduce any compound, including metals, carbonyls and radicals.

$$X\bullet + AH \longrightarrow X^{-} + AH\bullet$$

Relative reactivity is based on deprotonation and IP of the reactive functional group so SET is pH dependent. In general, IP decrease with increasing pH, reflecting increased electron-donating capacity with deprotonation. At acidic conditions, the reducing capacity may be restrained due to protonation on antioxidant compounds, whereas in basic conditions, proton dissociation of phenolic compounds would increase samples reducing capacity. SET methods can be relatively slow and involve two components, antioxidant and oxidant (probe).

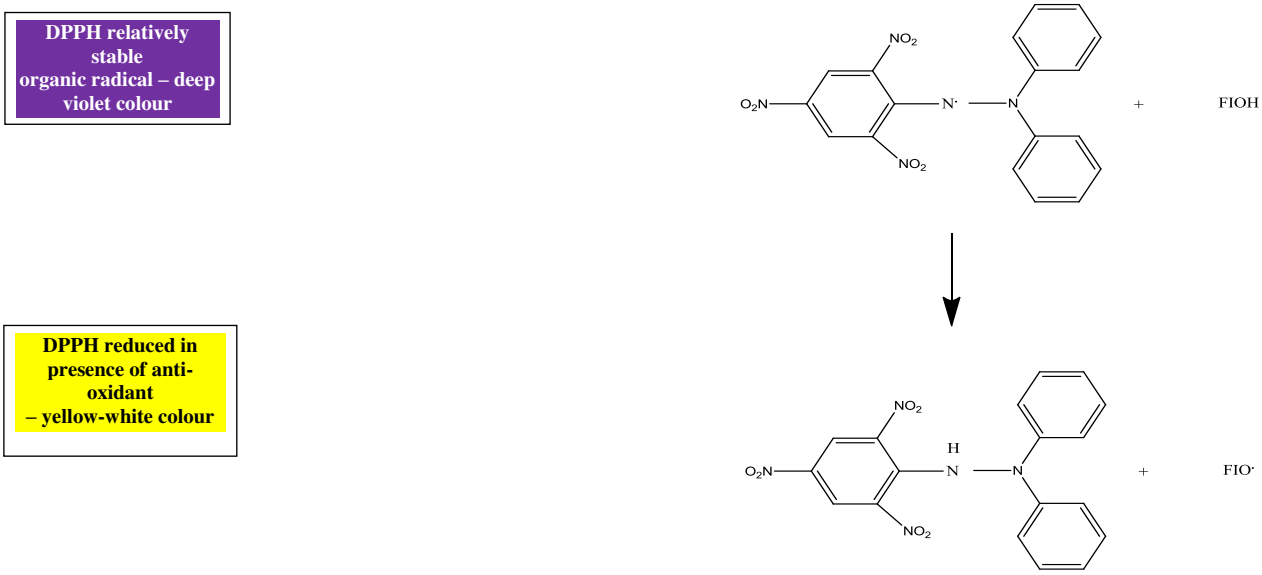
Probe (oxidant) + e (from antioxidant) ⟶ reduced probe + oxidised antioxidant

Huang *et al*. [69] broadly classified antioxidant capacity assays based on the reaction mechanisms as follows : SET [Folin-Ciocalteu (Folin-C), 1,1-diphenyl-2-picrylhydrazine (DPPH) radical scavenging, trolox equivalent antioxidant capacity (TEAC), ferric reducing ability of plasma (FRAP)]; and HAT [oxygen radical absorbance capacity (ORAC), β-carotene/linoleic acid model system and inhibition of phospholipid peroxidation]. Prior *et al.* [70] classified DPPH, TEAC and Folin-C antioxidant assays into a category that utilises both SET and HAT reaction mechanisms, though predominantly based on electron transfer [70][53].

4.2.2 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

4.2.2.1 Principle of the assay

The antioxidant activity of each of the plant extracts was determined using the colorimetric DPPH assay, as described by Juan Badaturuge [71], was employed to determine the radical scavenging activity of the plant extracts. 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 [72] (Figure 4.1). 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 550 nm, after which absorption decreases due to decolourisation to a yellow-white colour, in the event of reduction [73]. 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 4.1: Diagrammatic representation of chemical reaction of the reaction of DPPH in the presence of an electron donating antioxidant [74].

4.2.2.2 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 0.1 mM 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 550 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), quercetin and trolox was used as positive control. 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 (%) = [(Abscontrol - Abssample)]/ (Abscontrol)] x 100

Abscontrol is the absorbance of DPPH radical + methanol;

Abssample is the absorbance of DPPH radical + sample extract /standard.

4.2.3 2,2'-Azino-*bis*(3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS) assay

4.2.3.1 Principle of the assay

The antioxidant activity of each of the plant extracts was determined using the colorimetric ABTS assay, as described by Miller *et al*. [75], Rice-Evans [76] and Roberta *et al.* [77] was employed to determine the radical scavenging activity of the plant extracts. The ABTS anti-oxidant assay, also known as the Trolox equivalent anti-oxidant capacity (TEAC) assay, assesses the total radical scavenging capacity of the plant extracts. This is determined through the ability of these extracts to scavenge the long-lived specific ABTS radical cation chromophore in relation to that of Trolox, the water-soluble analogue of vitamin E [78]. The generation of the ABTS+ blue/green chromophore occurs through the oxidation of ABTS diammonium salt in the presence of potassium persulfate (Figure 3.4), with the absorption maxima occurring at wavelengths 645 nm, 734 nm and 815 nm. Anti-oxidants will reduce the pre-formed radical cation to ABTS, and in so doing bring about the decolourisation of ABTS+ to a colourless product [76]. The extent of this decolourisation is a measure of the ABTS+ radical cation that has been scavenged, after a fixed time period, and is relative to the Trolox standard.

ABTS- transparent

ABTS radical relatively stable organic radical- deep turquoise green

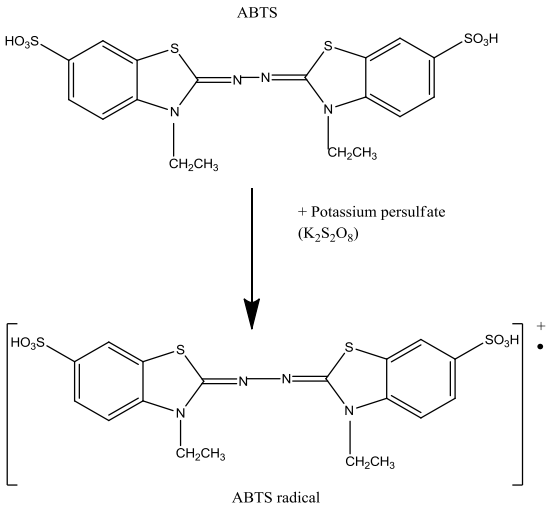


Figure 4.2: Diagrammatic representation of the formation of the ABTS radical after the addition of potassium persulphate.

4.2.3.2 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. At least three independent tests were performed for each sample. Trolox (6-hydroxy-2,5,7,8-tetramethylchromon-2-carboxylic acid, Sigma-Aldrich) Vitamin C (l-ascorbic acid) and quercetin was used as positive control and prepared in ethanol and serial dilutions of this positive control were also prepared. Ethanol was used as the negative control. The stock solution included 7 mM ABTS solution and 2.4 mM potassium persulfate solution. The working solution was then prepared by mixing the 2 stock solutions in equal quantities. This solution was then stored in the dark for 12 - 16 hours in order to stabilise it before use. It remains stable for 2 - 3 days in the dark.

The concentrated ABTS+ solution was diluted with cold ethanol shortly before conducting the assay, to a final absorbance of 0.70 ± 0.01 at 734 nm at 37 °C, in a 3 cm cuvette. The total scavenging capacity of the extracts was quantified through the addition of 100 µl ABTS+ to 100 µl of plant extract. The solutions were heated to 37 °C for 7 min, after which the absorbance was read at 734 nm on a spectrophotometer (Thermo Scientific Varioskan Flash microtiter plate reader, linked to a computer equipped with SkanIt Software 2.4.3). All experiments were done in triplicate. The percentage decolourisation was calculated using equation below and the extent of inhibition of the absorbance of the ABTS+ was plotted as a function of the concentration.

This activity is given as percent ABTS radical scavenging, which is calculated with the equation:

ABTS radical scavenging capacity (%) = [(Abscontrol - Abssample)]/ (Abscontrol)] x 100

Abscontrol is the absorbance of ABTS radical +ethanol;

Abssample is the absorbance of ABTS radical + sample extract /standard.

4.2.4. Ferric reducing ability of plasma (FRAP) assay

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4.2.4.1 Principle of assay

The antioxidant activity of each of the plant extracts was determined using the colorimetric FRAP assay, as described by Benzie & Strain [79] with slight modifications. The mechanism associated with this method is shown in Figure 4.3. This method was first developed to quantitate ascorbic acid in serum or plasma. When a Fe^{3+}-TPTZ complex is reduced to the Fe^{2+} form by an antioxidant under acidic conditions, an intense blue color with maximum absorption develops at 593nm. Therefore, the antioxidant effect (reducing ability) can be evaluated by monitoring the formation of a Fe^{2+}-TPTZ complex with a spectrophotometer. The FRAP assay gives fast and reproducible results.

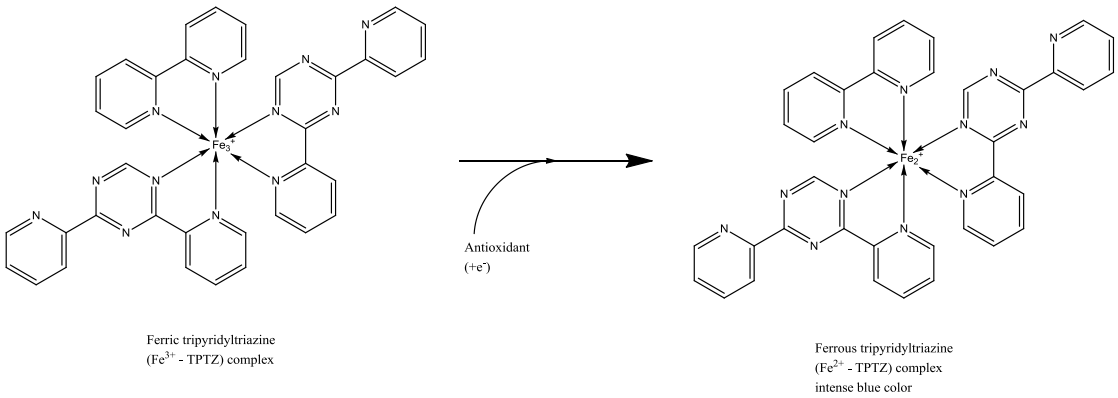


Figure 4.3: Formation of (Fe^{2+}-TPTZ) complex from (Fe^{3+}-TPTZ) complex by antioxidant.

4.2.4.2 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. Vitamin C (l-ascorbic acid), quercetin and trolox were used as positive control. At least three independent tests were performed for each sample. The working FRAP reagent was prepared just before assay by mixing 300 mM of acetate buffer (pH 3.6), 10 mM of 2,4,6-tripyridyl-s-triazine (TPTZ) and 20 mM of $FeCl_3.6H_2O$ in ratio of 10:1:1.

Briefly 180 µl of the FRAP reagent was mixed with 20 µl of the test sample so that the final dilution of the test sample in the reaction mixture was 1/10. After 30 minutes, the absorbance of the coloured product (ferrous triphridyltriazine complex) was recorded using a spectrophotometer (Thermo Scientific Varioskan Flash microtiter plate reader, linked to a computer equipped with SkanIt Software 2.4.3). Fe (II) concentrations in the range of 1 µM-100 µM ($FeSO_4.7H_2O$) was used as standard for calibration curve and equation of linearity is determined (y=ax+b). From the linearity equation, concentration of sample that produced same absorbance as 1mM of Fe (II) was determined (y of sample filled in equation to obtain x). The antioxidant activity was calculated as Ferrous Equivalents, the concentration of samples which produced an absorbance value equal to that of 1 mM $FeSO_4$.

4.2.5 β-carotene bleaching assay

4.2.5.1 Principle of assay

The β-carotene bleaching assay was conducted according to the method described by Habtemariam *et al.* [80] with some modifications. It has long been known that β-carotene reacts with the peroxy radical to produce that β-carotene epoxides. Therefore that β-carotene has received attention as a radical scavenger or antioxidant. Later an antioxidant assay using that β-carotene combined with lipids such as linolenic acid was established [80]. As shown in Figure 4.4, lipids such as linolenic acid forms as peroxy radical (LOO•) in presence of the ROS and O_2. This peroxy radical reacts with β-carotene to form a stable β-carotene radical subsequently, the amount of β-carotene reduces in the testing solution. If an antioxidant is present in the testing solution, it reacts competitively with the peroxy radical. Therefore, antioxidant effects are easily monitored by the bleaching of the colour of a test solution with a spectrophotometer at 470 nm, which is the typical absorbance by β-carotene [56].

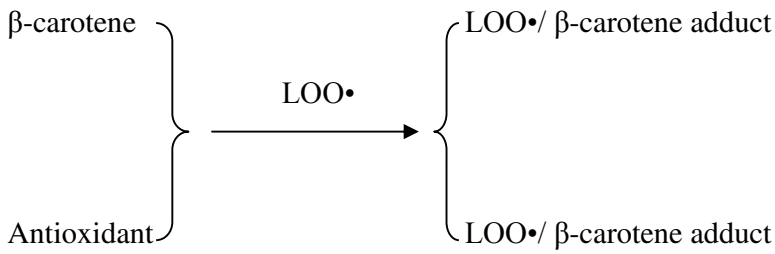


Figure 4.4: Formation of adducts from by β-carotene and antioxidant with lipid peroxide radical.

4.2.5.2 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. Vitamin C (l-ascorbic acid), quercetin and trolox were used as positive control. Briefly, 1 ml of a β-carotene solution in chloroform (2 mg in 10 ml) was pipetted into a round bottom flask containing 40 µl of linoleic acid and 500 µl of Tween 20. After the removal of chloroform using a rotary vacuum evaporator at 45 ^{o}C, 100 ml of deionised water were added with vigorous agitation. 180 µl of the emulsion was added to 20 µl of test samples at varying concentrations in 96-well microtitre plate. The absorbance was measured at 470 nm immediately against a blank consisting of the emulsion without β-carotene and after 3 hr of incubation at 50 ^{o}C using a spectrophotometer (Thermo Scientific Varioskan Flash microtiter plate reader, linked to a computer equipped with SkanIt Software 2.4.3). All determinations were carried out in triplicates.

The antioxidant activity of test agents was evaluated in terms of bleaching of β-carotene using the following formula:

$$\text{Antioxidant activity AA (\%)} = [1- (A_0-A_t)/(A_o' -A_t')]x\ 100$$

A_0 and A_o' are absorbances measured at zero time of incubation for the test sample and control, respectively; A_t and A_t' are the absorbances measured in the test sample and control, respectively, after incubation for 3 hr.

4.2.6 Superoxide dismutase assay

4.2.6.1 Principle of assay

This assay was conducted with Sigma SOD assay kit [81]. SOD Assay Kit-WST allows very convenient SOD assaying by utilizing Dojindo's highly water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)- 3-(4- nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) that produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of reduction with oxygen is linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD. Therefore, the IC_{50} (50% inhibition activity of SOD or SOD-like materials) can be determined by a colorimetric method. Since the absorbance at 450 nm is proportional to the amount of superoxide anion, the SOD activity as an inhibition activity can be quantified by measuring the decrease in the colour development at 450 nm.

4.2.6.2 Colorimetric spectrophotometric assay

Twenty microliters of sample was plated at different concentrations in a 96-well microtiter plate. Then, 200 µl of WST solution and 20 µl of enzyme was added and incubated for 20 mins at 37 ^{o}C. Absorbance was then recorded at 450nm using Thermo Scientific Varioskan Flash microtiter plate reader, linked to a computer equipped with (SkanIt Software 2.4.3) and SOD inhibition rate was determined. SOD enzyme (Nacalai Tesque) was used as standard control.

4.2.7 Total phenolic content determination

4.2.7.1 Principle of assay

The total phenolic content of the crude extract and fractions was determined with the Folin–Ciocalteu reagent, following the modified method of Singleton and Rossi [82]. The Folin-Ciocalteu reagent is sensitive to reducing compounds including polyphenols, thereby producing a blue colour upon reaction. This blue colour is measured spectrophotometrically, thus determining the total phenolic content.

4.2.7.2 Colorimetric spectrophotometric assay

Briefly, 20 µl of test samples dissolved in methanol (1 mg/ ml) were added to 100 µl of Folin–Ciocalteu reagent and 1.58 ml of deionised water. After allowing the mixture to stand at room temperature for 5 min, 300 µl of 20% (w/v) sodium carbonate were added. Reaction mixtures were further incubated at room temperature for 30 mins, following which absorbance at 765 nm was read against a blank, using a Jenway 6305 UV–Vis spectrophotometer (Jenway Ltd., Essex, UK). The standard calibration curve was plotted using gallic acid (50-250 µg/ml), from which total phenolic content was expressed as gallic acid equivalents (mg/g extract).

4.2.8 Total flavonoid content determination

4.2.8.1 Principle of assay

The total flavonoid content of the crude extract and fractions was determined with the aluminium chloride colorimetric method of Froehlicher *et al.* [83]. The principle of this assay is that aluminium chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. In addition, aluminium chloride forms acid labile complexes with the orthodihydroxyl groups in the A- or B- ring of flavonoids [84].

4.2.8.2 Colorimetric spectrophotometric assay

Briefly, 0.5 ml of test samples dissolved in methanol (1 mg/ ml) were added to 1.5 ml of 2% methanolic solution of aluminium chloride in sealed tubes and kept in dark for 15 mins. Absorbance was then read at 430 nm using UV-vis spectrophotometer against blank of methanolic aluminium chloride solution. The standard calibration curve was plotted using quercetin (50-250 µg/ml), from which total flavonoid content was expressed as quercetin equivalents (mg/g extract).

4.2.9 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. All data were expressed as mean ± standard deviation. SD was computed but values were not shown in the graphs via this software. Data were analyzed using one way Anova followed by Tukey test using GraphPad Prism5 software. A significant difference was considered at the level of $P < 0.01$.

4.3 Results

4.3.1 DPPH assay

In this test the ethanol extract of leaves and barks exhibited profound antioxidant activities (Figure 4.5). The antioxidant activities were highest for ethanol extract of barks (77.80±0.01 %) followed by the ethanol extract of leaves (65.80±0.01 %), the hexane extract of leaves (38.90±0.02 %), the chloroform extract of barks (8.00±0.02 %), the hexane extract of barks (5.00±0.01 %) and the chloroform extract of leaves (3.00±0.01 %) against standard ascorbic acid which was 97.90±0.00 % at the dose of 10 µg/ml (Table 4.1). The EC_{50} (µg/ml) for the ethanol extract of barks (2.33 µg/ml) and leaves (2.93 µg/ml) were as good as ascorbic acid (1.88 µg/ml) as shown in Figure 4.6 and Table 4.2.

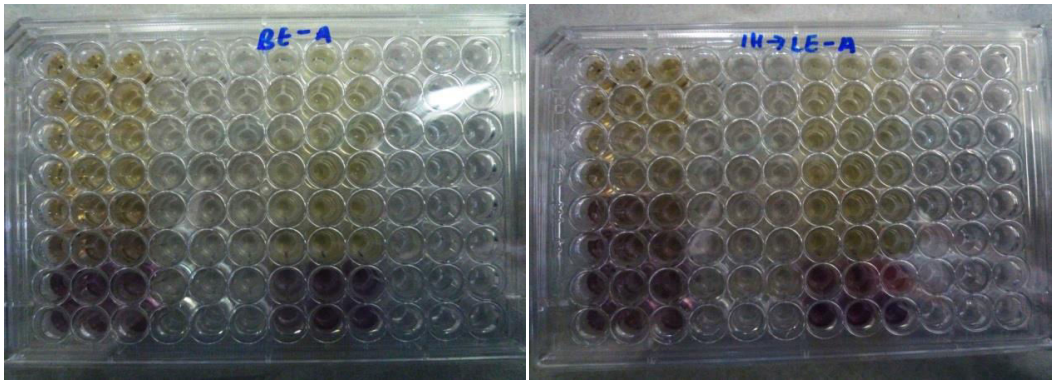


Figure 4.5: DPPH test result with 96 well microtiter plate results for bark (left) and leaf (right) ethanol extract.

4.3.2 ABTS assay

In this test the ethanol extract of leaves and barks exhibited good antioxidant activities (Figure 4.7). The EC_{50} (µg/ml) for the ethanol extract of barks (0.93±0.01 µg/ml) and leaves (2.28±0.01 µg/ml. The EC_{50} for ethanol extract of bark was significantly lower than ascorbic acid (1.54±0.06 µg/ml) (Table 4.2).

4.3.3 FRAP assay

The FRAP values are represented in terms of concentration of sample that produced same absorbance as 1 mM of Fe (II) was determined (y of sample filled in equation to obtain x). The antioxidant activity was calculated as Ferrous Equivalents, the concentration of samples which produced an absorbance value equal to that of 1 mM $FeSO_4$.

Table 4.1: DPPH radical scavenging activity at 10 µg/ml of extract

Extracts	% DPPH radical scavenging
LH	38.90±0.02
LC	3.00±0.01
LE	65.80±0.01
BH	5.00±0.01
BC	8.00±0.02
BE	77.80±0.01
AA	97.90±0.00

LH-leaf hexane extract, LC- leaf chloroform extract, LE-leaf ethanol extract
BH- bark hexane extract, BC- bark chloroform extract, BE- bark ethanol extract
AA- ascorbic acid

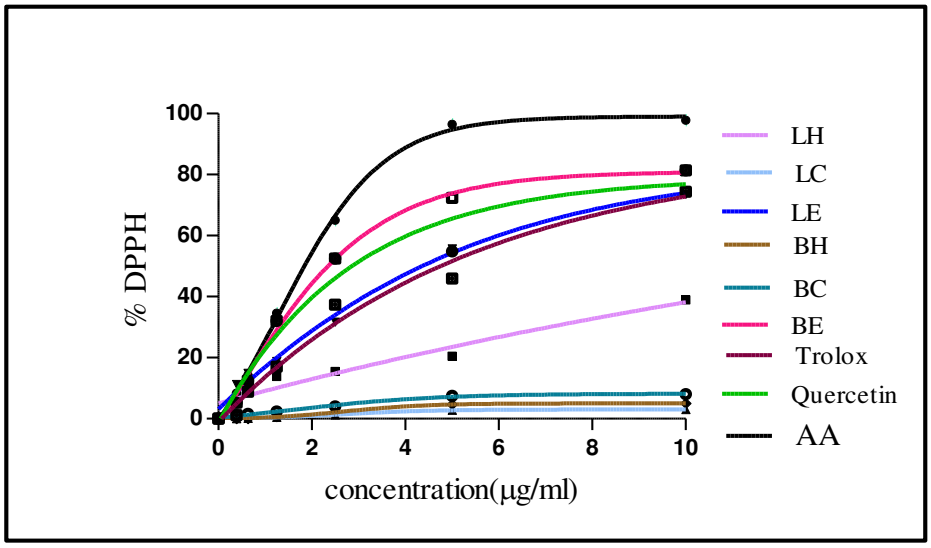


Figure 4.6: DPPH scavenging activity (%) of *Canarium patentinervium* Miq.

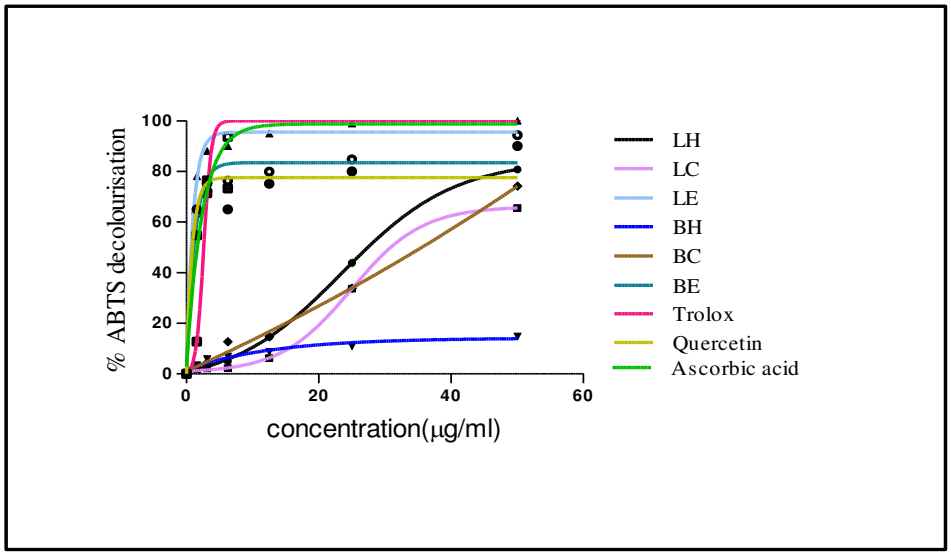


Figure 4.7: ABTS scavenging activity (%) of *Canarium patentinervium* Miq.

Linear regression curve for all samples and positive controls are shown in Figure 4.9-4.17. From the standard graph of ferrous sulphate (Figure 4.8), the values for six extracts were determined using the standard formula (Table 4.2). The FRAP activity was calculated from regression equation of ferrous sulphate calibration curve ($y=0.0105x+0.0136$, r^2- 0.9817). The ethanol extract of bark showed significant FRAP activity of 67.00±0.32 µg/ml compared to quercetin and ascorbic acid. The ethanol extract of the leaves showed significant FRAP activity of 200.00±0.07 µg/ml compared to ascorbic acid. The poorest activity was shown by hexane extract of barks that correlates with the low r^2 in its regression equation (Figure 4.12).

4.3.4 β-carotene bleaching assay

The β-carotene decolourisation (Figure 4.18) was most prominent in ethanol extract of leaves and barks with EC_{50} (µg/ml) of 6.04±0.02 µg/ml and 7.04±0.04 µg/ml respectively (Table 4.2)

4.3.5 Superoxide dismutase assay

The ethanol extract of the leaves shows highest SOD-like activity with IC_{50} of 3.05±0.01 µg/ml. (Table 4.2).

4.3.6 Total phenolic content determination

The total phenolics in the extracts was determined with the Folin-Ciocalteu assay, calculated from regression equation of calibration curve of gallic acid

(y=0.1762x+0.0047, r^2- 0.9994) (Figure 4.19) and is expressed as gallic acid equivalents, GAE (mg/g). The highest phenolic content was in ethanol extract of leaves followed by barks (Table 4.2).

4.3.7 Total flavonoid content determination

The total flavonoids in the extracts was determined with the aluminium chloride assay, calculated from regression equation of calibration curve (y=0.09330x+0.0023, r^2-0.9992) (Figure 4.20) of quercetin and is expressed as quercetin equivalent, (mg/g). The highest flavonoid content was in ethanol extract of leaves followed by barks (Table 4.2).

4.3.8 The antioxidant values, total phenolic and flavonoid content and their correlations

The total antioxidant activity investigated with the relevant total phenolic and flavonoid content are summarized in table 4.3.

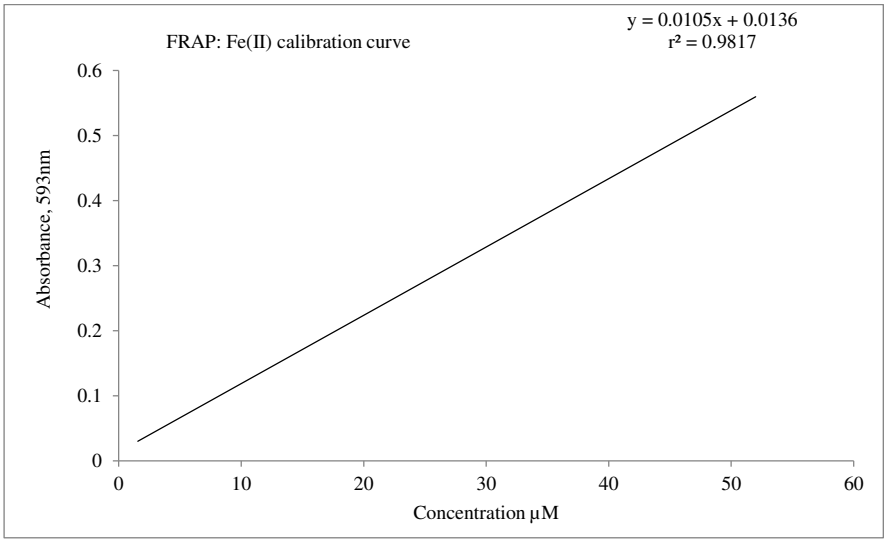


Figure 4.8: Fe (II) calibration curve in FRAP assay.

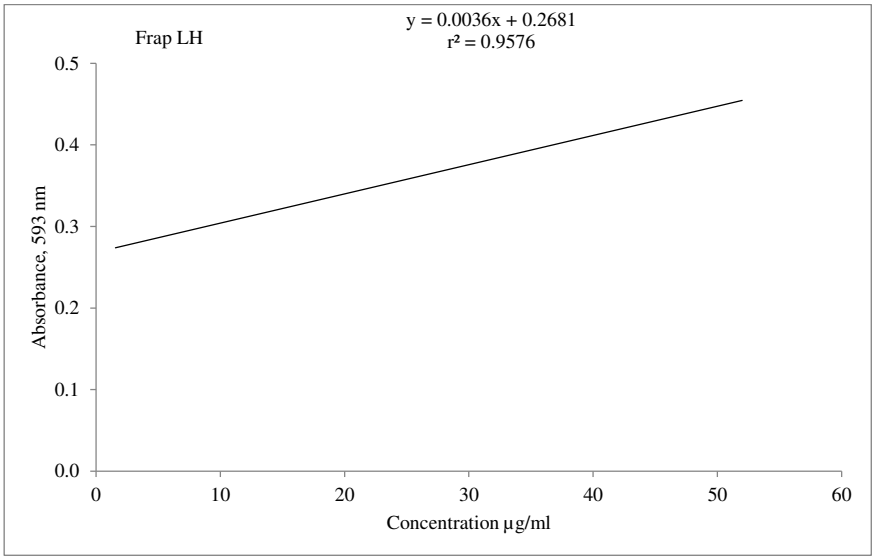


Figure 4.9: Leaf hexane (LH) extract calibration curve in FRAP assay.

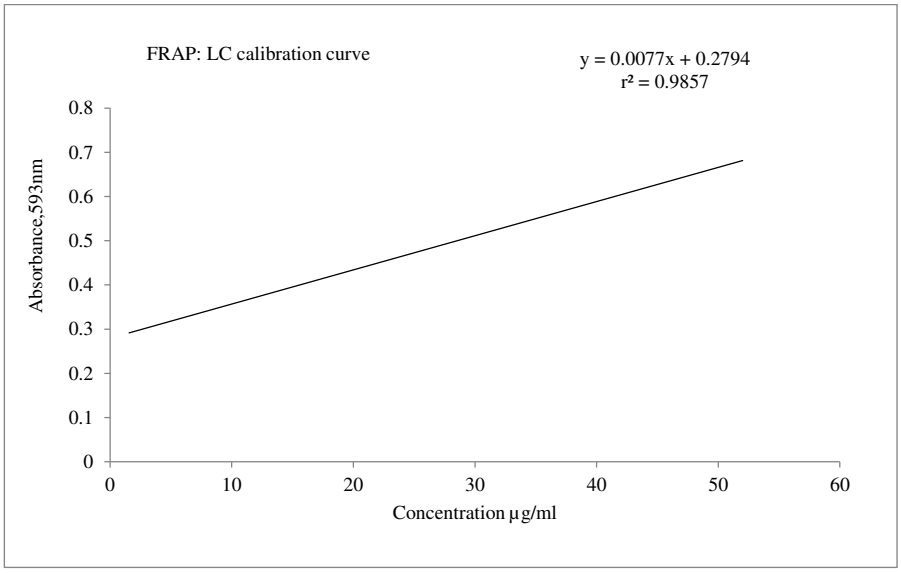


Figure 4.10: Leaf chloroform (LC) extract calibration curve in FRAP assay.

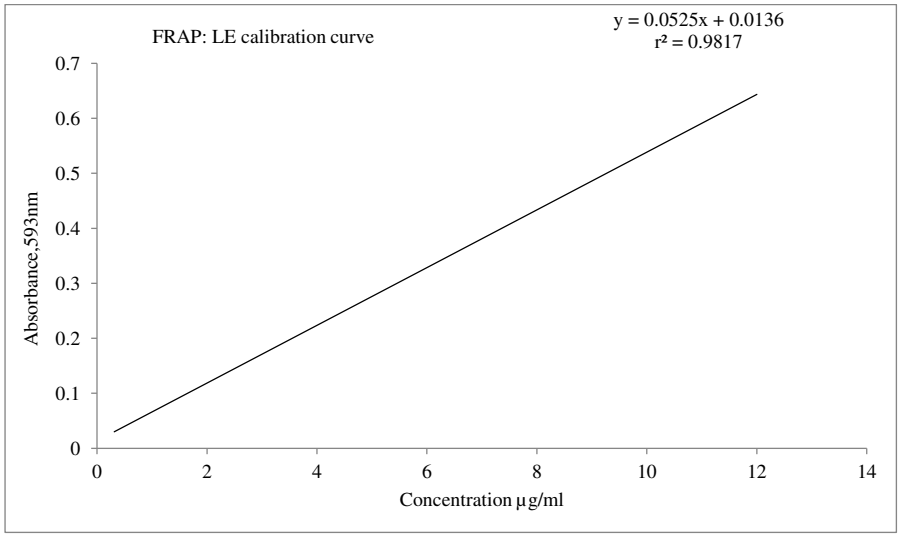


Figure 4.11: Leaf ethanol (LE) extract calibration curve in FRAP assay.

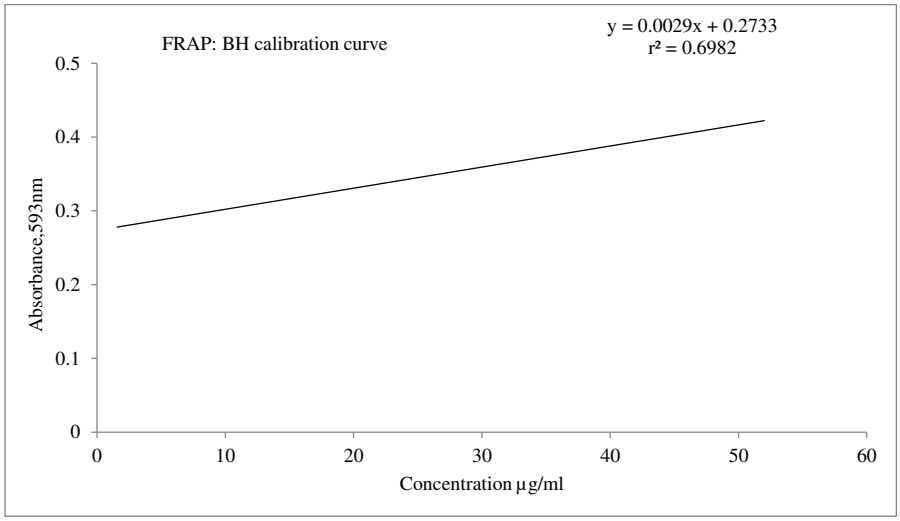


Figure 4.12: Bark hexane (BH) extract calibration curve in FRAP assay.

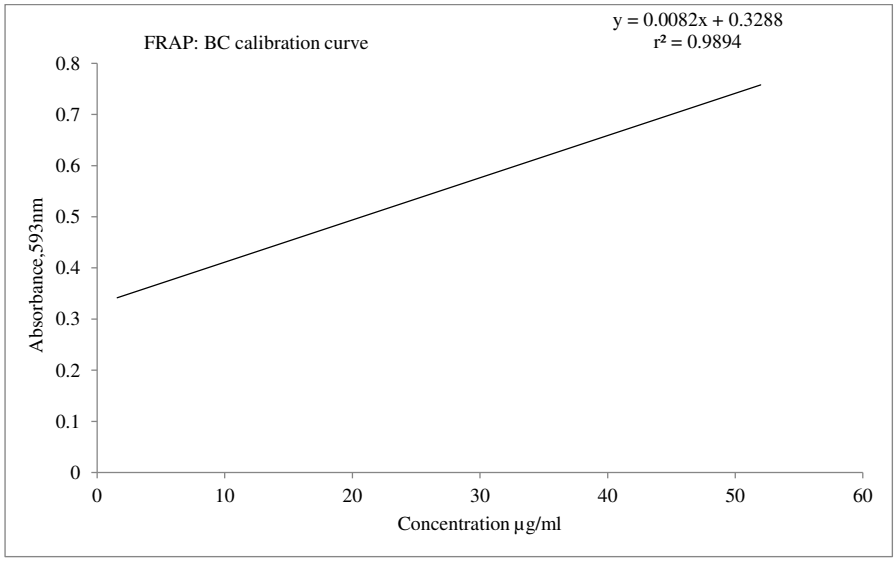


Figure 4.13: Bark chloroform (BC) extract calibration curve in FRAP assay.

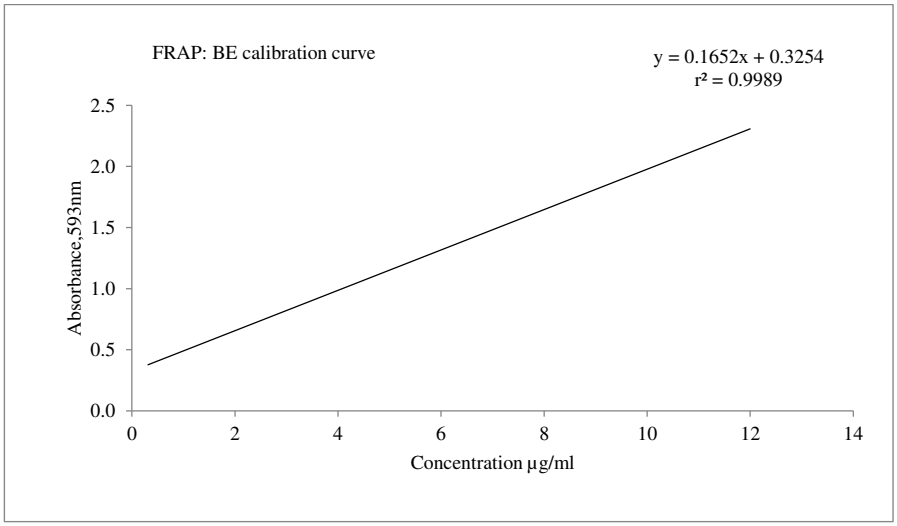


Figure 4.14: Bark ethanol (BE) extract calibration curve in FRAP assay.

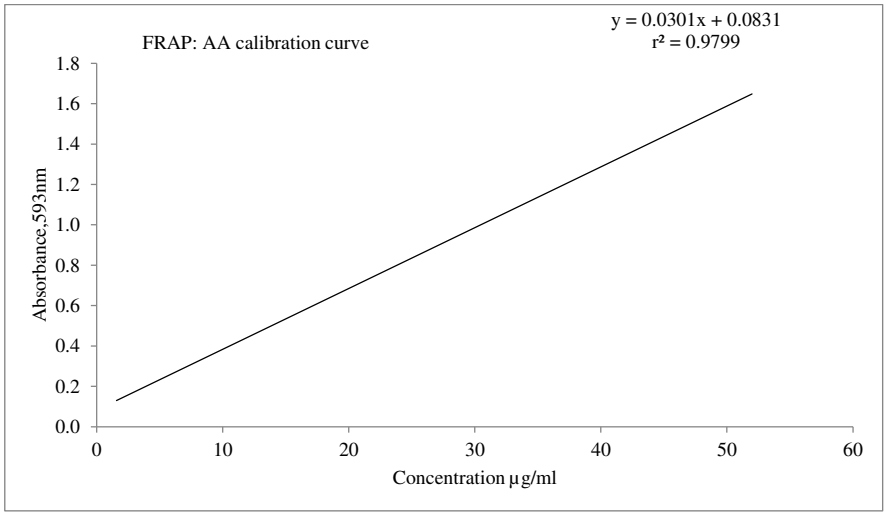


Figure 4.15: Ascorbic acid (AA) calibration curve in FRAP assay.

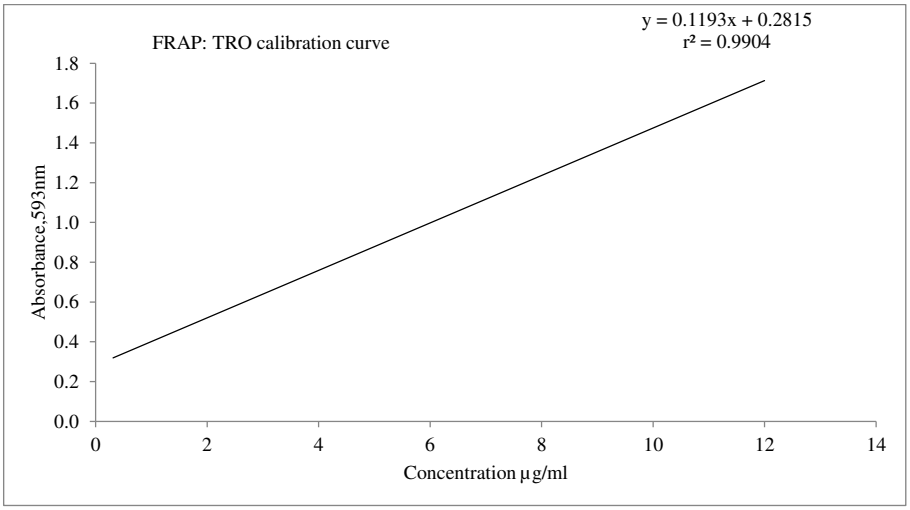


Figure 4.16: Trolox (TRO) calibration curve in FRAP assay.

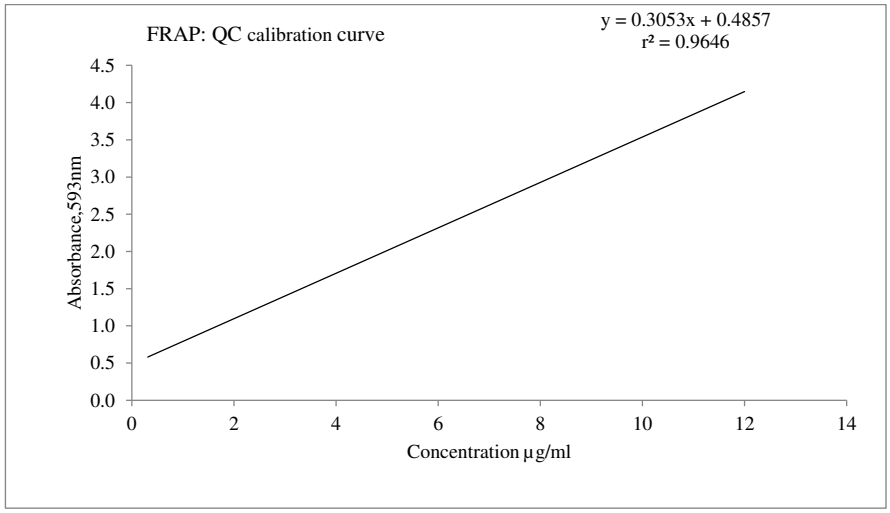


Figure 4.17: Quercetin (QC) calibration curve in FRAP assay.

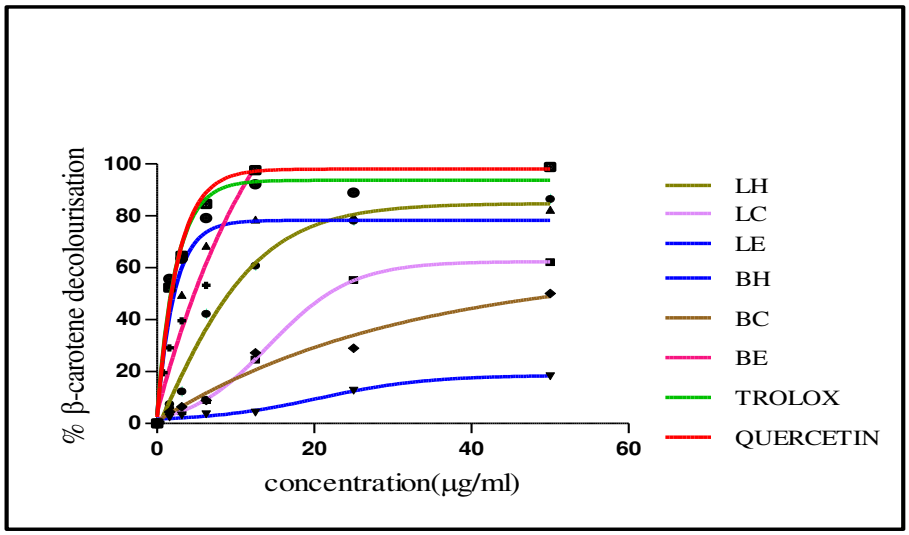


Figure 4.18: β-carotene decolourisation (%) of *Canarium patentinervium* Miq.

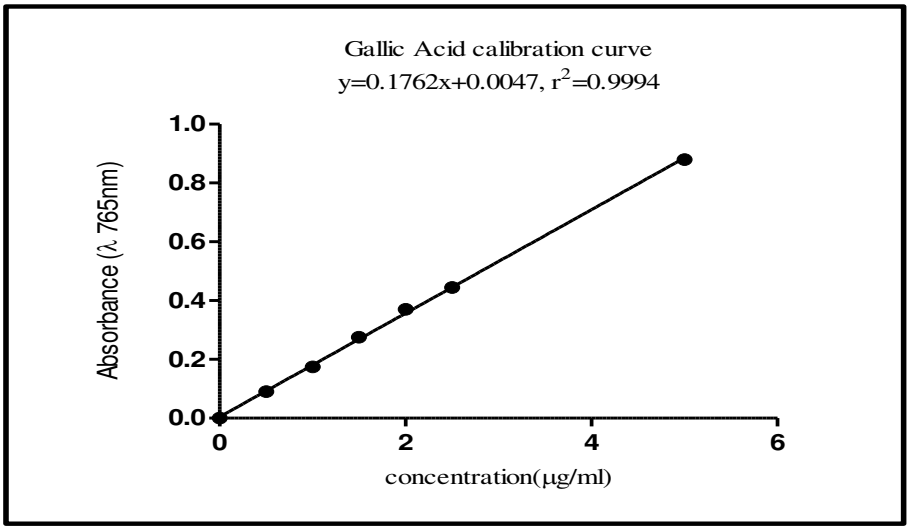


Figure 4.19: Gallic acid calibration curve in Folin-Ciocalteu assay.

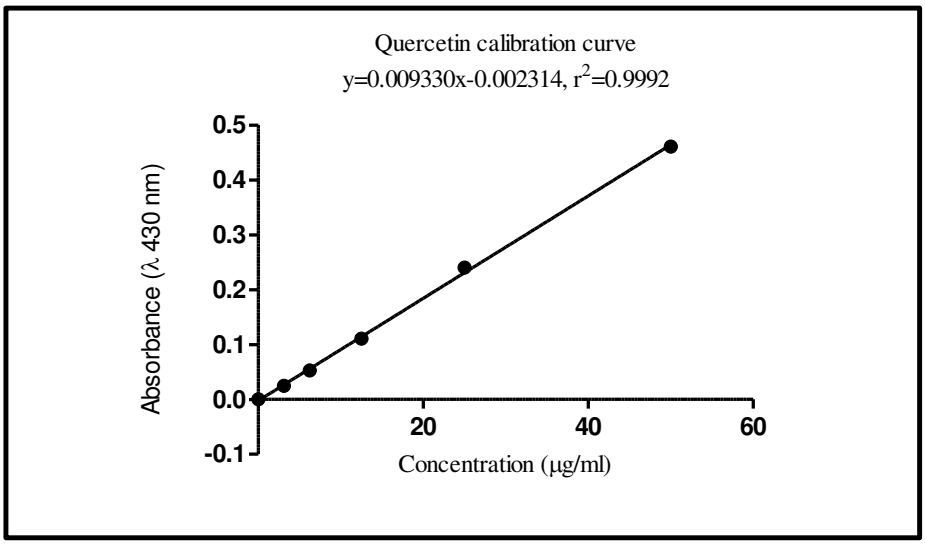


Figure 4.20: Quercetin calibration curve in total flavonoid content determination.

Table 4.2: Antioxidant values of *Canarium patentinervium* Miq.

Extracts	Total phenolic content, Gallic acid equivalent (mg/g)	Total flavonoid content, Quercetin equivalent (mg/g)	ABTS assay, EC_{50} (µg/ml)	DPPH assay, EC_{50} (µg/ml)	FRAP assay, FRAP value (µg/ml)	β-carotene bleaching assay, EC_{50} (µg/ml)	Superoxide dismutase assay IC_{50}(µg/ml)
LH	6.61±0.01	18.99±0.02	27.30±0.01	17.45±0.03	2845.00±0.15	9.39±0.05	80.54±0.03
LC	14.28±0.03	12.57±0.04	31.10±0.02	134.00±0.04	1329.00±0.18	21.81±0.04	26.84±0.03
LE	204.97±0.05	125.32±0.03	2.28±0.01	2.93±0.00^{B}	200.00±0.07	6.04±0.02	3.05±0.01
BH	5.19±0.01	1.55±0.02	521.00±0.01	1857.00±0.01	3531.00±0.14	125.00±0.08	238.00±0.04
BC	13.99±0.03	12.14±0.02	34.90±0.02	87.80±0.05	1242.00±0.19	50.40±0.03	20.13±0.02
BE	100.26±0.01	24.57±0.02	0.93±0.01^{A}	2.33±0.02	67.00±0.32	7.04±0.04	8.51±0.07
AA	NA	NA	1.54±0.06	1.88±0.01	347.00±0.23	NA	NA
QC	NA	NA	0.88±0.03^{A}	2.88±0.02^{B}	86.00±0.24	1.64±0.04^{C}	NA
TRO	NA	NA	0.68±0.02	4.77±0.04	33.00±0.54	1.65±0.03^{C}	NA
SOD	NA	NA	NA	NA	NA	NA	1.59±0.04

LH: leaf hexane extract, LC: leaf chloroform extract, LE: leaf ethanol extract, BH: bark hexane extract, BC: bark chloroform extract, BE: bark ethanol extract, AA: ascorbic acid, QC:quercetin, TRO: trolox and SOD: superoxide dismutase

Data were obtained from three independent experiments, each performed in triplicates (n=9) and represented as mean ± SD.

Values with the same capital letter are not significantly different (P<0.01) according to Tukey multiple comparison test.

Table 4.3: The mean r^2 values for correlation between total phenolics contents, total flavonoid contents and antioxidant activities (EC_{50})

	ABTS	**DPPH**	**FRAP**	**BC**
Total phenolic contents	0.1410	0.1285	0.5246	0.2287
Total flavonoid contents	0.1388	0.1319	0.3179	0.2146
ABTS	na	0.9977***	0.5398	0.9008**
DPPH	0.9977***	na	0.5029	0.8977**
FRAP	0.5398	0.5029	na	0.4770
BC	0.9008**	0.8977**	0.4770	na

** significant with $p<0.01$

*** significant with $p<0.0001$, na- not applicable

4.4 Discussion

In vitro antioxidant capacity can be determined by hydrogen atom transfer (HAT) method and single electron transfer (SET) method [56]. HAT based methods measure the ability of an antioxidant to scavenge free radical by hydrogen donation to form a stable compound. SET based methods detect the ability of the antioxidant to transfer one electron to reduce compound including metals, carbonyls and radicals [70][85]. β-carotene bleaching assay involves HAT method, FRAP assay involves SET method while DPPH and ABTS assay involves both method predominantly via SET method [55][57].

FRAP is the ferric reducing power of antioxidants by the reduction of the ferric ions to the ferrous ions, which form a blue colored ferrous-tripyridyltriazine complex (ferric TPTZ) which is detected at 593 nm. Deeper blue colour indicates higher antioxidant potential [86]. The samples absorbance equivalent to 1 mM $FeSO_4$ was calculated from equation of linearity of individual sample. Total FRAP value was determined from the absorbance obtained from samples above using the standard Fe (II) calibration curve equation ($y=0.0105x+0.0136$, $r^2=0.9817$). Ethanol extract of barks displayed significant ($p< 0.01$) FRAP value (67.00±0.32 μg/ml) compared to ascorbic acid (347.00±0.23 μg/ml) and quercetin (86.00±0.24 μg/ml).

In the β-carotene/linoleic model, linoleic acid reacts with ROS and O_2 to form an unstable peroxy radical. β-carotene being an antioxidant will react with this radical to form stable epoxide causing the bleaching of yellow solution. Competition reaction occurs with the presence of another antioxidant (sample) to react with the peroxy

radical resulting in slower bleaching of solution detected at 470 nm spectrophotometrically [87]. Ethanol extract of leaves and barks displayed best EC_{50} value of 6.04±0.02 μg/ml and 7.04±0.04 μg/ml respectively. Our results are in agreement to the previous reports which pointed out that ascorbic acid did not show its antioxidant activity under similar assay [88] Such phenomenon was termed as "polar paradox" whereby polar antioxidants remaining in the aqueous phase of the emulsion are more diluted in lipid phase and thus, they are less effective [89].

ABTS assay involves the reduction of the blue-green 2,2-azino-bis(3-thylbenzothiazoline-6- sulfonate) radical cation ($ABTS^{\cdot+}$) by antioxidants to its original colourless ABTS form. Greater discolourisation results in lower absorbance at 734 nm indicating higher antioxidant capacity [75][76]. Ethanol extract of the bark displayed significant ($p<0.01$) EC_{50} (0.93±0.01 μg/ml) compared to ascorbic acid (1.54±0.06 μg/ml).

The DPPH radical has a deep purple colour and absorbs strongly at a wavelength of 550 nm, whereas the yellowish reduction product $DPPH_2$ does not. In DPPH assay (another SET method), ethanol extract of leaves and barks displayed significantly lower EC_{50} value of 2.93±0.00 μg/ml and 2.33±0.02 μg/ml respectively compared to trolox (EC_{50} = 4.77±0.04 μg/ml) [90]. 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 [91]. 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 [92].

The DPPH and ABTS assays have the same mechanism of action, but, in most cases, the results obtained from the ABTS assay are higher than those from DPPH assay. It has been documented that results reported for the ABTS assay do not only take into account the activity of the parent compound, but also the contribution of reaction products and other individual compounds on the activity, which is not the case in the DPPH assay [93][94].

SOD which catalyses the dismutation of the superoxide anion (O_{2-}) into hydrogen peroxide and molecular oxygen, is one of the most important anti-oxidative enzymes. Preventive antioxidants, such as SOD are described either as preventing introduction of initiating radicals or as inhibiting the rate which new chains are set up. The analytical methods for determination of O_{2-} scavenging capacity employ enzymatic or nonenzymatic O_{2-} generation systems. In this study the enzymatic system that uses xanthine oxidase (XOD) to generate O_{2-} radicals was used [57]. XOD was used for formation of O_{2-} that is the reduced to oxygen by SOD-like activities in samples. The WST-1 is used as a probe that undergoes competitive reaction with SOD-like samples to reduce O_{2-} to oxygen forming a formazan coloured dye. Thus higher rate of SOD activity results in less dye formation and less absorbance. The ethanol extract of the leaves shows highest SOD-like activity with IC_{50} of 3.05±0.01µg/ml.

In the above experiments, the ethanol extract of leaves and barks displayed superior antioxidant capacities. The EC_{50} values of the samples were consistently low in SET

methods (ABTS, DPPH and FRAP) superior to standard as opposed to HAT method (β-carotene bleaching) assay. The present study demonstrates that *Canarium patentinervium* Miq. is a potent source of antioxidants that exhibits its antioxidant activity predominantly via the SET method. Several reports emphasized on the fact that there is a positive relationship between total phenolic contents and antioxidant activity [95][96]. Our study demonstrated the existence of a low positive correlation between the total phenolic contents and the FRAP values ($y = -0.0125x + 2.2564$, $r^2 = 0.5246$) and total phenolic contents and β-carotene bleaching values ($y = -0.2747x + 52.42$, $r^2 = 0.2287$), respectively. Therefore, one could draw an inference that non phenolic by itself or in synergy with the phenolics and flavonoids impart *Canarium patentinervium* Miq its antioxidant properties.

4.5 Conclusion

The present study demonstrates that *Canarium patentinervium* Miq. is a potent source of antioxidants that exhibits its antioxidant activity predominantly via the SET method. 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, phenolic acids, tocopherol, and cathecheses. Tannins are, at least in part, responsible for the free radical scavenging activities working synergistically with other antioxidant substances [86].

CHAPTER 5

ANTI-INFLAMMATORY ACTIVITY

5.1 Introduction

5.1.1 Inflammation and arachidonic system

Over the last 10 years, a significant body of evidence has emerged indicating that chemically diverse classes of naturally-occurring substances derived from higher plants are of potential interest for therapeutic interventions in several inflammatory diseases [97]. Inflammation response is a critical protective reaction to irritation, injury, or infection, characterised by redness, heat, swelling, loss of function and pain. Redness and heat result from an increase in blood flow, swelling is associated with increased vascular permeability, and pain is the consequence of activation and sensitisation of primary afferent nerve fibres. In normal conditions, these changes in inflamed tissue serve to isolate the effects of the insult and thereby limit the threat to the organism [97].

In recent years, roles have been identified for several inflammatory cells and for a large number of inflammatory mediators in important pathologies not previously linked to inflammation, such as Alzheimer's disease and cardiovascular disorders including atherosclerosis, as well as cancer [98]. These findings have significantly increased the importance of understanding the cellular and molecular mechanisms forming the basis of the inflammatory process and in identifying new targets for the

development of innovative and safe therapeutic strategies for the management of inflammatory diseases. Most plant-derived secondary metabolites are known to interfere directly or indirectly with the following molecules and/or mechanisms [98][99]:

1. various inflammatory mediators (eg arachidonic acid metabolites, peptides, cytokines, excitatory amino acids etc).
2. the production and/or action of second messengers (such as cGMP, cAMP, various protein kinases, and calcium among others).
3. the expression of transcription factors such as AP-1, NF-κB, and proto-oncogenes (*c-jun, c-fos, and c-myc*),
4. the expression of key pro-inflammatory molecules such as inducible NO synthase (iNOS), cyclooxygenase (COX-2), cytokines (IL=1β, TNF-α etc), neuropeptidases and proteases.

In this study we reviewed and investigated the contribution of naturally occurring substances from higher plants acting on the arachidonic acid pathway. Inflammation in injured cells is both initiated and maintained by the overproduction of prostaglandins and leukotrienes, which are produced by separate enzymatic pathways, viz. the cyclo-oxygenase (COX) and lipoxygenase (LOX) pathways, respectively. Both the prostaglandins as well as the leukotrienes are biosynthesised on demand from arachidonic acid, which is a 20-carbon fatty acid, derived from the breakdown of cell membrane phospholipids by any number of phospholipase A_2 (PLA_2) isoforms [100] (Figure 5.1).

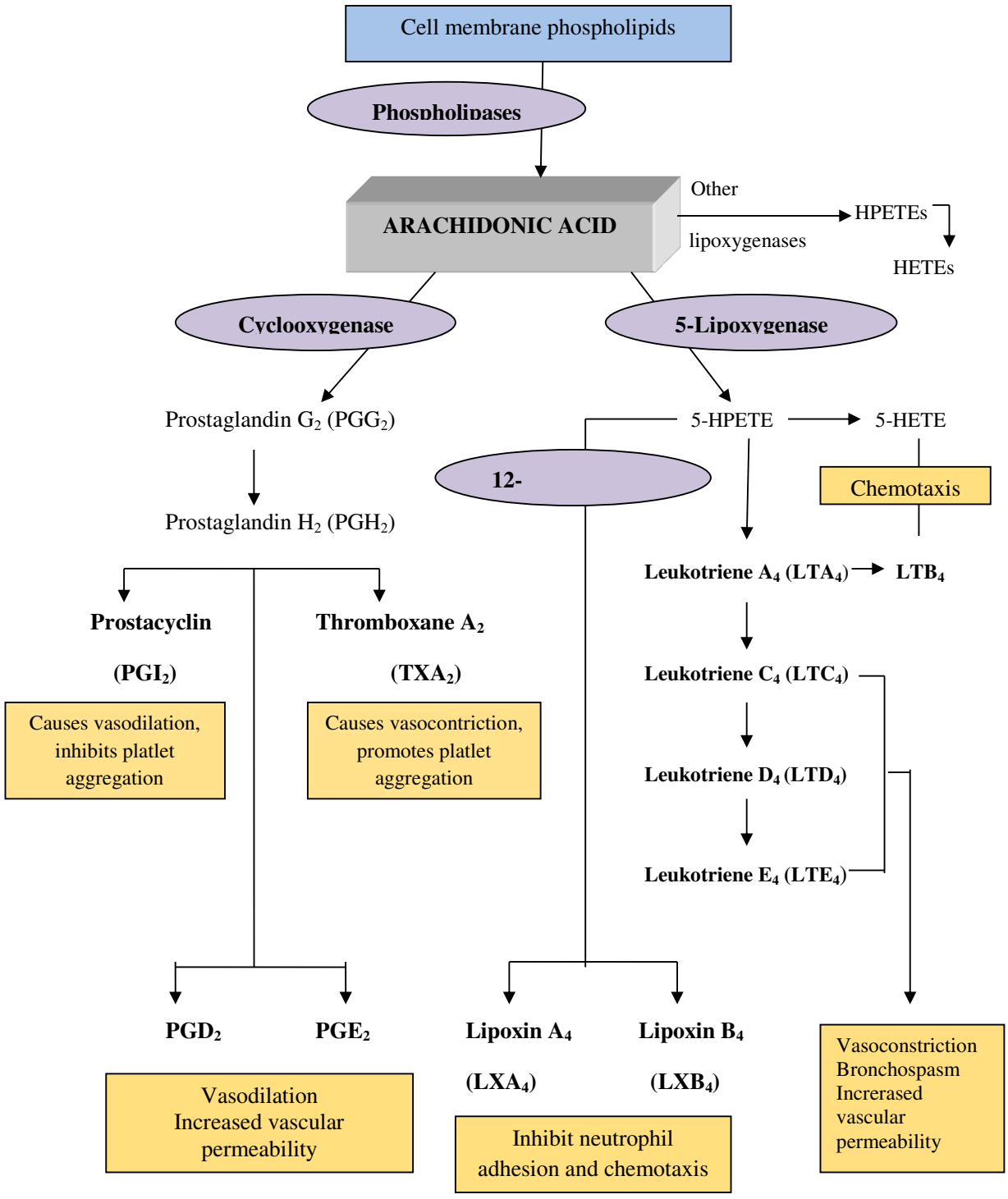


Figure 5.1: Arachidonic acid metabolites and inflammation [101].

Arachidonic acid is normally stored in membrane-bound phospholipids and released by the action of phospholipases. Enzymatic conversion of released arachidonic acid into biologically active derivatives proceeds through one of several routes [99]. Cyclooxygenase converts arachidonic acid to unstable cyclic endoperoxides from which prostaglandins, prostacyclin and thromboxanes are derived. Formation of the leukotrienes from arachidonic acid is initiated by the action of 5-lipoxygenase producing leukotriene A_4. Hydrolysis of leukotriene A_4, or the incorporation of glutathione results in the formation of leukotriene B_4 and C_4, respectively [102]. In addition, 12- and 15-lipoxygenase can catalyse arachidonic acid conversion and lipoxins A and B are amongst the possible products. Many of these metabolites of arachidonic acid feature prominently in the development of inflammation. Prostaglandin E_2 and prostacyclin are potent vasodilators, while leukotriene D_4 causes cellular adhesion, chemotaxis of neutrophils and degranulation. Leukotrienes C_4, D_4 and E_4 contribute to inflammation by increasing vascular permeability. Leukotrienes are also believed to play an important pathophysiological role in allergic broncho-constriction of asthma [103].

Through pharmacological intervention in the arachidonic acid cascade various anti-inflammatory agents have been developed. These include aspirin-like drugs, which inhibit cyclo-oxygenase. Corticosteroids appear to indirectly inhibit phospholipases thus preventing release of arachidonic acid. Future progress in this field is likely to produce drugs which antagonise arachidonic acid derivatives or inhibit the enzymes involved in their synthesis with greater specificity [99][103].

5.1.2 The lipoxygenase system

5-LOX presents either in the cytosol or the nucleus of a resting cell (depending on the cell), as a soluble enzyme. Upon cellular stimulation, 5-LOX and cytosolic phospholipase A_2 ($cPLA_2$) co-migrate to the nucleus. It is here that cPLA2 liberates arachidonic acid from the membrane phospholipids [104] (Figure 5.2). Arachadonic acid is the main substrate for the lipoxygenase enzyme, and is presented to 5-LOX for metabolism by the 5-lipoxygenase activating protein (FLAP). The metabolism of arachidonic acid produces leukotrienes and lipoxins via the LOX pathway [105]. 5-LOX, catalysing the oxidation of arachidonic acid, produces 5(*S*)-hydroxyperoxyeicosatetraenoic acid (5-HPETE), a hydroperoxide intermediate, which undergoes dehydration, resulting in the formation of leukotriene A_4 (LTA_4). Enzymatic hydrolysis of LTA_4, as well as conjugation with other substances, leads to the formation of inflammatory mediators [106]. These inflammatory mediators are responsible for the powerful chemo-attractive effects on the eosinophils, neutrophils and macrophages, as well as the increased release of pro-inflammatory cytokines by macrophages and lymphocytes.

Two other pathways in the LOX system, 15-LOX and 12-LOX, are responsible for the production of lipoxins, the latter having the potential to counteract the pro-inflammatory effects of the leukotrienes. 5-Lipoxygenase is inhibited by quinones, hydroxyquinones, and a variety of phenolic compounds, including certain flavonoids such as, quercetin, kaempferol, morin, myricetin and cirsiliol [107]. At the onset of the inflammatory process, arachidonic acid is converted to eicosanoids and leukotriene B_4 (LTB_4) by LOX [104], which is coupled with the production of

prostaglandins and thromboxanes by cyclooxygenase (COX). The leukotrienes present at the onset of inflammation are also responsible for the maintenance thereof. Leukotrienes have been identified as mediators of a number of inflammatory and allergic reactions [103]..

These include rheumatoid arthritis, inflammatory bowel disease, atopic dermatitis, psoriasis, chronic urticaria, asthma [108] and allergic rhinitis [109][103]. The 5-LOX pathway has also recently been linked to the development of atherosclerosis, osteoporosis and certain types of cancers [105]. As a result of the pathophysiological implications of 5-LOX products and the potential benefits of anti-leukotriene therapy, different strategies have been employed (targeting PLA_2, 5-LOX, FLAP, LTA_4 hydrolase and leukotriene C_4 (LTC_4) synthase with 5-LOX being the ideal and most promising target [105].

5.1.3 The cyclooxygenase system

Cyclooxygenase (COX) is a key enzyme that catalyzes the transformation of arachidonic acid to several biologically active eicosanoids, such as prostaglandins (PGs), thromboxane (e.g., TxA_2), and prostacyclin (e.g., PGI_2). This enzyme actually catalyzes two major reactions, as shown in Figure 5.3. Arachidonic acid, the most important physiological precursor of eicosanoids biosynthesis, can be released from the membrane phospholipid by activation of phospholipase A_2 (PLA_2) and/or phospholipase C (PC). Cyclooxygenase then catalyzes the addition of molecular oxygen at the C-11 of arachidonic acid to form the 11-peroxy intermediate, following

by rearrangement to a cyclic endoperoxide and introduction of a second molecule of oxygen at C-15 to form prostaglandin G_2 (PGG_2).

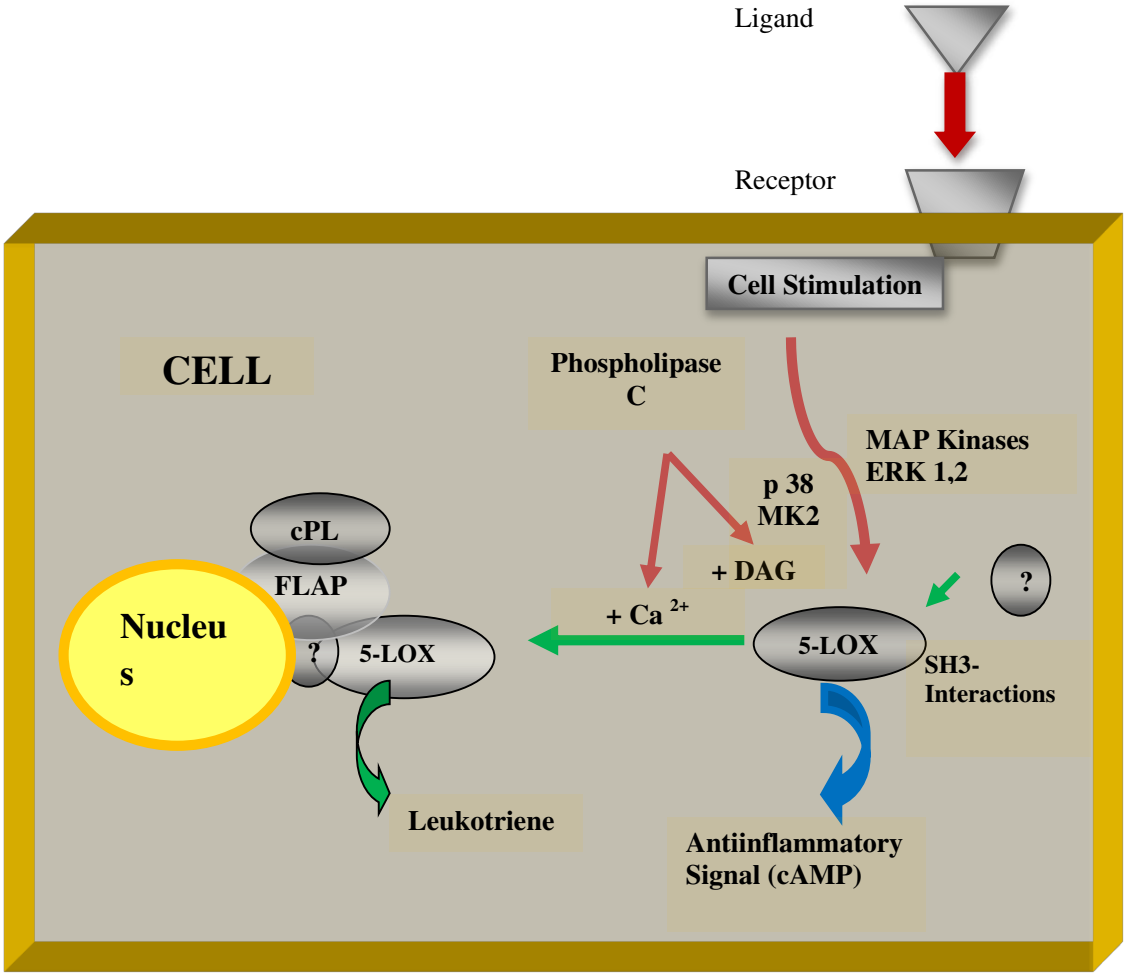


Figure 5.2: The translocation of 5-lipoxygenase and cytosolic phospholipase A_2, upon cellular stimulation, to the nuclear membrane, followed by substantial generation of leukotrienes [105]. 5-LOX = 5-lipoxygenase, $cPLA_2$ = cytosolic phospholipase A_2, FLAP = 5-lipoxygenase activating protein, PKA = protein kinase A, cAMP = cyclic adenosine monophosphate, MK2 = kinases, ERK1,2 = extracellular signal-regulated kinase 1,2.

The hydroperoxidase activity reduces PGG_2 to generate PGH_2 which is the immediate precursor for production of eicosanoids, such as PGD_2, PGE_2, PGF_{2a}, PGI_2, and TxA_2, through corresponding synthase activities. These arachidonic acid metabolites derived from PGH_2 are important mediators of inflammatory responses, immunological effects, and tumour development [100].

Cyclooxygenase (COX) exist as three isoforms: cyclooxygenase-1 (COX-1), expressed in most cells constitutively, inducible cyclooxygenase-2 (COX-2), triggered by pro-inflammatory stimuli, and COX-3, present mainly in the cerebral cortex and human heart [110][111] . COX-1 is a constitutive form of the enzyme that has been linked to the production of physiologically important prostaglandins that may play a role in homeostasis (gastric, renal, etc). COX-2 exists in a constitutive form in kidney and brain and is inducible by cytokines and growth factors. Induction of the COX-2 is linked to inflammatory cell types and tissues and COX-2 is believed to be the target for the anti-inflammatory actions of NSAIDs. Thus there is an on-going effort to identify compounds that will inhibit COX-2 in preference to COX-1 since such an agent may be safer and perhaps more efficacious [112].

Despite of the undesired side effects such as increased cardiovascular risk of COX-2-selective inhibitors, much research spanning many years has been focused on finding COX-2 selective inhibitors, owing to the remarkable reduction of adverse gastrointestinal and renal effects associated with conventional non-steroidal anti-inflammatory drugs (NSAIDs) as well as to their potential therapeutic benefits in several diseases, including certain types of cancer [113]. Thus, it is of value to study cyclooxygenase inhibitors.

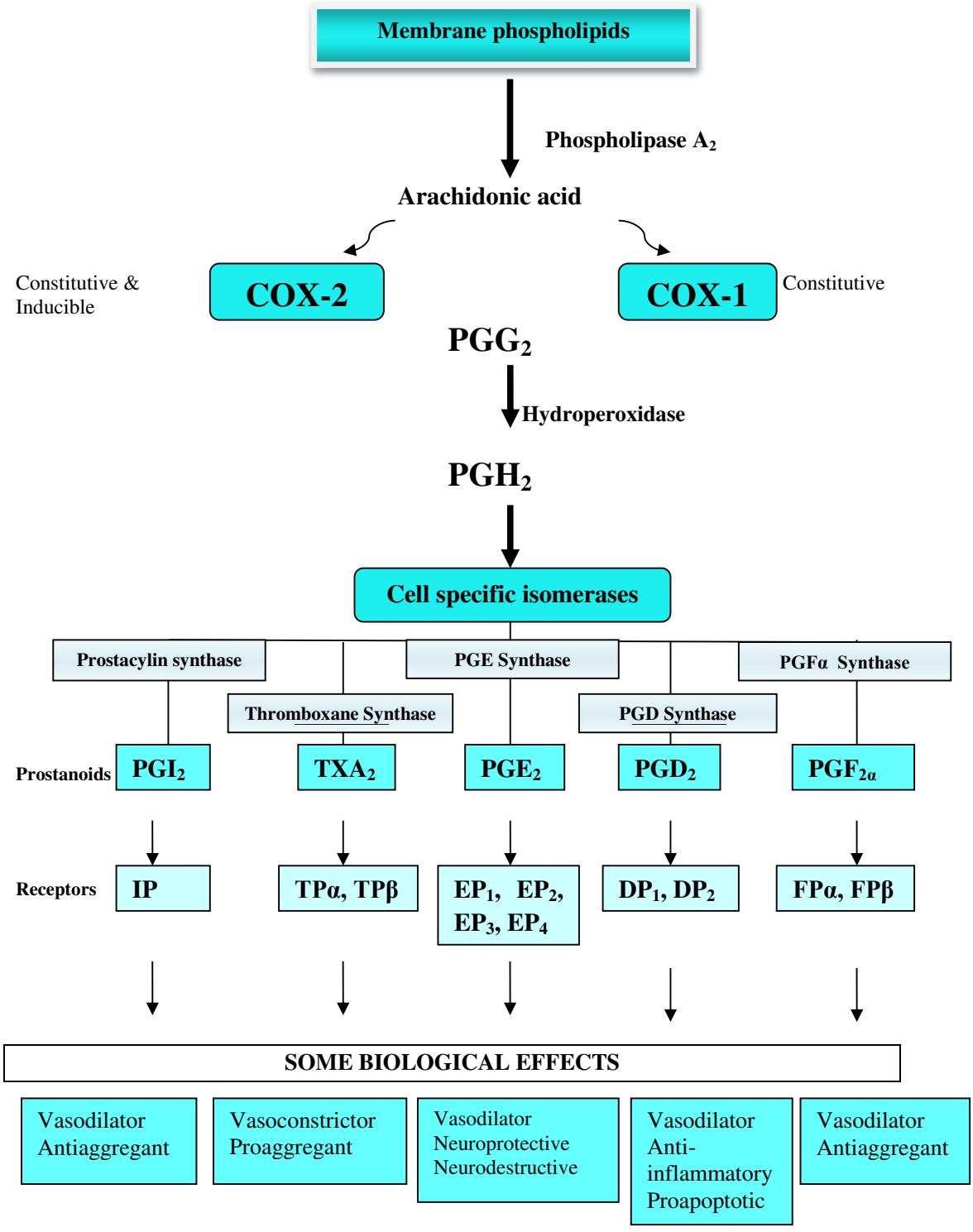


Figure 5.3: Cyclooxygenase and arachidonic acid metabolism.

5.1.4 Dual inhibitors

Conventional pharmacological management of inflammatory disease like osteoarthritis involves treatment with non-steroidal anti-inflammatory drugs (NSAIDs) or selective COX-2 inhibitors that block the formation of PGs without modulating 5-LOX enzyme activity. Inhibition of one or both of the COX enzymes may "shunt" AA metabolism down the 5-LOX pathway, which can aggravate toxicity associated with the lack of PGs and excess production of LTs. For example, NSAID-induced gastric ulcers have been shown to have high concentrations of LTB_4 in their walls, which attracts leukocytes to the stomach and may contribute to ulceration [114][115].

Recently, reports have appeared regarding so-called "dual inhibitors," agents that inhibit not only COX-1 and COX-2, but also 5- LOX [116][117][118][119]. These agents with anti-oxidative properties may be particularly effective for managing the metabolic processes underlying inflammatory conditions and reducing both gastric and cardiovascular side effects by balancing AA metabolism in the body. This dual inhibition activity will be investigated for this plant.

5.1.5 Anti-inflammatory potential of *Canarium* L.

The essential oil of *Canarium schweinfurthii* Engl. collected from the region of Cameroon inhibited the enzymatic activity of lipoxygenase with an IC_{50} value of 62.6 ppm [19]. However in a separate study of the same species collected from central African region did not show any activity in the cotton pellet induced granuloma

formation experiment [13]. This may suggest possible evidence of variety between secondary metabolites constituents according to regions. The essential oil of *Canarium schweinfurthii* Engl. at a dose of 1, 2 and 3 mg/ kg i.p. displayed potent analgesic effect in the acetic acid-induced writhing and hot plate experiments [13].

5.2 Materials and methods

5.2.1 Anti-inflammatory determination assays

The anti-inflammatory activity of the plant extracts was determined using the *in vitro* 5- LOX assay. This assay measures the inhibitory activity against the 5-LOX enzyme, which is a key enzyme in the metabolism of arachidonic acid that is responsible for the formation of leukotrienes (which play a pivotal role in the pathophysiology of chronic inflammatory and allergic diseases) as first determined by Sircar *et al*. [120] and later modified by Evans [121].

There are three ways to measure COX activity, one is to measure the final product of the reaction, PGE_2; another is to measure directly the oxygen uptake that occurs during the first step of the reaction using the oxygen sensor and third is to measure the second reaction (peroxidase function) spectrophotometrically [112]. The peroxidase co-substrate oxidation assay can detect cyclooxygenase inhibitors which may directly interfere with enzyme activity or scavenge the free radical intermediates to block the reaction process. However, the oxygen consumption assay is mainly reflective of inhibitors which block the interaction of enzyme and arachidonic acid. We currently

describe a peroxidase co-substrate oxidation assay system adapted to a 96-well plate format. This requires less enzyme and reaction material and yields savings in time and money. The peroxidase co-substrate oxidation assay is useful as a pre-screening method [100][112].

5.2.2 The 5-LOX inhibition assay

5.2.2.1 Principle of the assay

The 5-LOX assay was conducted according to the method described by Baylac & Racine [122] with some modifications. Lipoxygenases (LOX) are dioxygenases that catalyse the addition of molecular oxygen to polyunsaturated fatty acids containing a 1,4-pentadiene group. The 5-LOX enzyme thus converts its substrate, arachidonic acid, to the conjugated diene product 5-hydroxy- 6,8,11,1-eicosatetraenoic acid (5-*S*-HETE), which, in turn, is converted to LTA_4, and then to LTB_4, by LTA_4 hydrolase. For the purposes of this experiment, linoleic acid was used as the substrate, as it shares a high degree of structural similarity to arachidonic acid (containing the 1,4-pentadiene group in question), it is far easier to handle as well as having a stronger affinity for the 5-LOX enzyme resulting in greater UV absorbance readings [123].

The experiment specifically determines increases in absorbance at 234 nm as a result of the formation of conjugate double bonds in linoleic acid hydroperoxide (from a 1,4-diene to a 1,3-diene), as used in the biochemical evaluation of the LOX pathway of soybean plants submitted to wounding [124].

5.2.2.2 Protocol

5-LOX enzyme (human recombinant from Calbiochem) was used. Ice-cold buffer (potassium phosphate) at 4 ^{o}C was mixed with 100 U of the thawed enzyme. Twenty microliters of samples dissolved in DMSO was plated out in triplicate in a 96-well microtiter plate at different concentrations, followed by 160 µl of 0.1 M potassium phosphate buffer (pH 6.3) maintained at 25 ^{o}C and 20 µl of enzyme solution. Mixture was agitated and 10 µl of linoleic acid was added and incubated for 10 mins at 25 ^{o} C. Absorbance was recorded at 234 nm using Thermo Scientific Varioskan Flash microtiter plate reader, linked to a computer equipped with (SkanIt Software 2.4.3). 5-Lipoxygenase is known to catalyse oxidation of unsaturated fatty acids containing 1-4 diene and the modification of linoleic acid (1-4-diene into 1- 3-diene) can be detected at 234 nm. Percentage inhibition of enzyme was determined by comparison of rates of reaction of samples relative to blank sample using the formula (E-S)/E x 100, where E is the activity of enzyme without test sample and *S* is the activity of enzyme with test sample. The experiments were done in triplicate. Nordihydroguaiaretic acid (NDGA) was used as positive control.

5.2.3 The peroxidase endpoint assay for COX-1 and COX-2

5.2.3.1 Principle of the assay

The COX-1 and COX-2 peroxidase endpoint assay was conducted according to method described by Gierse *et al.* [112]. The peroxidase endpoint assay system is based on sequential reactions of the cyclooxygenase (generating PGG_2) and the

peroxidase (converting PGG_2 to PGH_2) activities. Its success relies on the peroxidase co-substrate being transformed to a comparatively stable chromophore in a predictable stoichiometric relationship to the reduction of PGG_2. *N,N,N',N'*-tetramethyl-p-phenylenediamine (TMPD) is a convenient co-substrate for use in the assay.

5.2.3.2 Protocol

COX-1 and COX-2 enzyme from Calbiochem was used. Twenty microliters of sample dissolved in DMSO was plated in triplicates at different concentrations in a 96-well microtiter plate followed by 20 µl of enzyme solution. Then, 160 µl of endpoint assay mix was added and incubated for 10 mins at room temperature. Absorbance was recorded at 611 nm using Thermo Scientific Varioskan Flash microtiter plate reader, linked to a computer equipped with (SkanIt Software 2.4.3). Percentage inhibition of enzyme was determined by comparison of rates of reaction of samples relative to blank sample using the formula (E-S)/E x 100, where E is the activity of enzyme without test sample and *S* is the activity of enzyme with test sample. Indomethacin was used as positive control.

5.2.4 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. All data were expressed as mean ± standard deviation. SD was computed but values were not shown in the graphs via this software. Data were analyzed using one way Anova followed by Tukey test using GraphPad Prism5 software. A significant difference was considered at the level of $P < 0.01$.

5.3 Results

5.3.1 The 5-LOX inhibition assay

In the 5-LOX assay, chloroform extract of the barks displayed potent enzyme inhibition (IC_{50}=29.53±0.03 µg/ml) which was close to nordihydroguaiaretic acid (IC_{50}=29.19±0.02 µg/ml). Ethanol extract of leaves and barks follow through with IC_{50}=49.66±0.02 µg/ml and IC_{50}=59.06±0.07 µg/ml respectively. Thus the potency of activity against 5-LOX was BC > LE > BE (Table 5.1.). According to the 5-LOX enzyme inhibition activity measurement guide by Kamatou *et al*. [94], [IC_{50} <30 µg/ml: good activity; 30 < IC_{50} <80 µg/ml : moderate activity; IC_{50} >80 µg/ml : poor activity] chloroform extract of barks of *Canarium patentinervium* Miq displays good 5-LOX enzyme inhibition activity followed by moderate activity by ethanol extract of leaves and barks.

5.3.2 The peroxidase endpoint assay for COX-1 and COX-2

In the COX assay, only ethanol extract of leaves and barks exhibited IC_{50} below 100 µg/ml. Ethanol extract of leaves had superior COX-1 inhibition (IC_{50} = 0.60±0.01 µg/ml) compared to COX-2 inhibition (IC_{50} = 1.07±0.01 µg/ml) (Figure 5.5) whereas the barks had superior COX-2 inhibition (IC_{50} = 9.39±0.03 µg/ml) as opposed to COX-1 (IC_{50} = 11.41±0.03 µg/ml) (Figure 5.4) .

5.4 Discussion

These assay measures the inhibitory activity against 5-LOX and COX enzymes. At the onset of the inflammatory process, arachidonic acid is converted to eicosanoids and leukotriene B4 (LTB_4) by LOX [104], which is coupled with the production of prostaglandins and thromboxanes by cyclo-oxygenase (COX). 5-LOX is the key enzyme in the metabolism of arachidonic acid that is responsible for the formation of leukotrienes which play a pivotal role in the pathophysiology of chronic inflammatory and allergic diseases. Lipoxygenase catalysing the oxidation of arachidonic acid, produces 5(*S*) - hydroxyperoxyeicosatetraenoic acid (5-HPETE), a hydroperoxide intermediate, which undergoes dehydration, resulting in the formation of leukotriene A_4 (LTA_4). Enzymatic hydrolysis of LTA_4, as well as conjugation with other substances, leads to the formation of inflammatory mediators [106]. These inflammatory mediators are responsible for the powerful chemo-attractive effects on

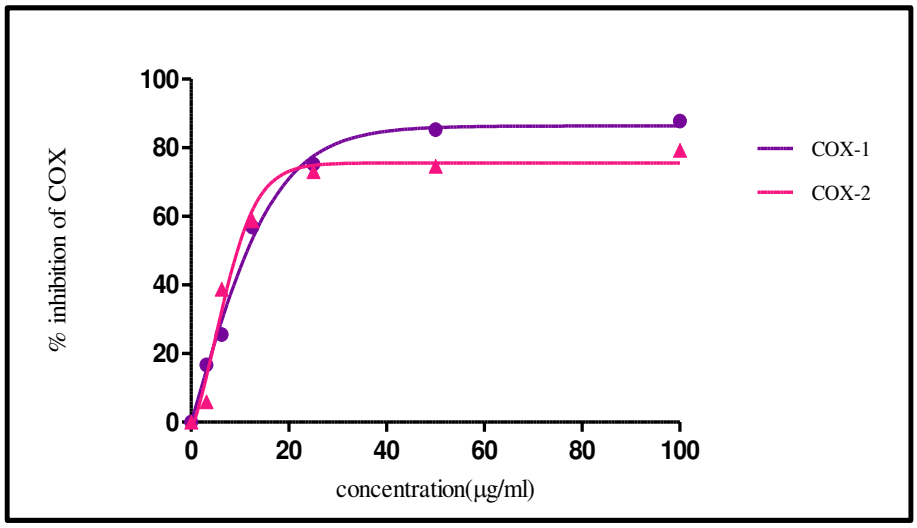


Figure 5.4: COX inhibition by BE.

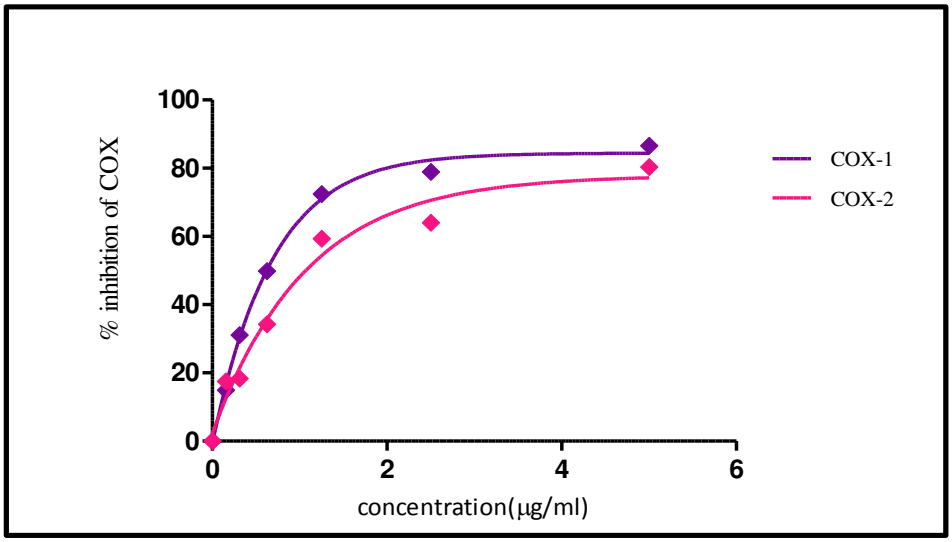


Figure 5.5: COX inhibition by LE.

Table 5.1.Anti-inflammatory values of *Canarium patentinervium* Miq.

Extracts	Anti-inflammatory assay 5-LOX, IC_{50} (µg/ml)	COX-1 inhibition assay IC_{50}(µg/ml)	COX-2 inhibition assay IC_{50}(µg/ml)	COX-1/COX-2 ratio
LH	206.00±0.02	>100	>100	NA
LC	104.69±0.04	>100	>100	NA
LE	49.66±0.02	0.60±0.01	1.07±0.01	0.56
BH	110.07±0.04	>100	>100	NA
BC	29.53±0.03	>100	>100	NA
BE	59.06±0.07	11.41±0.03	9.39±0.03	1.22
NDGA	29.19±0.02	NA	NA	NA
INDO	NA	0.29±0.04	0.26±0.03	1.11

LH: leaf hexane extract, LC: leaf chloroform extract, LE: leaf ethanol extract, BH: bark hexane extract, BC: bark chloroform extract, BE: bark ethanol extract, AA: ascorbic acid, QC: quercetin, TRO: trolox and INDO: indomethacin.

Data were obtained from three independent experiments, each performed in triplicates (n=9) and represented as mean ± SD. Values with the same capital letter are not significantly different (P<0.01) according to Tukey multiple comparison test.

the eosinophils, neutrophils and macrophages, as well as the increased release of pro-inflammatory cytokines by macrophages and lymphocytes [104].

Other inflammatory mediators such as histamine and immunoglobulin E, cause spasms in the smooth muscle of bronchi and blood vessels, playing an eminent role in asthmatic attacks. As leukotrienes thus play a major role in the pathophysiology of chronic inflammatory diseases, it has been suggested that 5-LOX inhibitors may thus be useful in the treatment of various conditions [125]. The 5-LOX pathway has also been implicated in cardiovascular disease, including atherosclerosis, stroke, myocardial infarction and the weakening of large artery walls and the formation of aneurysms [126][105]. The presence of the 5-LOX pathway, the production of leukotrienes and presence of the enzymes concerned, as well as leukotriene receptors is expressed in diseased tissue. Genetic studies have been carried out on mice linking the 5-LOX pathway to atherosclerosis, and population genetic studies involving humans, correlates genotypes of 5-LOX, FLAP and LTA_4 hydrolase to cardiovascular disease, as shown in studies by Dwyer *et al*. [127] and Helgadottir *et al*. [128][129][130].

The results obtained from the leaves and barks of the plant indicate good anti-inflammatory activity via 5-LOX inhibition cascade in the chloroform extract of the barks (IC_{50}=29.53±0.0 3µg/ml) when compared to NDGA (IC_{50}=29.19±0.02 µg/ml). Moderate activity was shown in BE and LE while poor activity was depicted in LH and BH in accordance with measure of activity by Kamatou *et al.* [94], [IC_{50} <30 µg/ml: good activity; 30 < IC_{50} <80 µg/ml : moderate activity; IC_{50} >80 µg/ml : poor activity].

COX (prostaglandin H_2 synthase) inhibition is the mechanism of action of most NSAIDS. COX-1 is a constitutive form of the enzyme that has been linked to the production of physiologically important prostaglandins that may play a role in homeostasis (gastric, renal, etc). COX-2 is a form of the enzyme that is inducible by cytokines and growth factors. Induction of COX-2 is linked to inflammatory cell types and tissues. There is an ongoing effort to identify compounds that might inhibit COX-2 in preference to COX-1 since such an agent may be safer and perhaps more efficacious. In this study, COX activity is measured by utilizing peroxidase activity and the electron donor TMPD, which turns blue upon reduction as a co-substrate. Arachidonic acid is used as a substrate which must first be converted to hydroperoxide thus yielding an indirect measure of COX activity. This assay has been noted to be a high-thorough put method [112].

In this study, only the ethanol extract of leaves and barks showed inhibition against COX-1 and COX-2 enzymes with IC_{50} below 100 µg/ml. Ethanol extract of leaves had superior COX-1 inhibition (IC_{50} = 0.60±0.01µg/ml) compared to COX-2 inhibition (IC_{50} = 1.07±0.01 µg/ml) whereas the barks had superior COX-2 inhibition (IC_{50} = 9.39±0.03 µg/ml) as opposed to COX-1 (IC_{50} = 11.41±0.03 µg/ml).

In order to determine the inhibitory selectivity on COX-1 and COX-2 enzymes, the COX-1/COX-2 ratio was applied. According to Burnett *et al.* [131], A COX-1 selective inhibitor will have ratio <1, whereas a COX-2 selective inhibitor will have ratio of > 1. In this study ethanol extract of bark was COX-2 selective (ratio 1.22) while the leaves were COX-1 selective (ratio 0.56). The COX-2 selective behaviour

of the ethanol extract of the barks was similar to positive control indomethacin (ratio 1.11). This indicates the presence of lead compound that would have a more potent COX-2 selectivity than indomethacin in the ethanol extract of barks. In view of dual inhibition activity, the ethanol extract of barks seemed to have moderate inhibition against 5-LOX and good inhibition against COX enzymes (selective COX-2). Chloroform extract of the bark though had good 5-LOX inhibition but did not have activity against COX enzymes below 100 µg/ml.

5.5 Conclusion

A combination of anti-inflammatory and antioxidant activity constitutes a good indication on potential anti-inflammatory activity of a drug [132][133]. Keeping this in mind the ethanol fraction of the plant warranted further studies. The pathway mechanism of the dual inhibition of the fraction against 5-LOX and COX needs to be investigated. However it would be interesting to investigate the bioactive component of the chloroform extract of barks as it exhibited an activity similar to our positive control. The clinical significance of these data is quite clear that they support a role for *Canarium patentinervium* Miq. (Burseraceae Kunth.) as a source of lead compounds in the management of inflammatory diseases.

CHAPTER 6

ANTI-ACETYLCHOLINESTERASE ACTIVITY

6.1 Introduction

6.1.1 Pathophysiology of Alzheimer's disease

Neurodegenerative disease is a term applied to a variety of conditions arising from a chronic breakdown and deterioration of the neurons, particularly those of the central nervous system [134]. Alzheimer's disease (AD) was first described in 1906 by a Bavarian neuropsychiatrist Alois Alzheimer [135]. It is a complex, multifactoral, progressive, neurodegenerative disease primarily affecting the elderly population and is estimated to account for 50 – 60 % of dementia cases in persons over 65 years of age [136]. The pathophysiology of AD is complex and involves several different biochemical pathways.

The first neurotransmitter defect discovered in AD involved acetylcholine (ACh), which plays an important role in memory and learning. In the CNS, ACh stimulation of the nicotinic receptors appears to be associated with cognitive function. Normally, ACh is stored in the nerve terminals, in structures called vesicles and is released from the nerve endings when the nerve terminal is depolarized, thereby entering the synapse and binding to the receptor [137]. However, in patients with AD, the ACh which is released has a very short half-life due to the presence of large amounts of the enzymes; acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), which are

both present in the brain and are detected among neurofibrillary tangles and neuritic plaques [138][139]. These enzymes hydrolyse the ester bond in the ACh molecule, leading to loss of stimulatory activity. Approaches to enhance cholinergic function in AD have included stimulation of cholinergic receptors or prolonging the availability of ACh released into the neuronal synaptic cleft by use of agents which restore the level of acetylcholine through inhibition of both AChE and BChE.

Recently, Hodges [140] demonstrated that the inhibition of AChE holds a key role not only to enhance cholinergic transmission in the brain but also to reduce the aggregation of β-amyloid and the formation of the neurotoxic fibrils in AD. Therefore, AChE and BChE inhibitors have become remarkable alternatives in treatment of AD [139]. Existing anticholinesterase drugs (example, tacrine, donepezil, physostigmine, galantamine and heptylphysostigmine) for the treatment of dementia are reported to have several dangerous adverse effects such as hepatotoxicity, short duration of biological action, low bioavailability, adverse cholinergic side effects in the periphery and a narrow therapeutic window [141][142]. This necessitates the interest in finding better AChE inhibitors from natural resources.

The history of drug discovery has shown that plants contain active compounds that have become new sources to investigate for the pharmaceutical industry. Plant constituents may not only act synergistically with other constituents from the same plant but may also enhance the activity of compounds or counteract toxic effects of compounds from other plant species [143]. In traditional practices, numerous plants have been used to treat cognitive disorders, including neurodegenerative diseases and different neuropharmacological disorders [144].

6.1.2 Acetylcholine and its functions

Acetylcholine (ACh), a neurotransmitter in the brain plays a critical role in the function of learning and memory. ACh is synthesized from acetyl-CoA and choline by choline acetyltransferase, and is released into the synaptic cleft which then is hydrolyzed by AChE to become choline and acetic acid (Figure 6.1). Choline is taken up again into the presynaptic neurons for use in ACh synthesis. Free choline within the nerve terminal is acetylated by a cytosolic enzyme, choline acetyltransferase (CAT), the source of the acetyl groups being acetyl-CoA [141].

The rate-limiting process in the Ach synthesis appears to be the choline transport, the activity of which is regulated according to the rate at which ACh is released. ACh has functions both in the peripheral nervous system (PNS) and in the central nervous system (CNS) as a neuromodulator [141]. Its receptors have very high binding constants. In the peripheral nervous system, acetylcholine activates muscles, and is a major neurotransmitter in the autonomic nervous system. In the central nervous system, ACh and the associated neurons form a neurotransmitter system, the cholinergic system, which tends to cause anti-excitatory actions. Cholinesterase is present in the presynaptic nerve terminals and Ach is continually being hydrolysed and re-synthesised [145][146].

6.1.3 Distribution and function of cholinesterase

There are two distinct types of cholinesterase both belonging to the family of serine hydrolases, namely acetylcholinesterase (AChE) and butyrylcholinesterase (BChE),

related closely in molecular structure but differing in their distribution, substrate specificity and functions [145].

AChE is widely distributed in the central nervous system (CNS) and the peripheral nervous system AChE is one of the most efficient enzymes of the nervous system which is bound to the basement membrane in the synaptic cleft at cholinergic synapses, where its function is to hydrolyse the released transmitter. AChE is quite specific to acetylcholine (Ach). BChE has a widespread distribution, being found in tissues such as liver, skin, brain and gastrointestinal smooth muscles, as well as soluble form in the plasma. It is not particularly associated with cholinergic synapses and has broader substrate specificity than AChE. It hydrolyses butyrylcholine more rapidly than ACh [145][147].

6.1.4 Cholinesterase inhibitors

The most important drugs that enhance cholinergic transmission act either by inhibiting cholinesterase or by increasing acetylcholine release by AChE. The choline released in the process is reused in synthesizing new ACh. Inhibition of AChE increases the amount of ACh available for neurotransmission.

There are three main effects of cholinesterase inhibition:

- effects on autonomic cholinergic synapses
- effects on neuromuscular junction
- effects on the central nervous system (CNS)

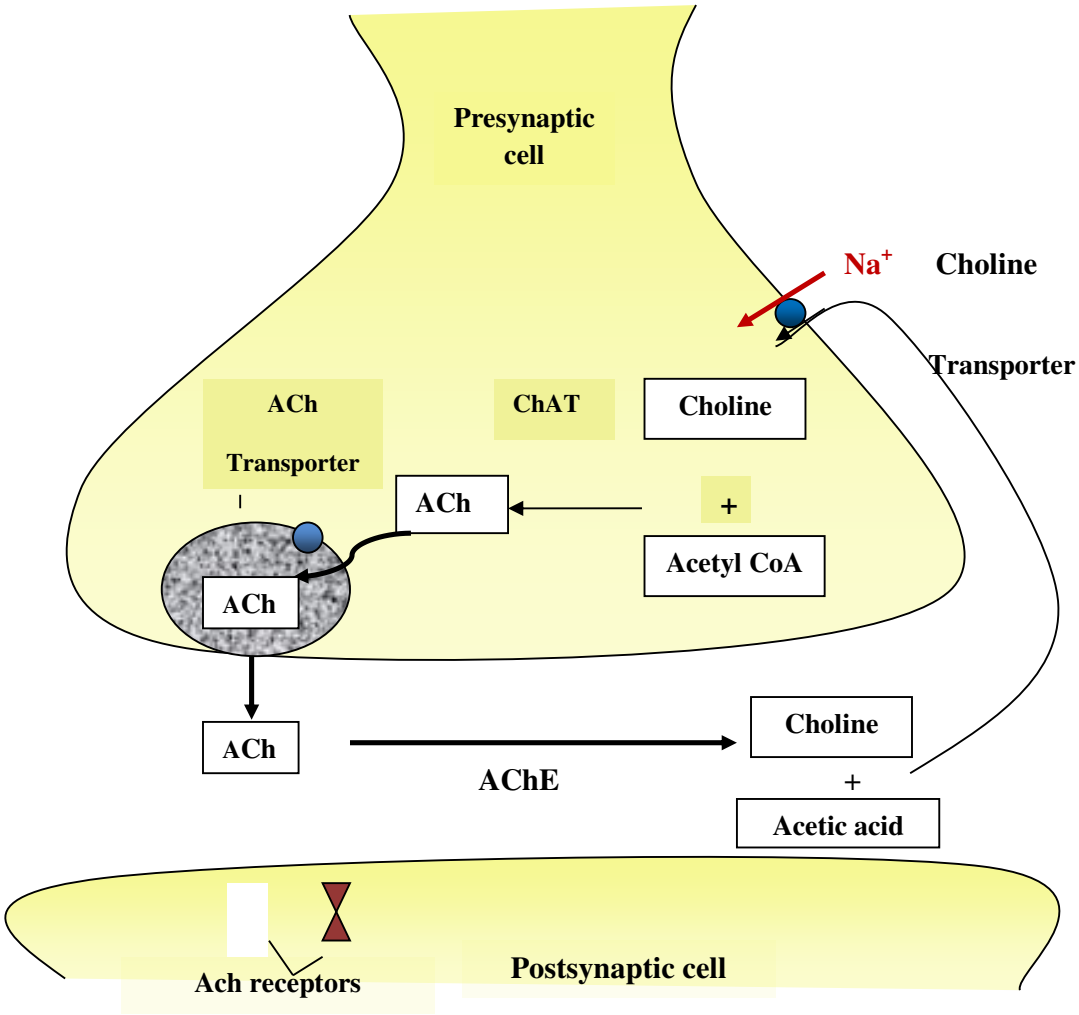


Figure 6.1: Cholinergic neurotransmission. The neurotransmitter Ach is synthesised in presynaptic cholinergic neurons by choline acetyltransferase (CAT or ChAT). The process entails transfer of an acetyl group from acetyl-coenzyme A to choline. Until needed, the ACh molecules are stored in discrete vehicles at the ends of the presynaptic neurons. Arrival of a nerve impulse triggers the release of Ca_{2+} ions, which activate actin microfilaments that in turn pull the storage vesicles into position for ACh release. In a single event the vesicles empty their contents into the synaptic cleft. Most of these molecules bind to cholinergic receptors on adjacent postsynaptic neurons. Any that remain unbound are rapidly hydrolysed by AChE. The choline released in the process is reused in synthesizing new ACh. Inhibition of AChE increases the amount of ACh available for neurotransmission.

Autonomic effects include bradycardia, hypotension, excessive secretions, bronchoconstriction, gastrointestinal hypermotility and decrease of intraocular pressure. This inhibition can be useful in the treatment of glaucoma that has increased intraocular pressure. Drugs that have been used for this condition are physostigmine and ecothiopate as eye drops. Neuromuscular action causes muscular fasciculation and increased twitch tension and can produce depolarisation block [147] .

The clinical uses of this action would be in myasthenia gravis where transmission fails due to too few ACh receptors due to immunological reasons, and cholinesterase inhibition improves transmission. Drugs targeting this action would be neostigmine and pyridostigmine taken orally. Cholinesterase inhibition at CNS has been used to alleviate the cholinergic deficiency associated with Alzheimers disease (AD). Although the overall AChE activity is reduced, it is increased in the neuritic plaque and neurofibrillary tangles at the early stages of a AD patient brain [146]. It has been suggested thay AChE may promote aggregation of Abeta (β-amyloid) into a more toxic amyloid form. Thus inhibiting AChE activity might increase ACh neurotransmission in the synaptic cleft of the brain and diminish the Abeta burden resulting in improved cognitive function and alleviating the process of amyloid deposition. Among several existing hypotheses explaining the origin of AD, including the cholinergic, tau and amyloid theories [148][149], the cholinergic one is most studied. Majority of drugs on the market are thus AChE inhibitors like donepezil, tacrine, rivstigmine and galathamine [147].

6.1.5 Plants as potential anticholinesterase inhibitors

In traditional practices, numerous plants have been used to treat cognitive disorders, including neurodegenerative diseases and different neuropharmacological disorders. Ethnopharmacological approach and bioassay-guided isolation have provided a lead in identifying potential AChE inhibitors from plant sources, including those for memory disorders. A variety of plants has been reported to show AChE inhibitory activity and so may be relevant to the treatment of neurodegenerative disorders such as AD [144].

Majority of studies have focused on the anticholinesterase alkaloids, such as physostigmine and galantamine. So far, more than 35 alkaloids have been reported to have AChE inhibitory activity [144]. The other major classes of compound reported to have such activity are the terpenoids, glycosides and coumarins. Plants belonging to families Acanthaceae, Apocynaceae, Amaryllidaceae, Angelicae, Araceae, Asclepiadaceae, Berberidaceae, Buxaceae, Combretaceae, Compositae, Coniferae, Cyperaceae, Ebenaceae, Ericaceae, Euphorbiaceae, Fumariaceae, Gentianaceae, Guttiferae, Lamiaceae, Leguminosae, Lilliaceae, Lycopodiaceae, Malvaceae, Magnoliaceae, Menispermaceae, Molluginaceae, Moraceae, Musaceae, Nelumbonaceae, Papaveraceae, Piperaceae, Rubiaceae, Rutaceae, Sapotaceae, Solanaceae and Tamaricaceae have been reported to have AChE inhibitory potential [149].

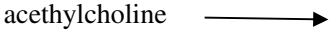
The present study was undertaken to screen the anti-acetylcholinesterase potential of *Canarium patentinervium* Miq. from family of Burseraceae. No anti-acetylcholinesterase studies have been reported on this species to date.

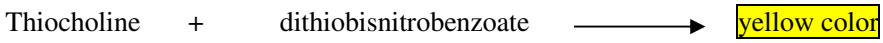
6.2 Materials and methods

6.2.1 Anti-acetylcholinesterase determination assays

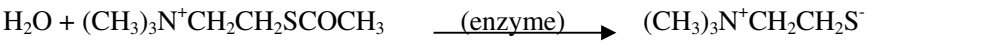
6.2.1.1 Principle of the assay

Acetylcholinesterase (AChE) inhibitory activity was measured by slightly modifying the spectrophotometric method developed by Ellman *et al*. [150]. Acethylcholine iodide which is the substrate is hydrolysed by acethylcholinesterase enzyme resulting in the formation thiocholine that reacts with dithiobisnitrobenzoate ion (DTNB) resulting in yellow 5-thio-2-nitrobenzoate anion observed at a wavelength of 412 nm.

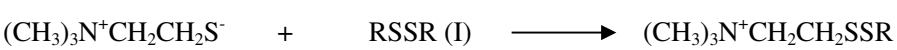




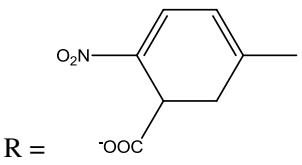
This is accomplished by the continuous reaction of the thiol with 5, 5' dithiobis-2-nitrobenzoate ion (I) to produce the yellow anion of 5-thio-2-nitro-benzoic acid (II).



$+ CH_3^+COO^- + 2H^+$



$+ RS^-$ (II)



The reaction with the thiol has been shown to be sufficiently rapid so as not to be rate limiting in the measurement of the enzyme, and in the concentrations used does not inhibit the enzymic hydrolysis.

6.2.1.2 Protocol

5,5´-Dithio-bis(2-nitrobenzoic) acid (DTNB), galanthamine, nordihydroguairetic acid (NDGA) and electric eel acetylcholinesterase (AChE) (Type-VI-S, EC 3.1.1.7) purchased from Sigma Aldrich^{R} was used. Samples were dissolved in dimethyl sulfoxide (DMSO, R&M) prior to assay and serial dilutions were done. In brief, 130 µL of 0.1 mM sodium phosphate buffer (pH 8.0), 20 µL of DTNB, 20 µL of test solution and 20 µL of AChE solution were added by multichannel automatic pipette (Eppendorf, Germany) in a 96-well microplate and incubated for 15 min at 25 °C. The reaction was then initiated with the addition of 10 µL of acetylthiocholine iodide. The hydrolysis of acetylthiocholine iodide was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines, catalyzed by enzymes at a wavelength of 412 nm utilizing a 96-well microplate Thermo Scientific Varioskan Flash microtiter plate reader, linked to a computer equipped with (SkanIt Software 2.4.3).

Percentage inhibition of AChE was determined by comparison of rates of reaction of samples relative to blank sample (ethanol in phosphate buffer pH=8) using the formula (E-S)/E x 100, where E is the activity of enzyme without test sample and S is the activity of enzyme with test sample. The experiments were done in triplicate. Galanthamine was purchased from Sigma (St. Louis, MO, USA) and used as reference.

6.2.2 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. All data were expressed as mean ± standard deviation. SD was computed but values were not shown in the graphs via this software. Data were analyzed using one way Anova followed by Tukey test using GraphPad Prism5 software. A significant difference was considered at the level of $P < 0.01$.

6.3 Results

In the anti-acethylcholinesterase assay, chloroform extract of the barks displayed the best activity (IC_{50} = 88.59±0.14 µg/ml) as opposed to galanthamine (IC_{50} = 0.74±0.06 µg/ml) (Figure 6.1). The ethanol extract of barks and leaves follow through with IC_{50} = 186.00±0.15 µg/ml and IC_{50} = 201.24±0.15 µg/ml respectively. Hexane extracts of bark and leaves and the chloroform extract of leaves had the lowest enzyme inhibition activity (IC_{50} = 570.00±0.08 µg/ml, IC_{50} = 842.00±0.25 µg/ml and IC_{50} = 1780.00±0.24 µg/ml respectively). Thus the potency of activity against AChE was BC > BE > LE > BH >LH > LC (Table 6.1).

Table 6.1. Anti-acetylcholinesterase values of *Canarium patentinervium* Miq.

Extracts	Anti-acetylcholinesterase assay, IC_{50} (µg/ml)
LH	842.00±0.25
LC	1780.00±0.24
LE	201.34±0.15
BH	570.00±0.08
BC	88.59±0.14
BE	186.00±0.15
Galantamine	0.74±0.06

LH: leaf hexane extract, LC: leaf chloroform extract, LE: leaf ethanol extract, BH: bark hexane extract, BC: bark chloroform extract, BE: bark ethanol extract. Data were obtained from three independent experiments, each performed in triplicates (n=9) and represented as mean ± SD.Values with the same capital letter are not significantly different (P<0.01) according to Tukey multiple comparison test.

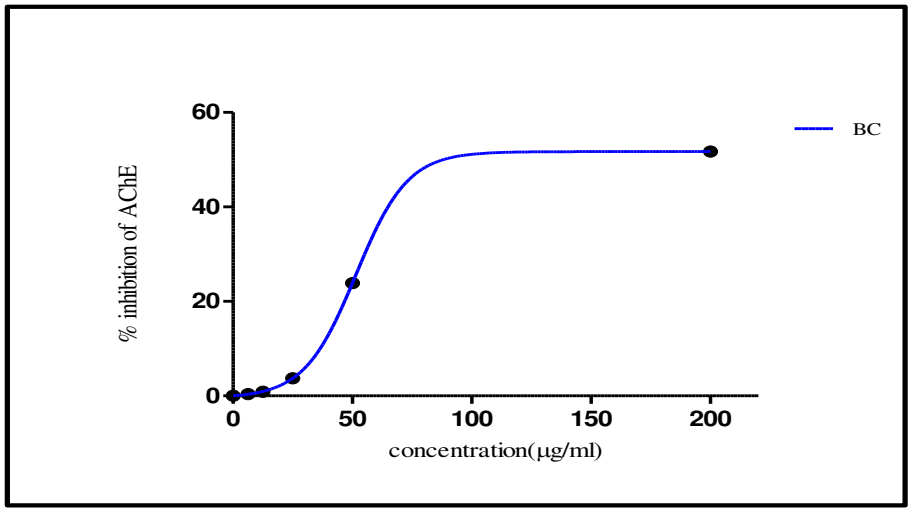


Figure 6.2: AChE inhibition by BC.

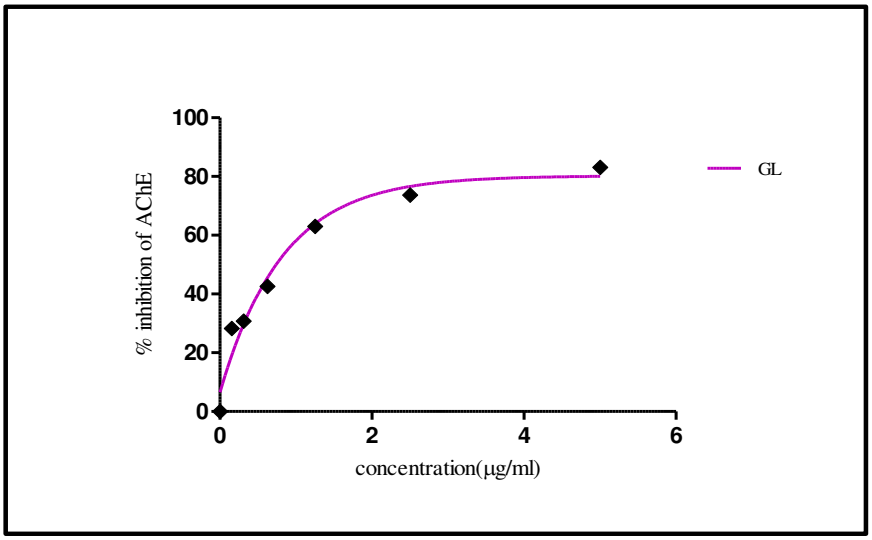


Figure 6.3: AChE inhibition by galanthamine.

6.4. Discussion

This assay measures the inhibition properties against AChE, which is the key enzyme in the hydrolysis of acetylcholine that is responsible for muscle and organ relaxations. Acetylcholinesterase inhibitors are used medicinally to treat myasthenia gravis to increase neuromuscular transmission and to treat Alzheimer's disease (deficiency in the production of acetylcholine). Furthermore, oxidative and inflammatory processes are among the pathological features associated with the central nervous system in Alzheimer's disease [151]. The brain of patients suffering from AD is said to be under oxidative stress as a result of perturbed ionic calcium balances within their neurons and mitochondria [152][153]. Accumulating evidence suggests that oxidative damage to neurons plays an important role in the AD pathogenesis [154].

Thus, efforts to reduce oxidative injury may prove beneficial in retarding or preventing the onset and progression of AD in patients. In this study (Chapter 4), BC had displayed good antioxidant potential via enzymatic and non-enzymatic assays. This in addition to its anti-acetylcholinesterase activity exhibited in this chapter suggest that chloroform extract of the barks hold lead compounds that inhibit acetylcholinesterase activity as well as reduce the oxidative stress with possible neuroprotective effects. Moreover, there is evidence that AChE inhibitors have an anti-inflammatory role through action against free radicals and amyloid toxicity, as well as through decreasing release of cytokines from activated microglia in the brain and blood [153].

There is an established link between the cholinergic system and inflammation as acetylcholine, the principle neurotransmitter, is reported to attenuate the release of

cytokines in the parasympathetic anti-inflammatory pathway by which the brain modulates systemic inflammatory responses to endotoxin [155]. Destruction of neurons due to inflammation around Abeta plaques is thought to be a major factor in the pathogenesis of AD [2]. In Chapter 5 it has been found that BC had an anti-inflammatory action against 5-LOX (IC50 = 29.53±0.03 µg/ml) against the positive control NDGA ((IC50 = 29.19±0.02 µg/ml). This in relation to the anti-acetylcholinesterase activity exhibited herein suggest that BC may also acts as an anti-inflammatory agent related to AD pathogenesis.

Because of the unclear pathogenesis of AD, there have been several hypothesis associated with the disease such as amyloid-β peptide-containing plaque formation, excess metal ions, oxidative stress, and reduced acetylcholine levels. As proposed by Zhang *et al.* [156], finding more than one approach with multifunction for AD treatment draws attention to researchers. Thus, BC that exhibits good antioxidant potential, anti-inflammatory and anti-acetylcholinesterase action warrants further study for isolation of its bioactive principle.

6.5 Conclusion

The creation of effective therapeutic agents for AD would be a major medical milestone. Different causation targets are being targeted and medicinal, pharmacological, clinical and pathological research is on-going around the world. The anti-acetylcholinesterase activity of *Canarium patentinervium* Miq. in agreement with its potent antioxidant and anti-inflammatory activity demonstrated in Chapter 4 and 5

respectively represents an exciting scaffold from which to develop leads for treatment of neurodegenerative disease.

CHAPTER 7

ANTIMICROBIAL ACTIVITY

7.1 Introduction

7.1.1 Infectious disease

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. The widespread use of these compounds is thought to further encourage the emergence of antimicrobial resistance [157]. The number of resistant pathogenic bacteria grows at 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 [158][159].

7.1.2 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 [160].

The efficacy of antimicrobial agents is influenced by a number of factors. Firstly, it is of 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 [160].

7.1.3 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 [157].

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 [161].

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 [162].

7.1.4 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 6-7 decades have been discovered through screening of soil samples, of which the antimicrobial efficacies were determined first *in vivo* and later *in vitro* [162].

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 thus no 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 [163]. An increase in the isolation and identification of such compounds may thus contribute greatly to the success in antibiotic discovery.

7.1.5 *Canarium* species and their known antimicrobial activity

Studies conducted on *Canarium* species have suggested that these species may well be active against micro-organisms. Antibacterial activities was previously reported in *Canarium schweinfurthii* Engl. Dichloromethane extract of *Canarium schweinfurthii* Engl. had bactericidal activity against Gram-negative *Vibrio cholerae* with minimum inhibitory concentration (MIC) of 0.62 mg/ml while the ethylacetate extract was active against Gram-positive and Gram-negative bacteria namely *Staphylococcus aureus* and *Proteus vulgaris* with MIC values of 10 mg/ml and 5 mg/ml respectively. Ethanol extract was active against Gram-negative *Vibrio cholerae* and *Proteus vulgaris* with MIC values of 0.62 mg/ml and 10 mg/ml respectively. The oil was also fungicidal against *Candida albicans* with an inhibition zone of 23 mm [28]. In a separate disc diffusion assay, the essential oil of *Canarium schweinfurthii* Engl. abrogated the survival of Gram-negative *Salmonella enterica*, Gram positive *Streptococcus pyogenes* and *Staphylococcus aureus* with an inhibition zone of 27 mm, 25 mm and 18 mm respectively [25]. *Canarium patentinervium* Miq. have been used traditionally in healing wounds [164], as such this study will attempt to validate these ethnopharmacological claims.

7.2 Materials and Methods

The *in vitro* antimicrobial properties of *Canarium patentinervium* Miq. was evaluated using the disc diffusion assay, minimum inhibitory concentration (MIC) assay, minimum bactericidal assay (MBC) and death kinetic assay. The hexane, chloroform and ethanol extracts of *Canarium patentinervium* Miq. were prepared (see Chapter 2 for the extraction process). The following bacterial strains were employed in the screening:

i. 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).

ii. Ten clinical bacterial strains and 3 clinical strain of yeasts obtained from the Bacteriology Unit, Department of Microbiology and Immunology of UKM Medical Centre. The assays involving these clinical strains were also perfomed at HUKM.

7.2.1 Disc diffusion assay

7.2.1.1 Principle of method

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 to be put up as a routine due to the large number of tests it requires [165]. 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 [165]. With the introduction of a variety of antimicrobials it became necessary to perform the antimicrobial susceptibility test as a routine.

In this assay, 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 years to come [165].

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 [165]. 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 [165][166].

7.2.1.2 Protocol [166][167][168]

i. Preparation of Mueller Hilton Agar

Mueller Hilton Agar 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 ^{o}C 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 ^{o}C). A representative sample of each batch of plates was examined for sterility by incubation at 30-35 ^{o}C for 24 hrs or longer.

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

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

cotton swabs. Tryptic soy broth (TSB) (20 ml) was prepared as the manufacturer's instructions. About 5 ml was poured into a Bijou bottle and autoclaved. About 150 ml of sodium chloride solution of 0.9 % was prepared and autoclaved

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. Standards 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 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 petri dish. Bacterias are streaked on the tryptic soya agar (TSA) plates in 4 density level around the plates. Plates are then sealed with a parafilm and incubated for 18 hrs 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 are 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 bacteria into each Bijou bottle. The broth culture was incubated at 35 ^{o}C for about 2 hrs 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 mins 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}C. 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 mins after discs are applied.

viiii. Reading plates and interpretation of results

After 18 hrs of 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.

7.2.2 Minimum inhibitory concentration assay

7.2.2.1 Principle of method

The MIC is the lowest concentration being beyond the concentration where no growth inhibition of test organisms was observed. Minimum inhibitory concentration (MIC) assays investigate the *in vitro* susceptibility of organisms to antimicrobial agents. This method employs different dilutions of the antimicrobial agent and quantitatively investigates the lowest concentration at which visible microbial growth inhibition is achieved. Microbial growth is visualised through the addition of the tetrazolium salts, specifically *p*-iodonitrotetrazolium (INT, Sigma-Aldrich). The assay is based on the detection of dehydrogenase activity in living cells by being converted from a colourless solution to an intensely coloured formazan (red) product [169]. This method yields reproducible results within one doubling dilution of the end point of the activity.

7.2.2.2 Protocol

The MIC of the plant extracts was determined by serial dilution, as described by Eloff [169]. These dilution experiments were performed in sterile 96-well microtiter plates.

i. Preparation of sample

Stock solutions of the respective plant extracts were prepared in 1.5 ml microcentrifuge tubes (Eppendorff) by dissolving dry plant extract in

dimethylsulphoxide (DMSO, Saarchem) to a final concentration of 64 mg/ml. Aliquots of 100 µl of the stock solution were transferred aseptically into the top row of microtiter plate (row A), which already contained 100 µl aliquots of sterile water, thereby resulting in a 50 % dilution of the stock solution to 32 mg/ml. After adequate mixing of the contents of each well, 100 µl aliquots of row A were transferred from row A to the corresponding wells in row B (also containing 100 µl aliquots of sterile water), followed by mixing and resulting in yet another 50% dilution of the plant extract (to 16 mg/ml). This process was repeated for every row, resulting in 100 µl aliquots ranging in concentration from 32 mg/ml (row A) to 0.25 mg/ml (row H).

ii. Preparation of inocolums

In order to determine the range of antimicrobial activity of *Canarium patentinervium* Miq., four different microbial (reference) cultures and 10 clinical strains were used and are listed below.

i. Gram-positive bacteria

- *Staphylococcus aureus* (ATCC 11632)
- *Bacillus cereus* (ATCC 10876)
- Methicillin-resistant *Staphylococcus aureus* (ATCC 43300)
- *Staphylococcus aureus* MSSA
- *Staphylococcus aureus* MRSA
- Coagulase negative *Staph*: Oxacillin (S)
- Coagulase negative *Staph*: Oxacillin (R)
- *Enterococcus faecalis*

ii. Gram-negative bacteria

- *Pseudomonas aeruginosa* (ATCC 10145)
- *Escherichia coli*
- *Klebsiella sp*
- *Escherichia coli* ESBL
- *Klebsiella pneumoniae* ESBL

iii. Yeast

- *Candida parapsilosis*

Bacterias are streaked on TSA plates in 4 density level around the plate. Plates are then sealed with a parafilm and incubated for 18 hrs to obtain fresh single colonies. At least 3-5 well isolated colonies of the same morphological type are selected from the agar plate culture. The top of each colony was touched with a sterilized loop and the growth was transferred into Bijou bottle containing TSB/MHB. This was repeated for each bacteria placed into each Bijou bottle. Broth was incubated at 35 ^{o}C for about 2 hrs till it achieved 0.5 Mcfarland Standard/ 600 nm (OD-0.1 nm) to yield 1 x 10^8 cfu/ml [170]. Turbidity was adjusted with sterile normal saline. To obtain 10 x 10^5 cfu/ml, add 100 µl of adjusted broth to 9900 µl of TSB/MHB (1:100 dilution).

iii. MIC assay

This was followed by addition of 100 µl at concentration of 10 x 10^5 colony forming units (cfu)/ml, of liquid microbial culture grown in Mueller Hilton Broth (MHB) to each well. This yielded a final volume of 200 µl in each well and final extract

concentrations ranging from 16 mg/ml in row A to 0.125 mg/ml in row H (Figure 4.1) with bacteria 5 x 10^5 cfu/ml. The microtiter plates were incubated at 37 °C, overnight for bacteria and 48 hours for yeasts (as yeasts require a greater time period for growth). After incubation at 37 °C, 40 µl of a 0.4 mg/ml solution of INT was added to each well as an indicator of microbial growth. The plates were incubated at 37 ^{o}C for 30 min (bacteria) and 24 hr (yeast) and the MIC values visually determined. The lowest concentration of each extract displaying no visible growth was recorded as the minimum inhibitory concentration. The concentration that inhibited bacterial/yeast growth completely (the first clear well) was taken as the MIC value. MIC values were determined at least in duplicate and repeated to confirm activity

In order to determine the sensitivity of the microorganisms, positive control experiments were conducted: (1) for bacterial strains, ampicillin (Sigma-Aldrich) at a starting concentration of 0.10 mg/ml in sterile water, and (2) for yeast strains, Amphotericin B (Sigma-Aldrich), at a starting concentration of 0.10 mg/ml in DMSO and water (where 1.00 mg/ml was prepared in DMSO, and diluted to 0.10 mg/ml in sterile water thereafter). The final concentrations for these experiments ranged from 25.00 µg/ml (row A) to 0.19 µg/ml (row H). A negative control experiment was conducted using only DMSO.

7.2.3 Minimum bactericidal concentration (MBC)

7.2.3.1 Principle of method

Minimum bactericidal concentration (MBC) was recorded as a lowest extract concentration killing 99.9 % of the bacterial inocula after 24 hr incubation at 37 °C. Each experiment was repeated at least three times.

7.2.3.2 Protocol

The determination of MBC was performed using the method of Ozturk & Ercisli [171] only for the susceptible bacterias/ fungi from the MIC assay (Section 7.2.2). Ten microliters were taken from the well obtained from the MIC experiment (MIC value) and two wells above the MIC value well and spread on MHA plates. The number of colony was counted after 18-24 hrs of incubation at 37 °C. The concentration of sample that produces < 10 colonies was considered as MBC value.

7.2.4 Time-kill assay

7.2.4.1 Principle of method

Death kinetic studies are often referred to as time-kill studies, and are used to determine the rate at which the antimicrobial agent kills pathogens over time, as well as the extent at which the activity occurs. Upon introduction of a micro-organism into a new environment, its growth displays a lag phase during which no cell growth

occurs, after which its growth enters the exponential phase, where microbial cell multiplication occurs at an exponential rate (doubling in number at regular intervals). This exponential phase is followed by the stationary phase, the latter of which usually results from the depletion of nutrients (carbon, nitrogen and/or oxygen sources) [160]. However, upon exposure to a constant concentration of an antimicrobial agent, the organism will remain within the lag phase for a certain amount of time. This is followed by a log-linear killing phase, during which the number of microbial colonies are decreased until it enters into a second lag phase. Re-growth may occur after this second lag phase, but documentation of this phenomenon rarely occurs as time-kill studies are usually performed over a 24 hour period only [172][173].

7.2.4.2 Protocol

Only samples that have MIC values of lower or equivalent to 0.5 mg/ml (strong inhibitors) are tested for the time-kill [174]. Concentration of samples to be tested is determined base on the MIC values (up to 4 times the MIC value) [175]. Samples and incolums are prepared as in section 7.2.2.2 (i-ii). This was followed by addition of 100 µl of microbial broth at concentration of 10 x 10^5 colony forming units (cfu)/ml, of liquid microbial culture grown in Mueller Hilton Broth (MHB) to each well. This yielded a final volume of 200 µl in each well of the extract. The plates are then incubated at 37 °C and optical density was recorded at 1 hr intervals up to 18 hrs at wavelength of 600 nm. Graphs were plotted on the basis of the turbidity varying over a period of time. The growth rate thus obtained was studied for any signs of bactericidal effects of the plant extract. Ampicillin was used as positive control. A

solution of the solvent in which dried extract was dissolved served as negative control.

7.2.5 Statistical analysis

Growth curve of bacteria were analysed using MS-Excel and data were reported as mean and SD values obtained from a minimum of three determinations. Bacterial growth curve was plotted with SD and 95% confidence interval. All data were expressed as mean ± standard deviation. Data were analyzed using one way Anova followed by Tukey test. A significant difference was considered at the level of $P < 0.01$.

7.3 Results

7.3.1 Disc diffusion assay

The six extracts of *Canarium patentinervium* Miq. that were screened for antimicrobial activity using the disc diffusion assay displayed good activity (Table 7.1). The extracts were screened for activity against Gram-positive bacteria (*Staphylococcus aureus* ATCC 11632, MRSA ATCC 43300, *Bacillus cereus* ATCC 10876, clinical strains consisting of MSSA, MRSA, Coagulase-negative *Staphyloccocus*: oxacillin sensitive/resistant, *Enterococcus faecalis* and *Streptococcus pneumoniae*), Gram-negative bacteria (*Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 10145, clinical strains consisting of *Esherichia coli, Klebsiella sp*,

Esherichia coli ESBL and *Klebsiella pneumoniae* ESBL and yeasts (*Candida albicans, Candida glabrata and Candida parapsilosis*). All the bacterias and yeast were sensitive to the extracts except *Streptococcus pneumoniae, Eschericia coli* ATCC 10536, *Candida glabrata* and *Candida albicans*. The ethanol extract of leaves and the hexane extract of bark displayed good antibacterial activity against both Gram-positive bacteria and Gram-negative bacteria. The ethanol extracts and hexane extract of the bark had the best sensitivity to *Candida parapsilopsis* (inhibition zone 14 mm).

Both ATCC and clinical strains of MSSA and MRSA were sensitive to the ethanol extracts (9-12 mm) (Figure 7.1) and hexane extract of barks (8-14 mm). Coagulase-negative *Staphylococcus aureus*: oxacillin sensitive and resistant and *Enterococcus faecalis* were most sensitive to the ethanol extracts (inhibition zone 8-12 mm). Gram-negative bacteria *Klebsiella pneumoniae* ESBL (inhibition zone 9-14 mm) had sensitivity to all the bark extracts while *Klebsiella sp* showed sensitivity only towards the ethanol extracts (inhibition zone 11-13 mm). *Pseudomonas aeruginosa* ATCC 10145 was inhibited by the ethanol extract of leaves (inhibition zone 11 mm) (Figure 7.2) and hexane extract of barks (inhibition zone 12 mm) while poor activity was seen with *Esherichia coli* with all extracts.

7.3.2 MIC assay

All extracts displayed some activity against Gram-positive and Gram-negative bacteria and yeast (Table 7.2). The activity against MSSA reference and clinical strains ranged from 0.50 mg/ml to 2.00 mg/ml with the strongest activity seen with

the ethanol extract of leaves. The activity against MRSA reference and clinical strains ranged from 0.25 mg/ml to 2.00 mg/ml with the lowest MIC seen in the ethanol extract of leaves. All the extracts had activity against coagulase-negative *Staphylococcus aureus*: oxacillin resistant and sensitive with MIC ranging from as low as 0.50 mg/ml (ethanol extract of leaves) to 16.00 mg/ml (hexane extract of leaves). *Enterococcus faecalis* had activity only against the ethanol extracts with an MIC of 0.50 mg/ml. *Bacillus cereus* ATCC 10876 showed sensitivity only against the ethanol extracts (MIC 0.5 mg/ml) and hexane extract of barks (MIC 1.00 mg/ml).

All the bark extracts showed activity against Gram-negative *Klebsiella sp*. with an MIC range of 0.50 mg/ml to 4.00 mg/ml while only the ethanol extracts of leaves and barks showed activity against *Klebsiella pneumoniae* ESBL with MIC of 0.5 mg/ml. *Pseudomonas aeruginosa* ATCC 10145 was sensitive towards ethanol extract of leaves and hexane extract of barks with an MIC of 1.00 mg/ml and 4.00 mg/ml respectively. *Esherichia coli* clinical strain was sensitive only towards ethanol extract of barks with an MIC of 0.50 mg/ml while *Escherichia coli* ESBL was inhibited by the chloroform extracts and hexane extract of barks with MIC of 8.00 mg/ml. The ethanol extracts and hexane extract of bark both had activity against yeast *Candida parapsilopsis* with the MIC of 2.00 mg/ml and 8.00 mg/ml respectively.

7.3.3 MBC assay and MBC/MIC ratio

The action of an antibacterial on the bacterial strains can be characterized with two parameters such as minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). According to the ratio MBC/MIC, we appreciated

antibacterial activity. If the ratio MBC/MIC ≤ 4, the effect was considered as bactericidal but if the ratio MBC/MIC > 4 , the effect was defined as bacteriostatic [176][177]. The MBC and MBC/MIC ratio are displayed in Table 7.3 and 7.4 respectively.

Both the ethanol extract of leaf and bark were bactericidal against *Bacillus cereus* ATCC 10876 (MBC of 1.00 mg/ml), MSSA ATCC 11632 (MBC range of 1.00 to 2.00 mg/ml), *Klebsiella* sp and *Klebsiella pneumonia* ESBL (MBC of 2.00 mg/ml) and yeast *Candida parapsilopsis* (MBC 8.00 mg/ml). The hexane extract of barks was bactericidal against MSSA, MRSA (both strains) and *Pseudomonas aeruginosa* ATCC 10145 with MBC range of 2.00 mg/ml to 8.00 mg/ml. Chloroform extract of barks showed bactericidal activity against MSSA (ATCC 11632 and clinical strains) with MBC of 1.00 mg/ml and 4.00 mg/ml respectively. Both hexane and chloroform extract of barks were bactericidal against *Klebsiella* sp with MBC/MIC ratio of 4. Rest of the MBC as displayed in Table 7.3 were all having bacteriostatic action with an MBC/MIC ratio > 4.

7.3.4 Time-kill assay

Only samples that have MIC values of lower or equivalent to 0.5 mg/ml (strong inhibitors) are tested for the time-kill [174]. The results obtained for the time-kill study of *Canarium patentinervium* Miq. are shown in Figure 7.3-7.22. The MBC values (Table 7.3) was consistent with the cidal concentration as displayed in the growth curve of this time-kill study. Blank control (broth alone) was the curve marked as 0 mg/ml of extract. The antibacterial activity was observed at different times upon

exposure of bacterias to the different concentration of extracts. This observation was made by varying turbidity over time for a period of 18 hrs.

7.4 Discussion

7.4.1 Disc diffusion assay

According to Gislene *et al*. [178], 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 *Canarium patentinervium* Miq. showed varying degrees of antibacterial activity against human pathogenic bacteria such as *Staphyloccocus aureus*, MRSA, *Bacillus cereus* (ATCC strain respectively), coagulase-negative *Staphylococcus aureus*, *Enterococcus faecalis*, *Klebsiella pneumoniae* ESBL and yeast *Candida parapsilopsis* (clinical strain respectively) cultured *in vitro*. Out of six extracts, the ethanol extract of leaves and hexane extract of barks exhibited significant antibacterial activity against both Gram-positive and Gram-negative bacteria. The best sensitivity to the ethanol extract of the leaves at 1 mg/disc was obtained against *Staphyloccocus aureus* (12 mm), MRSA (10 mm), *Bacillus cereus* (12 mm) and *Enterococcus faecalis* (12 mm), *Klebsiella pneumoniae* ESBL (13 mm) and yeast *Candida parasilopsis* (14 mm). The hexane extract of the barks showed best activity against MSSA (14 mm), *Klebsiella* sp (14 mm) and yeast *Candida parapsilopsis* (14 mm).

This is of special interest since most Gram-negative bacteria are more resistant to plant extracts [179]. Results from our phytochemical analysis revealed that the ethanol extract of leaves and barks of *Canarium patentinervium* Miq. accumulate substantial amounts of flavonoids and tannins which could be well correlated with the activities measured.

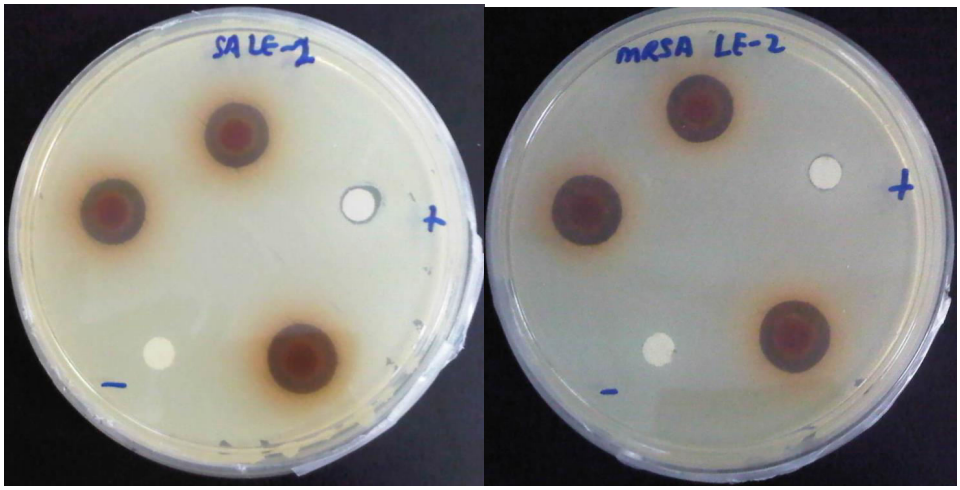


Figure 7.1: Zone of inhibition against *Staphylococcus aureus* (left plate) and methicillin resistant *Staphylococcus aureus* (right plate) for leaf ethanol extract at 1 mg/disc.

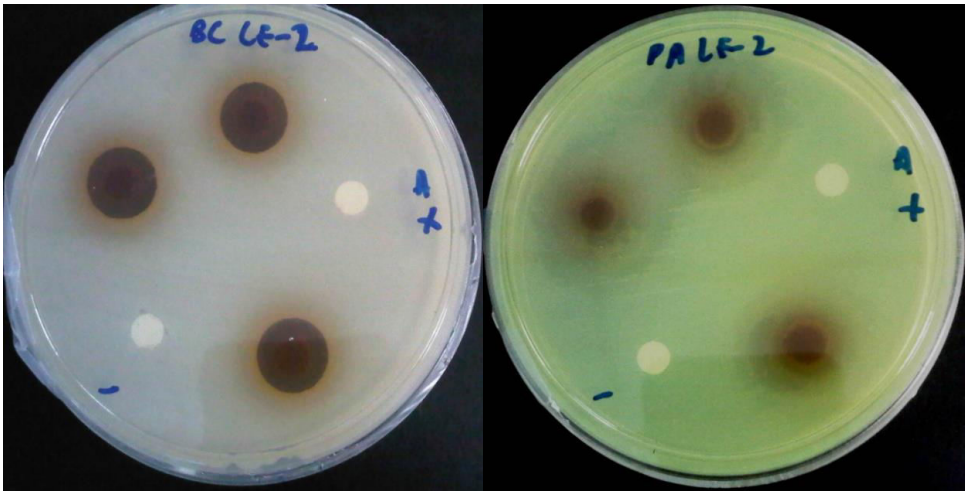


Figure 7.2: Zone of inhibition against *Bacillus cereus* (left plate) and *Pseudomonas aeruginosa* (right plate) for leaf ethanol extract at 1 mg/disc.

Table 7.1: Antibacterial activity of *Canarium patentinervium* Miq. (1 mg/disc) vs. ampicillin against 5 ATCC bacterias and 10 clinical bacterial strains and 3 clinical yeast strains tested by disc diffusion assay

Bacteria/ yeast	**Plant extract (Zone of inhibition) (mm) [a]**							
	LH	**LC**	**LE**	**BH**	**BC**	**BE**	**Ampicilin[c]**	**Amphotericin[c]**
MSSA *Staphylococcus aureus ATCC 11632*	-	-	12.00±0.21	11.00±0.25	7.00±0.29	9.00±0.58	10.00±0.66	NA
MRSA *Staphylococcus aureus ATCC 43300*	-	-	10.00±0.42	9.00 ± 0.25^{A}	-	9.00 ± 0.00^{A}	-	NA
Bacillus cereus ATCC 10876	-	-	12.00 ± 0.25^{B}	12.00 ± 0.45^{B}	-	9.00±0.00	8.00±0.00	NA
Eschericia coli ATCC 10536	-	-	-	-	-	-	7.00±0.00	NA
Pseudomonas aeruginosa ATCC 10145	-	-	11.00±0.34	12.00±0.47	-	-	-	NA
MSSA *Staphylococcus aureus*	-	-	11.00±0.23	14.00±0.43	13.00±0.17	10.00±0.45	7.00±0.26	NA
MRSA *Staphylococcus aureus*	-	-	10.00 ± 0.37^{C}	8.00±0.29	-	10.00 ± 0.47^{C}	-	NA
Eschericia coli	-	-	-	-	-	8.00±0.57	-	NA
Eschericia coli ESBL	-	7.00±0.18	-	8.00 ± 0.38^{D}	8.00 ± 0.26^{D}	-	-	NA
Coagulase-negative *Staphylococcus aureus:* O (R)	-	7.00±0.59	10.00±0.00	8.00 ± 0.34^{E}	8.00 ± 0.36^{E}	9.00±0.34	-	NA
Coagulase-negative *Staphylococcus aureus:* O (S)	7.00 ± 0.37^{F}	7.00 ± 0.16^{F}	12.00 ± 0.29^{G}	-	-	12.00 ± 0.23^{G}	10.00±0.27	NA
Enterococcus faecalis	-	-	12.00±0.46	-	-	8.00 ± 0.15^{H}	8.00 ± 0.28^{H}	NA
Streptococcus pneumoniae	-	-	-	-	-	-	-	NA
Klebsiella sp	-	-	11.00 ± 0.38^{I}	14.00±0.00	11.00 ± 0.34^{I}	9.00±0.42	10.00±0.14	NA
Klebsiella pneumoniae ESBL	-	-	13.00±0.25	-	-	11.00±0.27	-	NA
Candida parapsilosis	-	-	14.00 ± 0.12^{K}	14.00 ± 0.24^{K}	-	14.00 ± 0.10^{K}	NA	11.00±0.23
Candida glabrata	-	-	-	-	-	-	NA	9.00±0.15
Candida albicans	-	-	-	-	-	-	NA	-

LH-leaf hexane extract, LC- leaf chloroform extract, LE-leaf ethanol extract, BH- bark hexane extract, BC- bark chloroform extract, BE- bark ethanol extract, NA-not applicable

a: inhibition zones are the mean including disc (6 mm) b: sample extract at the concentration of 100 mg/ml (1mg/disc), c: ampicillin/amphotericin at 100 µg/ml = 1 µg/disc, -: no activity noted

Data were obtained from three independent experiments, each performed in triplicates (n=9) and represented as mean ± SD.

Values with the same capital letter are not significantly different (P<0.01) according to Tukey multiple comparison test.

Table 7.2: MIC values for *Canarium patentinervium* Miq. extracts against 4 ATCC bacterias and 9 clinical bacterial strains and 1 clinical yeast strain

Bacteria/ yeast	**Plant extract minimum inhibitory concentration MIC (mg/ml) and positive control[a] MIC (µg/ml)**							
	LH	**LC**	**LE**	**BH**	**BC**	**BE**	**Ampicilin[a]**	**Amphotericin[a]**
MSSA *Staphylococcus aureus ATCC 11632*	-	-	0.50±0.10	2.00±0.10	1.00±0.00^{A}	1.00±0.00^{A}	1.56±0.00	NA
MRSA *Staphylococcus aureus ATCC 43300*	-	-	0.50±0.00	1.00±0.00^{B}	-	1.00±0.00^{B}	-	NA
Bacillus cereus ATCC 10876	-	-	0.50±0.15^{C}	1.00±0.00	-	0.50±0.00^{C}	6.25±0.00	NA
Pseudomonas aeruginosa ATCC 10145$^{\#}$	-	-	1.00±0.00	4.00±0.05	-	-	-	NA
MSSA *Staphylococcus aureus*	-	-	0.50±0.10	2.00±0.00^{D}	2.00±0.00^{D}	0.50±0.00	>25.00±0.00	NA
MRSA *Staphylococcus aureus*	-	-	0.25±0.00^{E}	2.00±0.10	-	0.25±0.00^{E}	-	NA
Eschericia coli	-	-	-	-	-	0.50±0.00	-	NA
Eschericia coli ESBL	-	8.00±0.00^{F}	-	8.00±0.00^{F}	8.00±0.00^{F}	-	-	NA
Coagulase-negative *Staphylococcus aureus:* O (R)	-	8.00±0.00	1.00±0.00	0.50±0.00	2.00±0.00	0.50±0.00	-	NA
Coagulase-negative *Staphylococcus aureus:* O (S)	16.00±0.00	8.00±0.00	0.50±0.00^{G}	-	-	0.50±0.10^{G}	8.00±0.00	NA
Enterococcus faecalis	-	-	0.50±0.00^{I}	-	-	0.50±0.00^{I}	3.13±0.00	NA
Klebsiella sp	-	-	0.50±0.00^{J}	4.00±0.00^{K}	4.00±0.00^{K}	0.50±0.00^{J}	>25.00±0.00	NA
Klebsiella pneumoniae ESBL$^{\#}$	-	-	0.50±0.00	-	-	0.50±0.00	-	NA
Candida parasilopsis	-	-	2.00±0.00^{K}	8.00±0.00	-	2.00±0.00^{K}	NA	0.78±0.00

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

Data were obtained from three independent experiments, each performed in duplicates (n=6) and represented as mean ± SD, -: no activity, NA-not applicable
Values with the same capital letter are not significantly different (P<0.01) according to Tukey multiple comparison test.
#: statistical analysis not performed due to insufficient data

Table 7.3: MBC values for *Canarium patentinervium* Miq. extracts against 4 ATCC bacterias and 9 clinical bacterial strains and 1 clinical yeast strain

Bacteria/ yeast	**Plant extract minimum inhibitory concentration,MBC (mg/ml) and positive control[a] MBC (µg/ml)**							
	LH	**LC**	**LE**	**BH**	**BC**	**BE**	**Ampicilin[a]**	**Amphotericin[a]**
MSSA *Staphylococcus aureus ATCC 11632*	-	-	2.00±0.00^{A}	2.00±0.00^{A}	1.00±0.00^{B}	1.00±0.00^{B}	6.25±0.00	NA
MRSA *Staphylococcus aureus ATCC 43300*	-	-	2.00±0.00	4.00±0.00^{C}	-	4.00±0.00^{C}	-	NA
Bacillus cereus ATCC 10876	-	-	1.00±0.10^{D}	8.00±0.00	-	1.00±0.00^{D}	>25.00±0.00	NA
Pseudomonas aeruginosa ATCC 10145	-	-	4.00±0.00	8.00±0.05	-	-	-	NA
MSSA *Staphylococcus aureus*	-	-	4.00±0.10^{E}	4.00±0.00^{E}	4.00±0.00^{E}	2.00±0.00	>25.00±0.00	NA
MRSA *Staphylococcus aureus*	-	-	2.00±0.00	8.00±0.10	-	1.00±0.00	-	NA
Eschericia coli[#]	-	-	-	-	-	2.00±0.00	-	NA
Eschericia coli ESBL[#]		>16.00±0.00	-	>16.00±0.00	>16.00±0.00	-	-	NA
Coagulase-negative *Staphylococcus aureus:* O (R)		>16.00±0.00	4.00±0.00^{F}	2.00±0.00	8.00±0.00	4.00±0.00^{F}	-	NA
Coagulase-negative *Staphylococcus aureus:* O (S)[#]	>16.00±0.00	>16.00±0.00	2.00±0.00	-	-	2.00±0.10	12.50±0.00	NA
Enterococcus faecalis[#]	-	-	4.00±0.00	-	-	4.00±0.00	6.25±0.00	NA
Klebsiella sp	-	-	2.00±0.00^{I}	16.00±0.00^{J}	16.00±0.00^{J}	2.00±0.00^{I}	>25.00±0.00	NA
Klebsiella pneumoniae ESBL[#]	-	-	2.00±0.00	-	-	2.00±0.00	-	NA
Candida parapsilopsis[#]	-	-	8.00±0.00	>16.00±0.00	-	8.00±0.00	NA	3.13±0.00

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

Data were obtained from three independent experiments, each performed in duplicates (n=6) and represented as mean ± SD, -: no activity, NA-not applicable
Values with the same capital letter are not significantly different (P<0.01) according to Tukey multiple comparison test.
#: statistical analysis not performed due to insufficient data

Table 7.4: Bacteriostatic (-) and Bactericidal (+) effects of *Canarium patentinervium* Miq. extracts

Bacteria/ yeast	**MBC/MIC ratio (+/-)**			
	LE	**BH**	**BC**	**BE**
MSSA Staphylococcus aureus ATCC 11632	4 (+)	1 (+)	1 (+)	1 (+)
MRSA Staphylococcus aureus ATCC 43300	4 (+)	4 (+)	-	4 (+)
Bacillus cereus ATCC 10876	2 (+)	8 (-)	-	2 (+)
Pseudomonas aeruginosa ATCC 10145	4 (+)	2 (+)	-	-
MSSA Staphylococcus aureus	8 (-)	2 (+)	2 (+)	4 (+)
MRSA Staphylococcus aureus	8 (-)	4 (+)	-	4 (+)
Eschericia coli	-	-	-	4 (+)
Coagulase-negative *Staphylococcus aureus*: O (R)	4 (+)	4 (+)	4 (+)	8 (-)
Coagulase-negative *Staphylococcus aureus*: O (S)	4 (+)	-	-	4 (+)
Enterococcus faecalis	8 (-)	-	-	8 (-)
Klebsiella sp	4 (+)	4 (+)	4 (+)	4 (+)
Klebsiella pneumoniae ESBL	4 (+)	-	-	4 (+)
Candida parapsilopsis	4 (+)	ND	-	4 (+)

LE-leaf ethanol extract, BH- bark hexane extract, BC- bark chloroform extract, BE- bark ethanol extract

-: no activity, ND: not determined

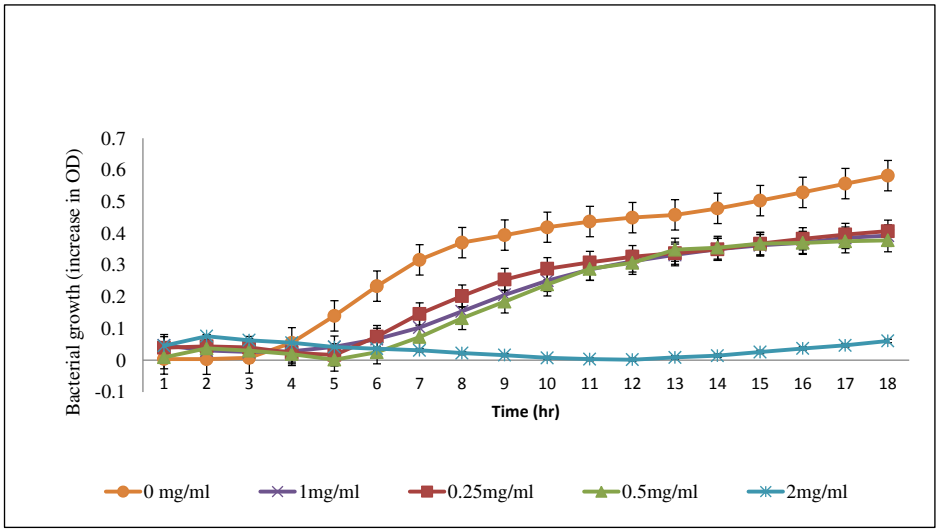


Figure 7.3: Time-kill plot for MSSA ATCC 11632 in presence of LE

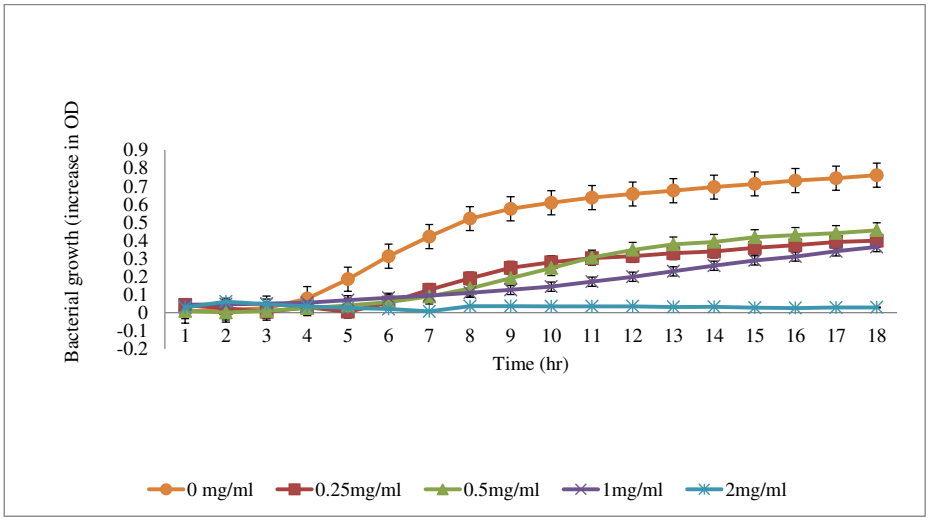


Figure 7.4: Time-kill plot for MRSA ATCC 43300 in presence of LE

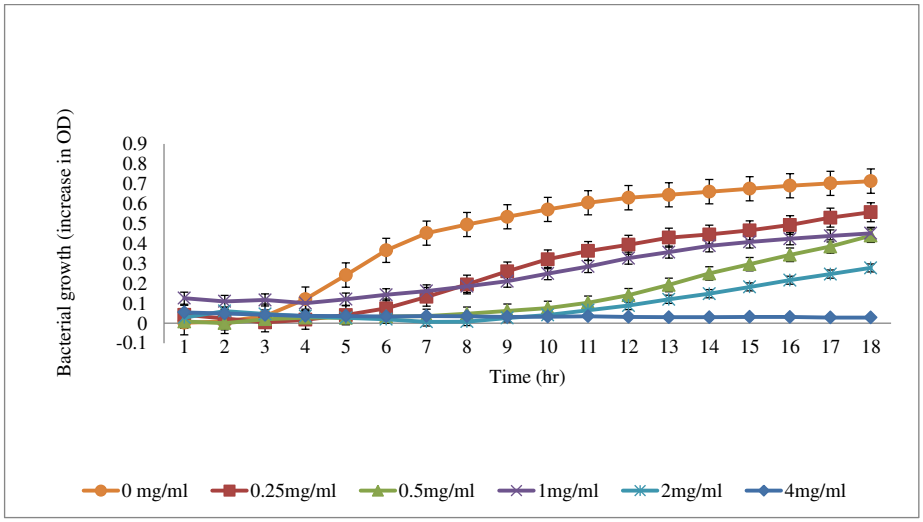


Figure 7.5: Time-kill plot for MSSA (clinical strain) in presence of LE

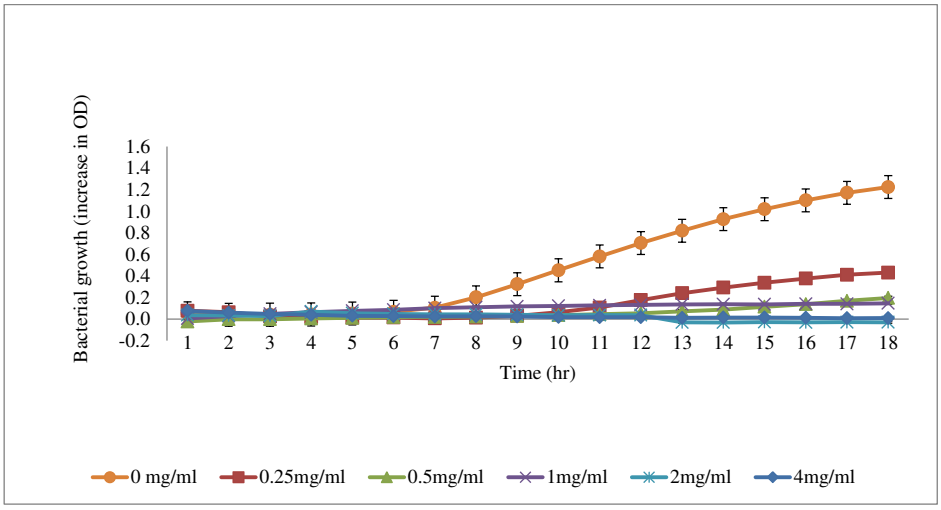


Figure 7.6: Time-kill plot for MRSA (clinical strain) in presence of LE

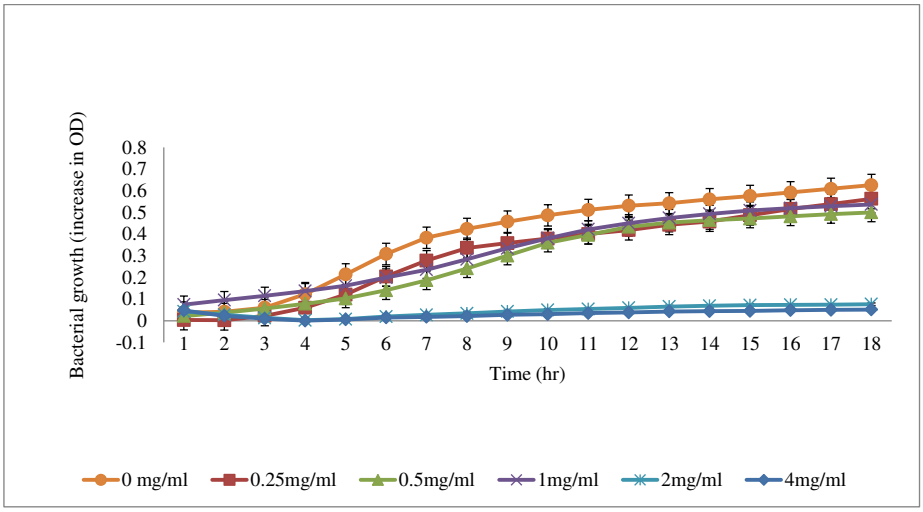


Figure 7.7: Time-kill plot for MSSA (clinical strain) in presence of BE

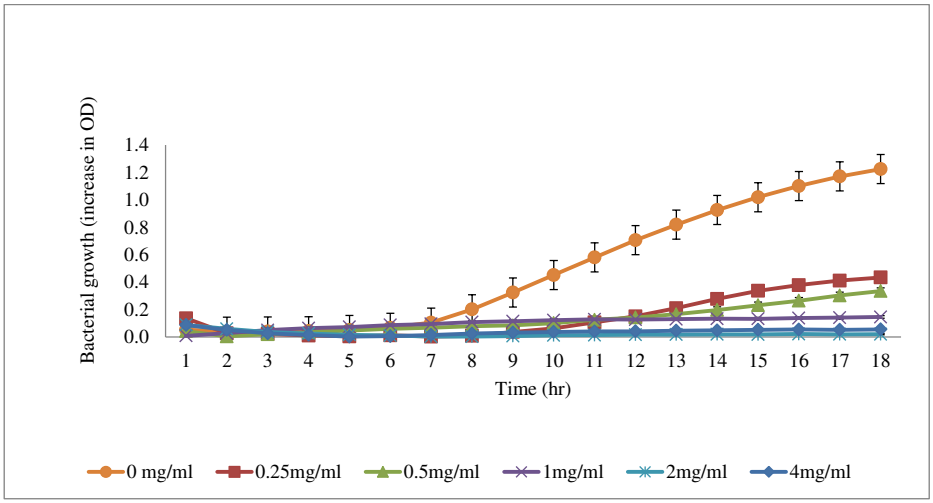


Figure 7.8: Time-kill plot for MRSA (clinical strain) in presence of BE

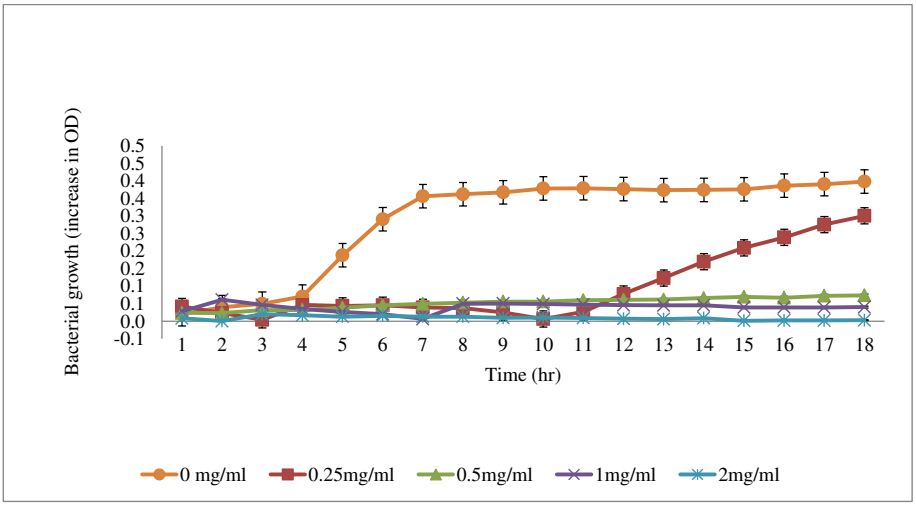


Figure 7.9: Time-kill plot for *Bacillus cereus* ATCC 10876 in presence of LE

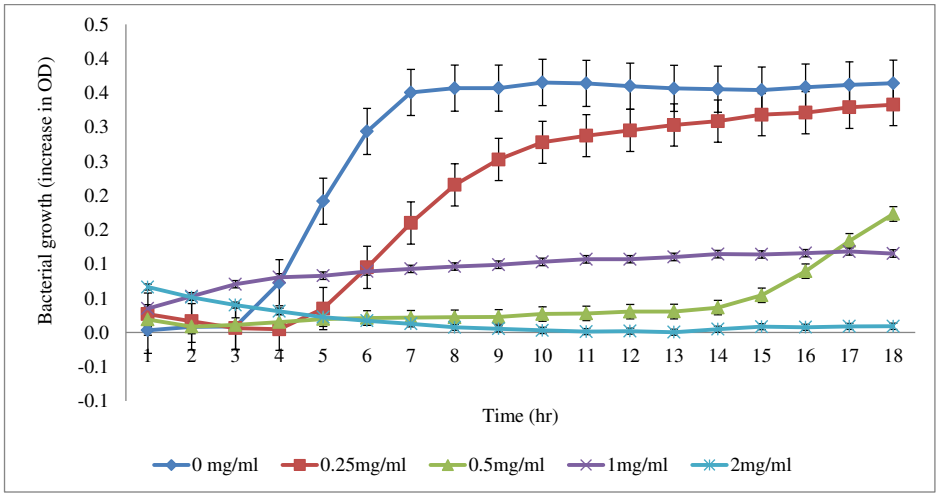


Figure 7.10: Time-kill plot for *Bacillus cereus* ATCC 10876 in presence of BE

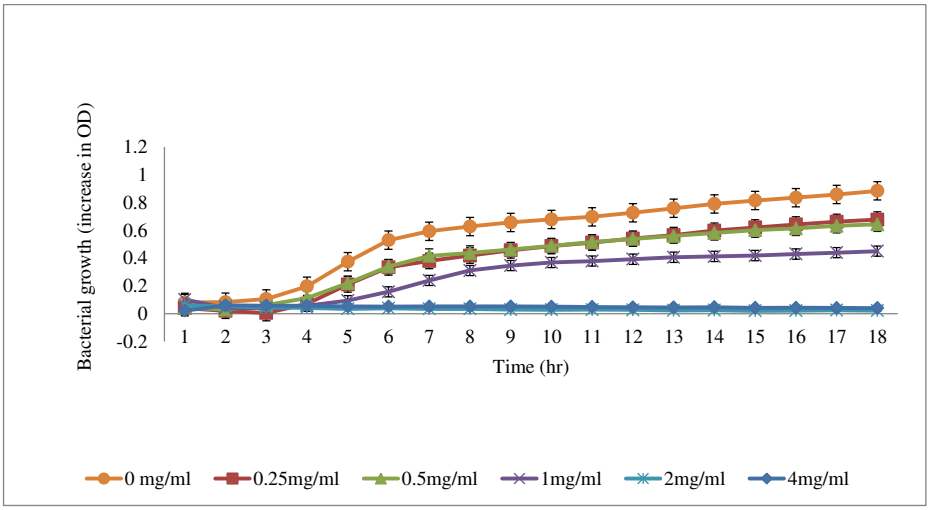


Figure 7.11: Time-kill plot for for *Escherichia coli* (clinical strain) in presence of BE

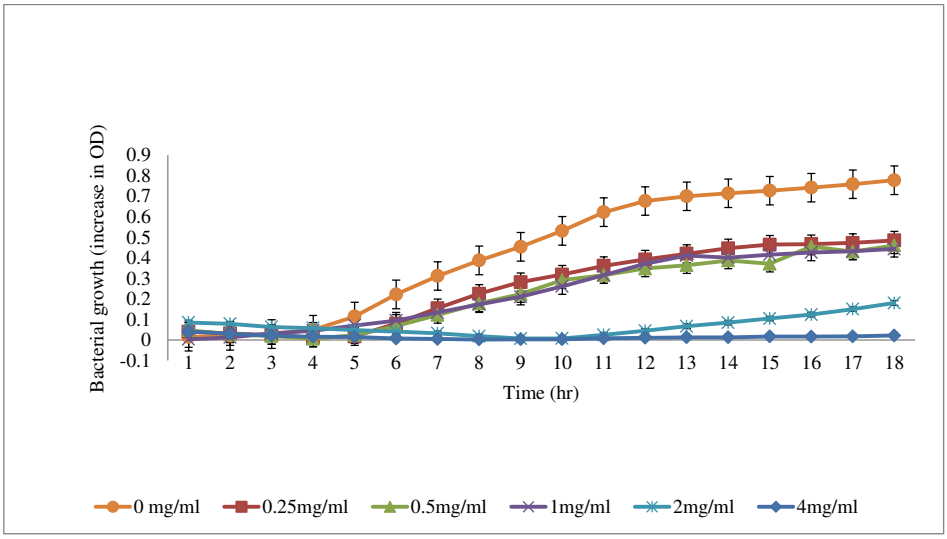
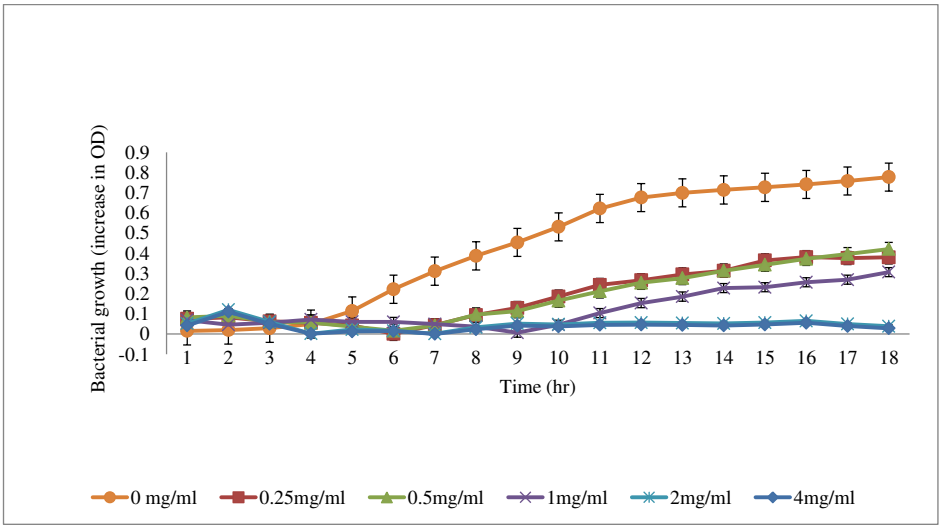


Figure 7.12: Time-kill plot for coagulase-negative *Staphylococcus aureus*: O (R) (clinical strain) in presence of LE



Fig**ure 7.13:** Time-kill plot for coagulase-negative *Staphylococcus aureu*s: O (R) (clinical strain) in presence of BH

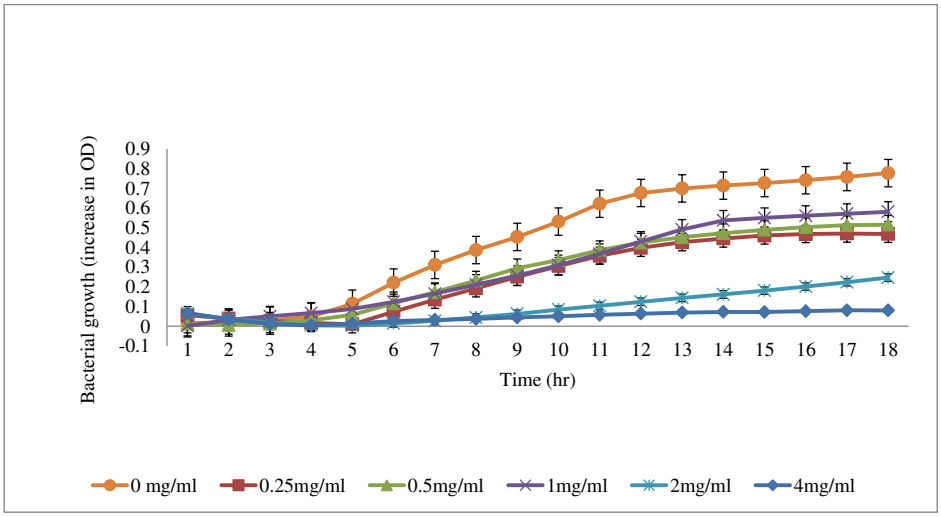


Figure 7.14: Time-kill plot for coagulase-negative *Staphylococcus aureu*s: O (R) (clinical strain) in presence of BE

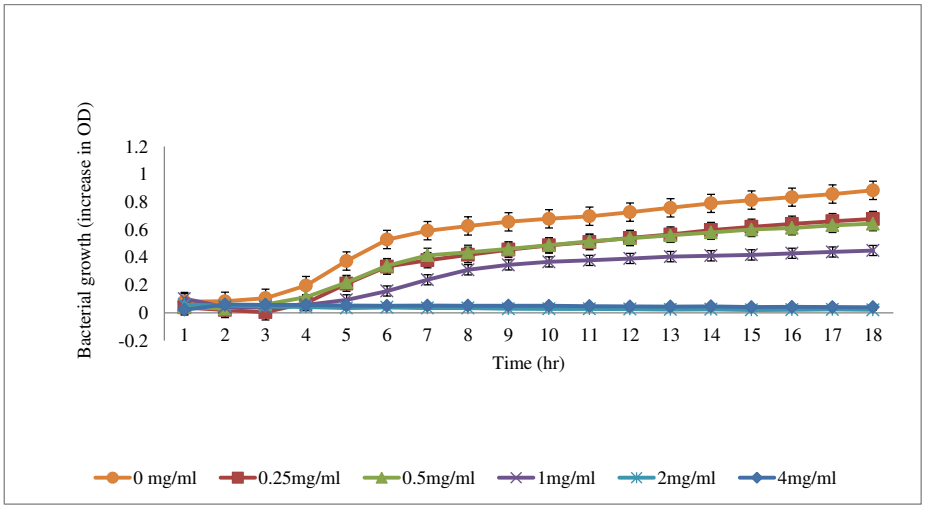


Figure 7.15: Time-kill plot for coagulase-negative *Staphylococcus.aureus*: O (S) (clinical strain) in presence of LE

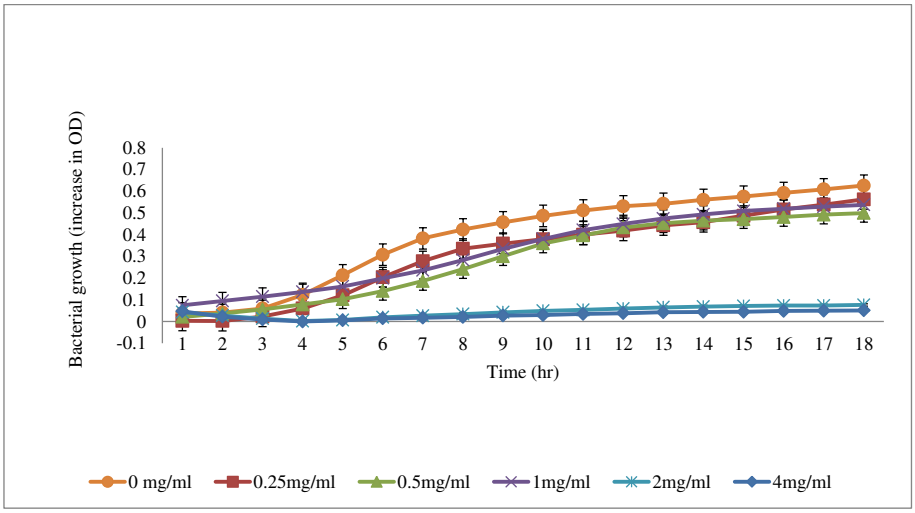


Figure 7.16: Time-kill plot for coagulase-negative *Staphylococcus aureus*: O (S) (clinical strain) in presence of BE

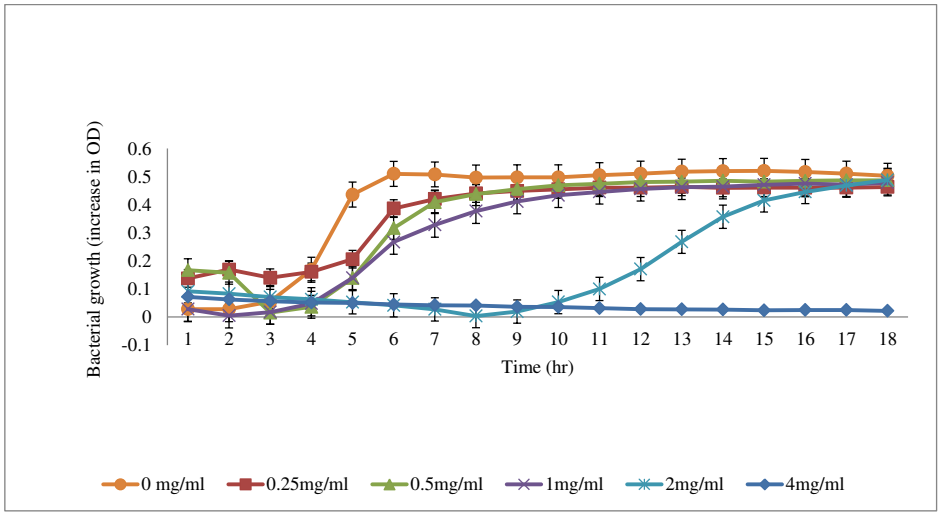


Figure 7.17: Time-kill plot for *Enterococcus faecalis* (clinical strain) in presence of LE

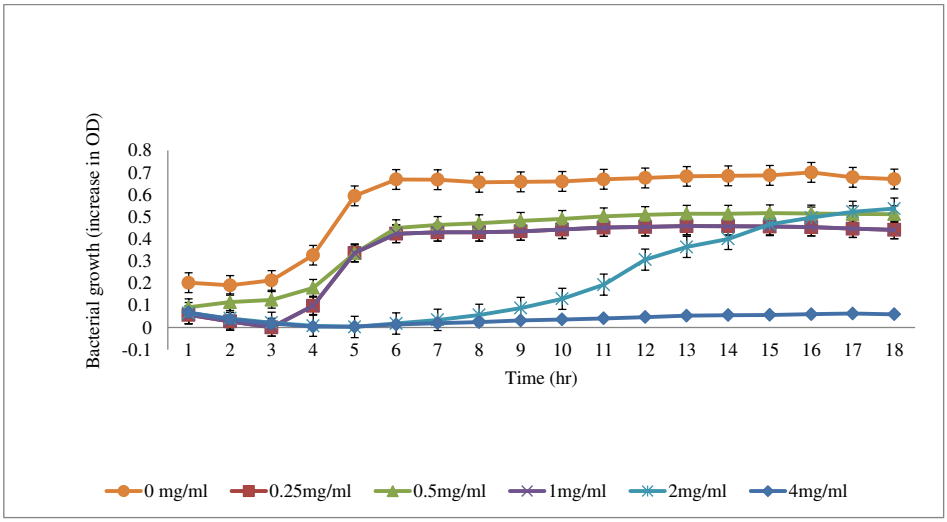


Figure 7.18: Time-kill plot for *Enterococcus faecalis* (clinical strain) in presence of BE

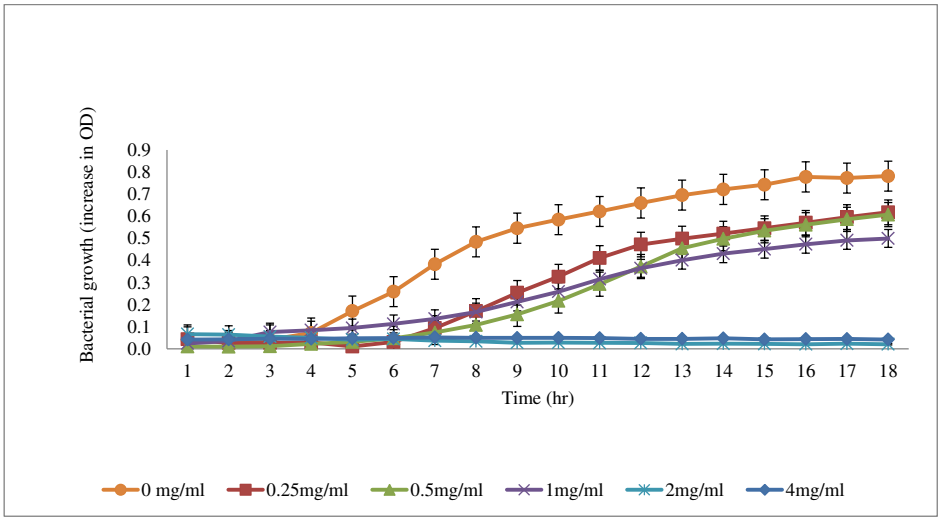


Figure 7.19: Time-kill plot for *Klebsiella sp.* (clinical strain) in presence of LE

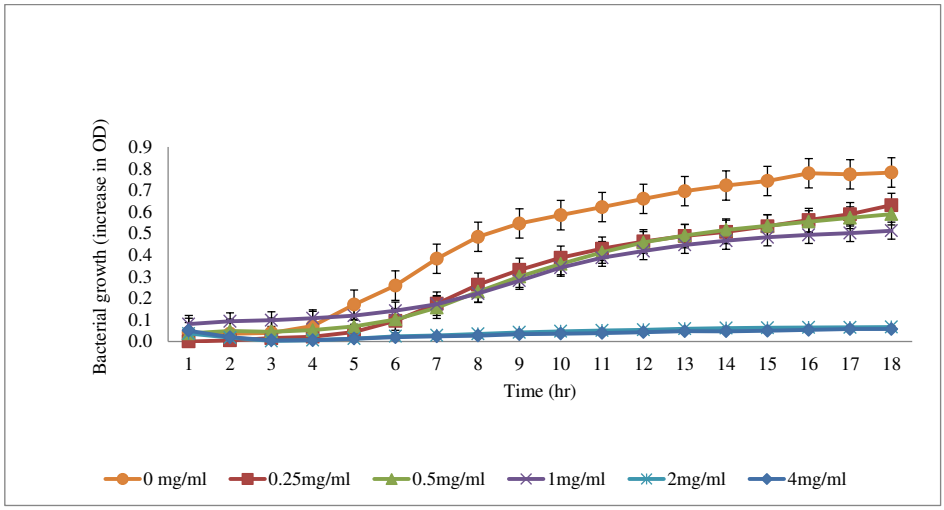


Figure 7.20: Time-kill plot for *Klebsiella sp.* (clinical strain) in presence of BE

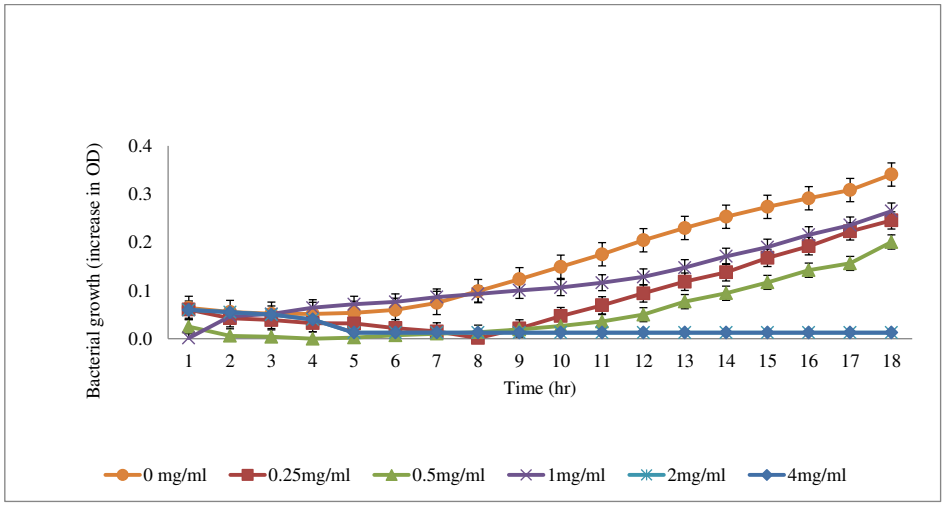


Figure 7.21: Time-kill plot for *Klebsiella pneumonia* ESBL (clinical strain) in presence of LE

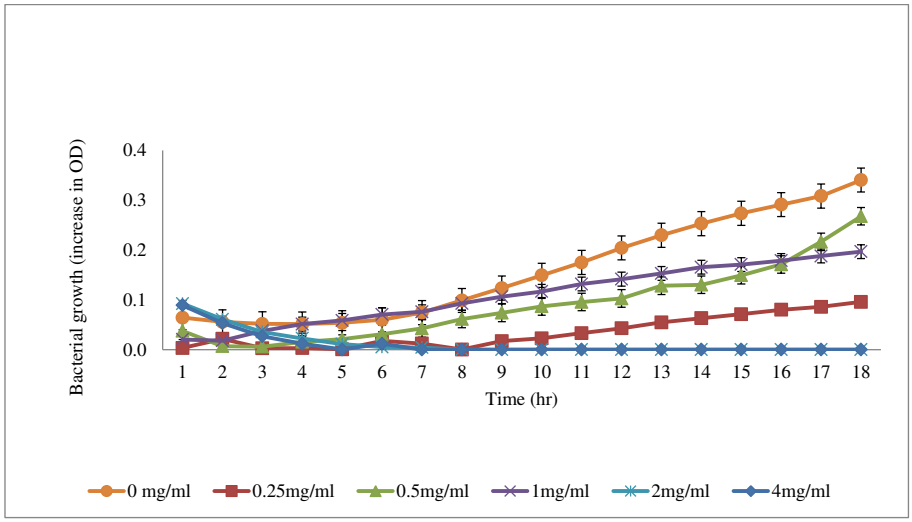


Figure 7.22: Time-kill plot for *Klebsiella pneumonia* ESBL (clinical strain) in presence of BE

7.4.2 MIC, MBC and MIC/MBC ratio

Upon identifying possible antimicrobial action with the disc diffusion assay, a more comprehensive investigation was done to evaluate the total bactericidal/ bacteriostatic activity of an extract against a microb using the MIC/ MBC and time-kill assay. All plant species with MIC values of up to 8 mg/ml are considered to possess at least some degree of inhibitory effect, and any concentration exceeding this should not be considered effective, according to Fabry *et al*. [180]. The majority of the crude bark and leaf extracts of the six *Canarium patentinervium* Miq. exhibited moderate to relatively good activity against Gram-positive and Gram-negative pathogens with the MIC values ranging from 0.25 mg/ml to 8.00 mg/ml. Only the hexane extract of leaves exhibiting weak antimicrobial activity. All active extracts displayed concentration-dependent antimicrobial activity. Aligiannis *et al.* [181] proposed a classification system based on MIC results obtained for plant materials, which was consequently described and implemented by Duarte *et al*. [182].

Strong microbial inhibitors possessed MIC values of equal or lower than 0.50 mg/ml; a clear indication that the MIC value of 0.25 mg/ml obtained for ethanol extracts (leaf and bark) against MRSA *Staphylococcus aureus* (clinical strain) indicates exceptional antimicrobial activity. The ethanol extract of leaves also resulted with the MIC of 0.50 mg/ml against MSSA (both strains) and MRSA (ATCC 43300). Coagulase-negative *Staphylococcus aureus*: oxacillin resistant and sensitive strains were both sensitive against both ethanol extracts with an MIC of 0.5 mg/ml for the bark extracts and MIC range of 0.5 mg/ml to 1.00 mg/ml for the leaves. *Staphylococcus aureus* is a Gram-positive bacteria that can cause a range of illnesses, from minor skin infections,

such as pimples, impetigo, boils, celluliti, folliculitis, carbuncles, scalded skin syndrome and abscesses to life threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, bacteremia and sepsis. Its incidence ranges from skin, soft tissue, respiratory, bone, joint, endovascular to wound infections [183]. MRSA is one of the greatly feared strains of *Staphylococcus aureus* which have become resistant to most β-lactam antibiotics and mostly hospital-accquired. Coagulase-negative *Staphylococcus aureus* are differentiated by their inability to produce free coagulase resulting in their inability to clot blood plasma [184].

The ethanol extracts (bark and leaves) were bactericidal against *Bacillus cereus* with a MIC of 0.50 mg/ml and MBC of 1.00 mg/ml. *Bacillus cereus* is a causative agent of both gastrointestinal infections (diarrheal and emetic type of food poisoning) and non-gastrointestinal infections (post traumatic wound and burn infections. ophthalmic infections, endocarditis, postoperative meningitis and urinary tract infections) [185]. The ethanol extracts were also bactericidal against Gram-negative *Klebsiella* sp and *Klebsiella pneumoniae* ESBL with MIC of 0.50 mg/ml and MBC of 2.00 mg/ml. *Klebsiella* is well known to most clinicians as a cause of community-acquired bacterial pneumonia, occurring particularly in chronic alcoholics which have a high fatality rate if untreated. Nosocomial *Klebsiella* infections are caused mainly by *Klebsiella pneumoniae*, the medically most important species of the genus. Due to the extensive spread of antibiotic-resistant strains, especially of extended-spectrum b-lactamase (ESBL)-producing strains, there has been renewed interest in *Klebsiella* infections. Since ESBL production frequently is accompanied by multiresistance to antibiotics, therapeutic options become limited [186].

The points above will make the discovery of an antimicrobial agent as effective as *Canarium patentinervium* Miq. against these pathogens highly significant. The ethanol extract of leaves displayed good microbial inhibition action against all microbs tested except for *Pseudomonas aeruginosa* and *Candida parapsilopsis* with moderate inhibition but bactericidal action with the MIC of 1.00 mg/ml and 2.00 mg/ml respectively. *Pseudomonas aeruginosa* is a Gram-negative bacterium and an opportunistic, nosocomial pathogen in immunocompromised individuals. It typically infects the pulmonary tract, urinary tract, burns, wounds and blood infections [187].

Moderate microbial inhibitors are described by Aligiannis *et al.* [181] as those plant extracts with MIC values ranging between 0.60 mg/ml and 1.50 mg/ml. Amongst the extracts investigated in the present study, hexane extracts of the barks seemed to have most of the moderate inhibitors with an interesting bactericidal action against MRSA ATCC 43300 strain (MIC of 1.00 mg/ml and MBC of 4.00 mg/ml). Weak microbial inhibitors are classified as those agents with MIC values of between 1.60 mg/ml and 8.00 mg/ml [181]. None of the extracts in the present study yielded MIC values in excess of 8.00 mg/ml against Gram-positive bacteria except with hexane extract of leaves. The results obtained against the Gram-positive and Gram-negative bacteria thus support the traditional use of *Canarium patentinervium* Miq. in wound healing[164] and hold potential in the treatment of colds, wound healing and as an antiseptic.

In general, the Gram-negative bacteria displayed the least sensitivity towards the extracts, and most of the plant extracts (except ethanol extract of barks) exhibited poor and unvaried activity against *Esherichia coli*, indicating the resistance of this

bacterium to the plant extracts. This was to be expected, as Gram-negative bacteria offer a much more complex barrier system against permeation of foreign substances (in this case, the antimicrobial agent). This is attributed to the specialised cell wall structure and especially the presence of the outer envelope resulting in the impermeability of these micro-organisms to biocides and antibiotics, and at times, resulting in regulation and prevention of their passage to the target region [188]. Resistance to the plant extracts is, thus, exhibited to a far greater extent by the Gram-negative bacteria than by Gram-positive bacteria [188]. The lipophilic or hydrophilic nature of compounds also plays a role in the activity, or lack thereof, against the micro-organisms. Compounds considered to be more effective against Gram-negative bacteria are considerably less lipophilic. This is as a result of the structure of the Gram-negative cell wall which also has a higher lipid content [189]. This could explain why in this study the ethanol extracts which contain more hydrophilic compounds showed more inhibition against Gram-negative bacteria such as *Esherichia coli* and *Klebsiella* species.

The ethanol extracts and hexane extract of barks also showed inhibition against *Candida parapsilopsis* with a cidal and static action respectively. Both ethanol extracts had MIC of 2.00 mg/ml (MBC of 8.00 mg/ml) and hexane extract of barks had an MIC of 8.00 mg/ml (MBC >16.00 mg/ml). More than 80% of all fungal bloodstream infections are a direct result from the Candida species and since the eighties there has been an explosive rise in the rate of Candidal infections. Over the past decade, the incidence of *Candida parapsilosis* has dramatically increased. In fact, reports indicate that *Candida parapsilosis* is often the second most commonly isolated

Candida species from blood cultures and *Candida parapsilosis* even outranks *Candida albicans* in some European, Asian, and South American hospitals [190].

The ethanol extracts have shown to have polyphenols such as tannins and flavonoids from the phytochemical analysis (chapter 2). Polyphenols, such as tannins and flavonoids, have important antibacterial activity [191]. The antimicrobial activity of flavonoids is due to their ability to complex with extracellular and soluble protein and to complex with bacterial cell wall while that of tannins may be related to their ability to inactivate microbial adhesions, enzymes and cell envelop proteins [163]. 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 [192].

Tannin is a general descriptive name for a group of polymeric phenolic substances capable of tanning leather or precipitating gelatin from solution, a property known as astringency. They are found in almost every plant part: bark, wood, leaves, fruits and roots. Tannins may be formed by condensations of flavan derivatives or by the polymerization of quinine units [191]. Many human physiological activities, such as stimulation of phagocytic cell, host mediated tumour activity and a wide range of anti-infective actions, have been assigned to tannins. Their mode of antimicrobial action as described for quinone, may be related to their ability to inactivate microbial adhesions, enzymes, cell envelope, transport-proteins etc [191]. They also form a

complex with polysaccharide. According to a number of studies, tannins can also be toxic to filamentous fungi, yeasts and bacteria [192]. However, it is important to note that, if tannins were solely responsible for the activity presented by these results, this activity would be observed against all organisms and would not be limited to Gram-positive bacteria or yeasts. The current hypothesis is thus that tannins are at least partially responsible for the antibiotic activity. Coumarins have also demonstrated antimicrobial activity [163].

7.4.3 Time-kill assay

All extracts with good inhibitors (MIC value ≤ 0.5 mg/ml) was tested for the time-kill efficacy. All extracts displayed killing (bactericidal) action except the ethanol extracts (leaves and barks) against *Enterococcus faecalis* which was only bacteriostatic (Figure 7.17 & 7.18) and ethanol extract of barks that was bacteriostatic against coagulase-negative *Staphylococcus aureus*: oxacillin resistant (Figure 7.14). The other extracts bactericidal effect was achieved in the concentration of 2.00 to 4.00 mg/ml. Killing rate was observed at varying times from range of 1 hr to 10 hrs all resulting in complete kill.

7.5 Conclusion

All crude extracts of *Canarium patentinervium* Miq. under investigation exhibited exceptional concentration-dependent antimicrobial activity against both Gram-positive and Gram-negative bacteria as well as against yeasts. The most promising

activity was displayed against Gram-positive bacteria *Staphylococcus aureus*, MRSA, *Bacillus cereus* and Gram-negative bacteria and *Pseudomonas aeruginosa, Klebsiella* sp, *Klebsiella pneumoniae* ESBL and yeast *Candida parapsilopsis*. This serves as a clear indication of the potential of these extracts for further chemical studies as antimicrobial agents. The antimicrobial activity exhibited by the bark extracts and ethanol extract of leaves against MSSA and MRSA indicates exceptional antimicrobial activity. All the tested bacterias and yeast were susceptible to either bark or leaf extracts.

In general, the Gram-negative bacteria *Escherichia coli* displayed the least sensitivity towards the extracts, being inhibited only by the ethanol extract of the bark indicating the resistance of this bacterium to the rest of the plant extracts. The activity against *Klebsiella pneumonia* ESBL and *Klebsiella sp.* was, however, far more promising with variability amongst the plant extracts. It is at this stage important to note that the failure of a plant extract to demonstrate *in vitro* activity during the general screening process does not necessarily imply a total absence of inherent medicinal value. The possible presence of synergistic interactions between the different plant constituents in crude preparations may result in activities that are not exhibited by isolated compounds, and should not be excluded.

CHAPTER 8

ANTIPARASITIC ACTIVITY

8.1 Introduction

8.1.1 Parasitic infection: causative factor and treatment

Parasitic infection is one of a major health problem in the developing world. Twenty-five percent of the world's population could be suffering from parasitic infection Highest prevalence is in underdeveloped agricultural and rural areas in the tropical and subtropical regions. In some areas incidence may reach 90 % of the population. During the evolution of humans a broad set of parasites have evolved, that use us as a host organism [193]. Usually a parasite will not kill its host (at least not immediately), as this would be an evolutionary dead end for a parasite. However, most parasites are either unpleasant for us (think of lice and fleas) or weaken our health (most internal parasites). However, some parasitic infections, such as malaria, trypanosomiasis or Chagas 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 [194].

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 1–2 billion infections, which lead to several million deaths every year [195]. An overview on important endoparasites under this study, their vectors, distribution, and disease symptoms is given in Table 8.1.

Table 8.1: Human endoparasites in study

Parasite	**Disease (estimated number of infections)**	**Vector (hosts); route of transmission**	**Distribution**	**Symptoms**
Trypanosomatida				
Leishmania donovani	Visceral leishmaniasis (kala-azar) (15 million)	Flies (Phlebotomus, Lutzomyia); bites	N.Africa, Eurasia, S America	Enlargement of liver and spleen, fever, dermal lesions, dermal nodules
Amoebida				
Entamoeba histolytica and other species	Amoebiasis (70000 death/year)	Infection from contaminated water or food	Worldwide	Dysentery, destruction of intestinal tissues, fever, liver and lung abscess
Diplomonadida				
Giardia lamblia	Giardiasis (2 million infections/ year in USA)	Infection from contaminated water	Worldwide	Infection of duodenal and jejunal mucosa; diarrhoea, fever

Leishmaniasis is a group of disease, caused by *Leishmania* species. The disease is considered as a major public health problem in 82 countries in the world causing morbidity and mortality [196]. Different modes of treatment are used in the treatment of cutaneous leishmaniasis. Pentavalent antimonial compounds 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 [193]. Leishmaniasis is caused by protozoan parasites of the genus *Leishmania* which invade macrophages of host

organisms. A distinction is made between cutaneous, mucocutaneous, visceral and diffuse leishmaniasis, of which visceral leishmaniasis is a fatal disease causing approximately 60,000 death per year [197]. Leishmaniasis and HIV infections often co-occur and these patients usually have a poor prognosis. Patients are treated with the synthetic drugs stibogluconate, meglumine and pentamidine (developed 70 years ago), which have severe side effects and fail to work in North Bihar (India) [198][199]. Also 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 [197]. Among natural products, berberine (which occurs in many TCM plants) had promising anti-leishmanial activities [198][199].

Entamoeba histolytica and *Giardia intestinalis* are parasite of large and small intestine of human, respectively. Although infection with *E. histolytica* is asymptomatic in 90% of cases, about 50 million people are estimated to suffer from the symptoms of amoebiasis such as haemorrhagic colitis and amoebic liver abscess. These infections result in 50,000 to 100,000 deaths annually [200]. *Giardia intestinalis* (Synonyms: *Giardia duodenalis*, *Giardia lamblia*) is one of the most common universal pathogenic intestinal protozoan parasites of humans [201]. It is also widely spread in various animals such as dogs [202], cats [203] and cattle [204]. It is frequently reported to be a significant cause of diarrhea in people traveling to tropical countries (traveler's diarrhea). Although the majority of infected humans shed *Giardia* cysts without any symptoms, the major clinical signs of giardiasis in humans include acute or chronic diarrhea, nutritional disorders and weight loss. Recent studies have shown that *Giardia* genotypes may be shared between human and numerous

other species of animal [205][206] indicating transmission between humans and animal vectors may occur, possibly through exposure to contaminated water.

Metronidazole, the current drug of choice, can cause mutagenicity in bacteria [207] and is carcinogenic in rodents [208][209]. It also possesses undesirable side effects and failures in treatment have been reported [210][211]. We therefore plan to evaluate the *in vitro* activity of *Canarium patentinervium* Miq., against *Giardia intestinalis* in the search for an alternative drug suitable for use in preventing and treating cases of diarrhoea.

8.1.2 Natural products and parasitic defence

We know that humans have used medicinal plants for several thousands of years to treat illness and health disorders [212]. Medicinal chemists have synthesized a number of drugs which can be used against many but by far not all endoparasites. A major problem is that many of these drugs were developed many years ago and some parasitic strains have become resistant to them. The development of new antiparasitic drugs has not been much of a priority for the pharmaceutical industry because many of the parasitic diseases occur in poor countries where the populations cannot afford to pay a high price for the drugs. Thus an investment in drug development against parasitic diseases is a risky affair. An alternative to synthetic drugs is the search for anti-parasitic plant extracts or secondary metabolites derived from them. Natural products still play an important role in therapy: between 1981 and 2006, 1,184 new drugs were registered of which 28% were natural products or their derivatives. Another 24% of the new drugs had pharmacophores (*i.e.*, functional groups with

pharmacological activity) derived from natural products [213]. A good starting point to find antiparasitic natural products would be traditional medicinal plants, such as those known from Asia, Africa or America [212] that have been employed to treat infections.

8.2 Materials and Methods

8.2.1 Principle of method

The antiprotozoal activity of the compounds was perform according to the standard methods as described by Sawangjaroen *et al*. [214][200]. The antileishmanial activity of the compounds was perform according to the standard methods as described by Mosmann [215].

8.2.2 Protocol

8.2.2.1 Antiparasitic assay against *Giardia intestinalis* and *Entamoeba histolytica*

8.2.2.1.1 Test organisms

Giardia intestinalis

A local Thai strain of *Giardia intestinalis*, originally described by Siripanth *et al*. [216] was used throughout this experiment.

Entamoeba histolytica

Entamoeba histolytica strain HM1: IMSS, purchase from ATCC was used throughout this experiment.

8.2.2.1.2 Cultivation of organisms

Both *Giardia intestinalis* and *Entamoeba histolytica* were cultured axenically in screw capped tubes at 37 ^{o}C, under anaerobic condition, on YI medium [217] supplemented with 10% heat-inactivated horse serum. Subculture was performed every 48 hr. For assays, trophozoites were harvested by chilling the tube on ice for 15 min to detach the monolayer and centrifuged at 300 g for 5 min. The supernatant was decanted and cells were re-suspended in fresh medium. The numbers of viable cells were calculated using a haemocytometer and 0.4 % (w/v) trypan blue. The criteria used for viability were motility and dye exclusion.

8.2.2.1.3 Antiprotozoal assay

Briefly, trophozoites, 2x10^{5} cells/ml, was incubated, in triplicate, in the presence of serial two fold dilutions of plant extracts that ranged from 31.25 to 500 μg/ml for crude extract in 96 well tissue culture plates (200 μl/well). Metronidazole and complete medium with added DMSO will be used as negative and positive control, respectively. After 24 hr of incubation at 37 ^{o}C under anaerobic conditions, the trophozoites from each well will examine with an inverted microscope. The appearance of trophozoites will score and presented as score values from 1 to 4 with 1 showing the most inhibition of growth and 4 showing no inhibition, according to

Upcroft and Upcroft [218]. The minimum inhibitory concentration (MIC) will be recorded (the lowest concentration at which >90% of the trophozoites rounded up). Each concentration was tested in triplicates and at least three experiments were performed on separate occasions.

8.2.2.2 Antiparasitic activity against *Leishmania donovani* promastigotes

Antileishmanial activity of tested plant extract, against *Leishmania donovani* (strain MHOM/IN/1983/AG83) promastigotes, was evaluated by a quantitative colorimetric assay using MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; Sisco Research Laboratory, Mumbai, India]. Amphotericin B (Sigma-Aldrich Co., MO, USA; IC_{50} =0.4±0.1µM) 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 ^oC. After 72 hr, 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] x 100.

8.2.3 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.

8.3 Results

The result of the *in vitro* effect of extracts on *Giardia intestinalis* and *Entamoeba histolytica* was summarized in Table 8.2. It was found that the ethanol extracts were both active against growth of *Giardia intestinalis in vitro* with MIC 500 µg/ml (Table 8.2). The MIC of metronidazole was 2.5 µg/ml and the remaining extracts showed no activity (MIC >500 µg/ml for crude extract). The appearance of trophozoites in negative, positive and with exposure to LE under microscope is shown in Figure 8.1.

The result of the *in vitro* effect of extracts on *Leishmania donovani* was summarized in Table 8.3. The potency of extracts against *Leishmania donovani* was LH > BH > BC > LC. Both ethanol extracts of leaves and barks had IC_{50} values of above 500 µg/ml. Hexane extract displayed lowest IC_{50} value of 257.40±0.30 µg/ml. Figure 8.2 shows the inhibition of the hexane and chloroform extracts.

8.4 Discussion

Parasites are eukaryotes and therefore share most molecular and biochemical properties with their eukaryotic hosts, making it often difficult to find antiparasitic drugs which are both effective and non-toxic for humans. This limitation always has to be kept in mind when discussing the numerous findings that some drug or extract from a medicinal plant is active against parasites *in vitro*. In order to be medicinally useful, such a drug must have bioavailability and should not intoxicate 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 [194].

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. 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. TCM and other traditional medicine systems employ several thousand of medicinal plants; some of which have known antiparasitic properties.

Table 8.2: MIC values of *Canarium patentinervium* Miq. incubated for 24 hr with *Giardia intestinalis* and *Entamoeba histolytica* growing *in vitro*.

Sample	**MIC (µg/ml)**	
	Giardia intestinalis	***Entamoeba histolytica***
LH	>500	>500
LC	>500	>500
LE	500	>500
BH	>500	>500
BC	>500	>500
BE	500	>500
Metronidazole	2.5	2.5

LH: leaf hexane extract, LC: leaf chloroform extract, LE: leaf ethanol extract, BH: bark hexane extract, BC: bark chloroform extract, BE: bark ethanol extract. Data were obtained from three independent experiments, each performed in triplicates (n=9) and represented as mean ± SD. Values with the same capital letter are not significantly different ($P<0.01$) according to Tukey multiple comparison test.

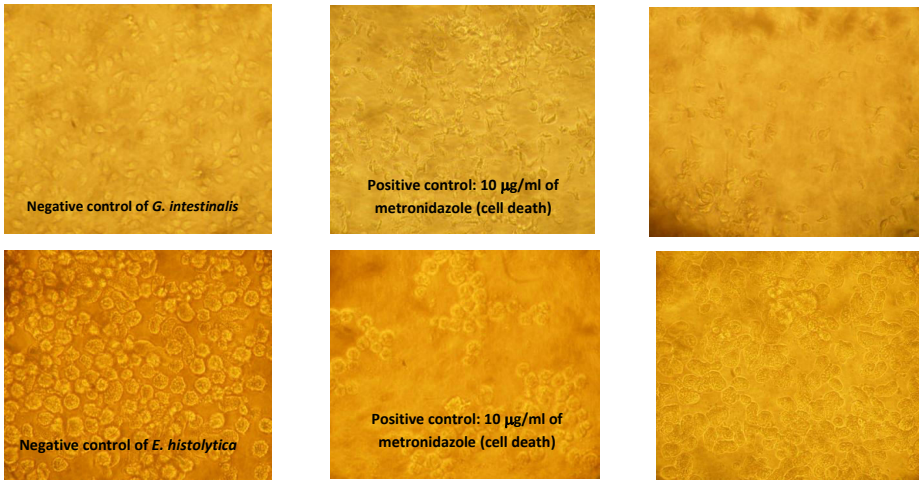


Figure 8.1: Appearance of trophozoites under microscope in negative control, positive control and with LE

Table 8.3: Antiparasitic values of *Canarium patentinervium* Miq. against *Leishmania donovani* (AG 83) promastigotes

Sample	**IC_{50} (µg/ml)**
LH	257.40±0.30
LC	457.70±0.25
LE	>500
BH	284.20±0.40
BC	359.90±0.20
BE	>500

LH: leaf hexane extract, LC: leaf chloroform extract, LE: leaf ethanol extract, BH: bark hexane extract, BC: bark chloroform extract, BE: bark ethanol extract.
Data were obtained from three independent experiments, each performed in triplicates (n=9) and represented as mean ± SD. Values with the same capital letter are not significantly different (P<0.01) according to Tukey multiple comparison test.

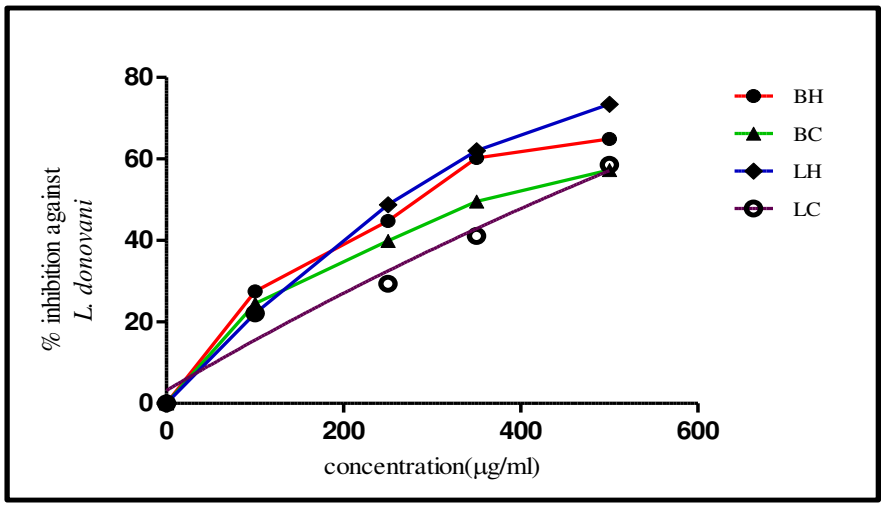


Figure 8.2: MTT assay *of Canarium patentinervium* Miq. against *Leishmania donovani.*

They offer a unique opportunity to identify natural products which could be potentially used to treat parasitic infections. The success story of artemisinin from *Artemisia annua* [213][219] can probably be repeated. A number of medicinal plants and secondary metabolites isolated from them have been screened for anti-trypanosomal activity [220]. Active natural products include several groups of alkaloids, phenolics, saponins, cardiac glycosides, other terpenoids, and polyacetylenes (common in Apiaceae, Asteraceae and Araliaceae). Although some natural products are active in the submicromolar range and show good selectivity, only few have been studied *in vivo* in an animal model.

In the present study, six different extracts of *Canarium patentinervium* Miq. (Burseraceae) leaves and barks were screened for their *in vitro* antiparasitic activities, among the different extracts tested, the hexane extract of leaves showed moderate antileshmanial activity with IC_{50} values of 257.40±0.30 µg/ml. This could be due to essential oils present in the hexane extracts as shown previously in the family of Burseraceae [221]. Only ethanol extracts showed activity against *Giardia intestinalis* and *Entamoeba histolytica* at concentration of 500 µg/ml. Other extracts were inactive against these two intestinal parasites.

Small lipophilic secondary metabolites, such as terpenoids or phenylpropanoids as found in the essential oil of many plants (especially in Lamiaceae, Myrtaceae, Rubiaceae, Apiaceae, Asteraceae, Lauraceae, Rutaceae, Burseraceae, Verbenaceae, Pinaceae, Cupressaceae), can dissolve in biomembranes and disturb their fluidity and the function of membrane proteins [221]. Therefore, many of the lipophilic mono- and sesquiterpenes, phenylpropanoids and isothiocyanates (as present in Brassicaceae,

Tropaeoleaceae) have a certain degree of antimicrobial and antiparasitic properties [222].

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. Preliminary antiparasitic screening of six extracts of *Canarium patentinervium* Miq. showed that the ethanol extract had promising growth inhibition on *Giardia intestinalis* and *Entamoeba histolytica*. The hexane extract of leaves was active against *Leshmanial donovani*. The results obtained suggest that further isolation studies can be performed on *Canarium patentinervium* Miq to identify the active constituents responsible for the antiparasitic activities.

CHAPTER 9

ANTICANCER ACTIVITY

9.1 Introduction

9.1.1 Carcinogenesis

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 [223] .

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.

Despite the therapeutic advances made in understanding the processes involved in carcinogenesis, cancer has become one of the most serious medical problems today. The worldwide mortality rate increases annually, with more than seven million deaths occurring per year. For this reason, cancer chemotherapy has become a major focus area of research. 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 [223]. 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 [224]. One of the most important contributions to the development of cancer is the oxidative damage to DNA [225]. 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 [224].

9.1.2 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 [226]. 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 treatment of 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 [227]. Of the 121 medications being prescribed for use in cancer theraphy, 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 tradition [228]. 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 9.1.

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 [227]. 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 [227] .

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 [224]. 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 via angiogenesis and metastasis.

The present study was undertaken to screen the anticancer potential *Canarium patentinervium* Miq. No anticancer studies have previously been reported on this species to date.

Table 9.1: 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	[229]
Etoposide, teniposide	*Podophyllum peltatum*	Inhibition of topoisomerase II	Testicular cancer, and small cell lung carcinoma, leukaemias, lymphomas	[229] [230] [231]
Paclitaxel, docetaxel	*Taxus brevifolia*	Promotion of tubulin stabilization	Ovarian and breast carcinoma	[229]
Irinotecan, topotecan, 9-aminocampothecin, 9-nitrocamptothecin	*Camptotheca acuminata*	Inhibition of topoisomerase I	Advanced colorectal cancer, also active in lung, cervix and ovarian cancer	[230]
Homoharingtonine	*Harringtonia cephalotaxus*	Inhibition of DNA polymerase	Various leukaemias	[229]
4-Ipomeanol	*Ipomoea batatas*	Cytochrome P-450-mediated conversion into DNA-binding metabolites	Lung cancer	[229]
Elliptinium	*Bleekeria vitensis*	Inhibition of topoisomerase II	Advanced breast cancer	[229]
Flavopiridol	*Amoora rohituka, Dysoxylum binectariferum*	Inhibition of cyclin-dependent 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	[229]

9.2 Materials and Methods

9.2.1 Principle of the MTT assay

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) [232]. Biological *in vitro* assay was determined as follows [233].

9.2.2 Protocol

9.2.2.1 Cell lines and cell culture

Human derived cell lines [HCT 116,colon cancer cell line and MCF-7 (ER+), MDA 468 (ER-) breast carcinoma] were routinely cultivated at 37 °C 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 hr to adhere before extracts were introduced (final concentration 200 µg/ml to 1 µg/ml).

9.2.2.2 Preparation of plant samples

Extracts were prepared as 400 µg/ml top stock solutions, dissolved in DMSO, and stored at 4 ^{o}C, protected from light for a maximum period of 4 weeks. Serial sample dilutions were prepared in medium immediately prior to each assay.

9.2.2.3 The MTT assay

At the time of extract addition and following 72 hr exposure, MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide) was added to each well. Incubation at 37 °C for 4 hr 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). Absorbance was read on an Anthos Labtec System plate reader at 550 nm as a measure of cell viability, thus cell growth or extract toxicity was determined. Doxorubicin was used as positive control.

9.2.3 Statistical analysis

Concentration–response curves were calculated using 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.

9.3 Results

A range of extracts were evaluated in MTT assays following a 3-day exposure against a panel of two human breast cancer cell lines, MCF-7 (ER+) and MDA 468 (ER-), and a colon cancer cell line, HCT 116. In general, the breast cancer cell lines were more sensitive to the extracts, and the MDA 468 line was the most sensitive of the three lines (Table 9.2) The best growth inhibition was observed with the chloroform and ethanol extract of barks, GI_{50} values of 23.44 µg/ml and 34.40 µg/ml respectively (Figure 9.1-9.3).

9.4 Discussion

In the present study, six different extracts of *Canarium patentinervium* Miq. (Burseraceae) leaves and barks were screened for their *in vitro* antitumor activities. Among the different extracts tested, the chloroform and ethanol extract of barks showed good antitumor activities with GI_{50} values of 23.44±0.05 µg/ml and 34.40±0.21 µg/ml respectively. The most susceptible cell lines were found to be the breast cancer cell line, MDA 468.

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.

Table 9.2: Growth inhibition of human cancer cell lines by crude plant extracts of *Canarium patentinervium* Miq.

Extracts	Cell line/ GI_{50} (µg/ml)		
	MDA 468	**HCT 116**	**MCF-7**
LH	>100	>100	>100
BH	>100	>100	>100
LC	>100	>100	>100
BC	23.44±0.05	62.46±0.23	64.50±0.27
LE	61.48±0.23	97.18±0.08	85.40±0.18
BE	34.4±0.21	46.53±0.12	39.06±0.16
Doxorubicin	0.06±0.12	0.70±0.18	0.34±0.26

LH: leaf hexane extract, LC: leaf chloroform extract, LE: leaf ethanol extract, BH: bark hexane extract, BC: bark chloroform extract, BE: bark ethanol xtract
Data were obtained from three independent experiments, each performed in triplicates (n=9) and represented as mean ± SD.
Values with the same letter are not significantly different (P<0.01) according to Tukey multiple comparison test.

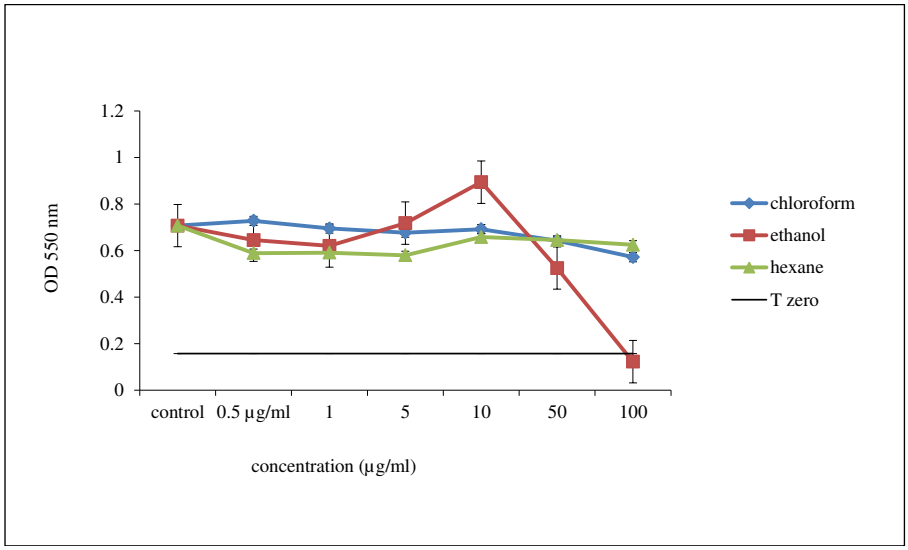


Figure 9.1a: Effects of extract of leaves against MDA 468 growth

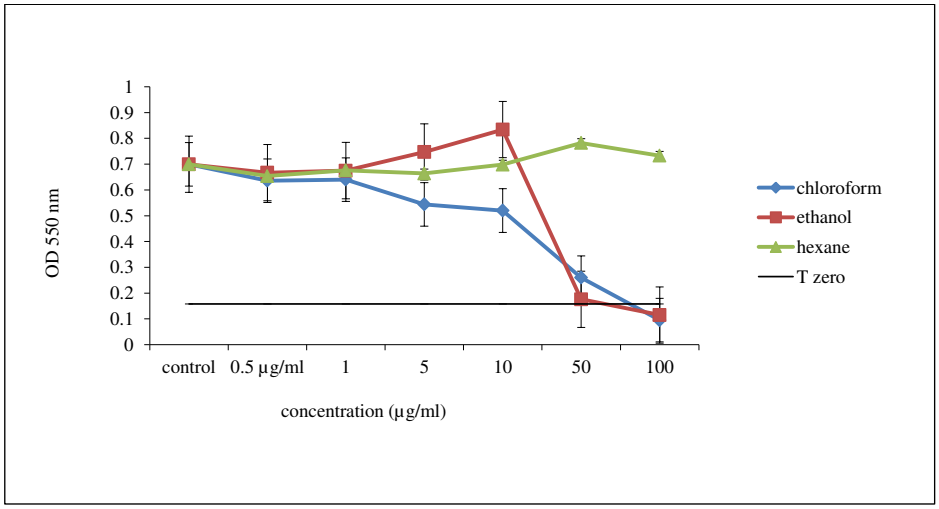


Figure 9.1b: Effects of extract of barks against MDA 468 growth

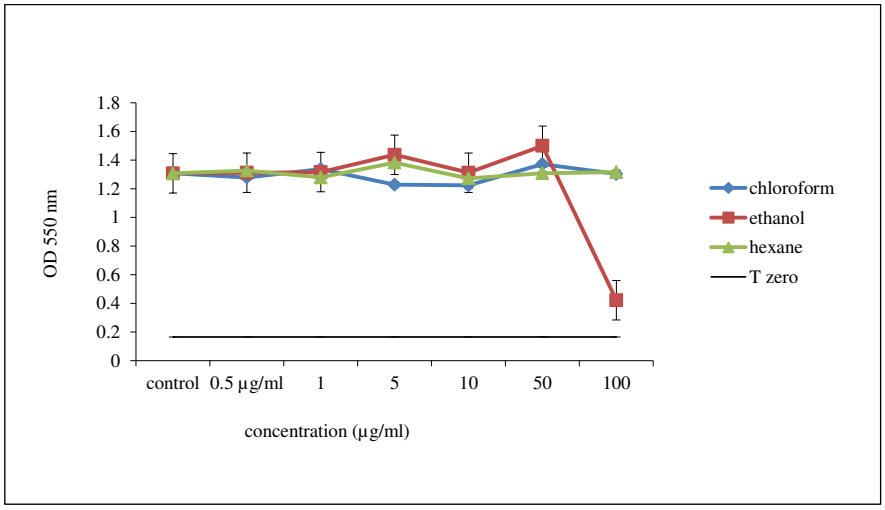


Figure 9.2a: Effects of extract of leaves against MCF-7 growth

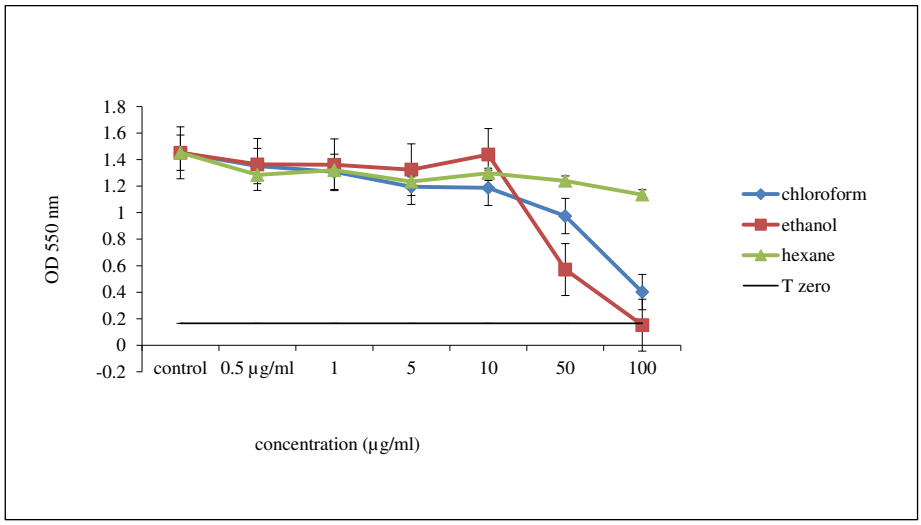


Figure 9.2b: Effects of extract of barks against MCF-7 growth

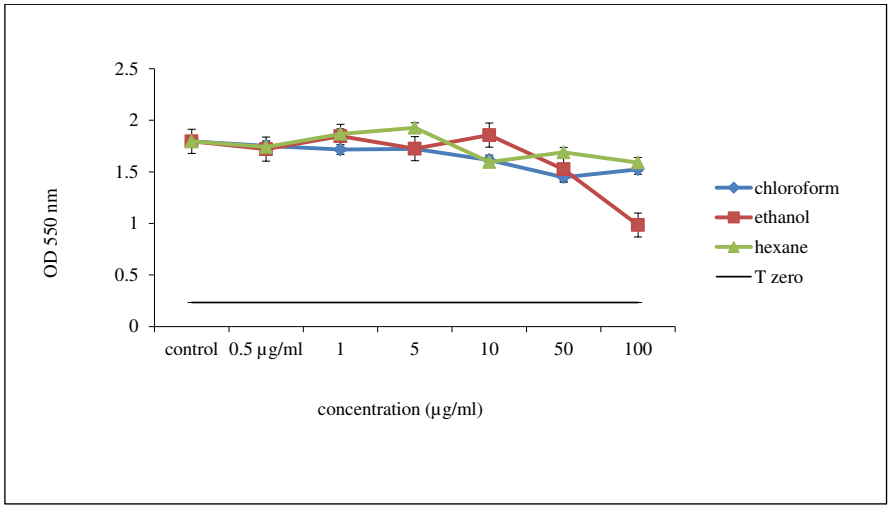


Figure 9.3a: Effects of extract of leaves against HCT 116 growth

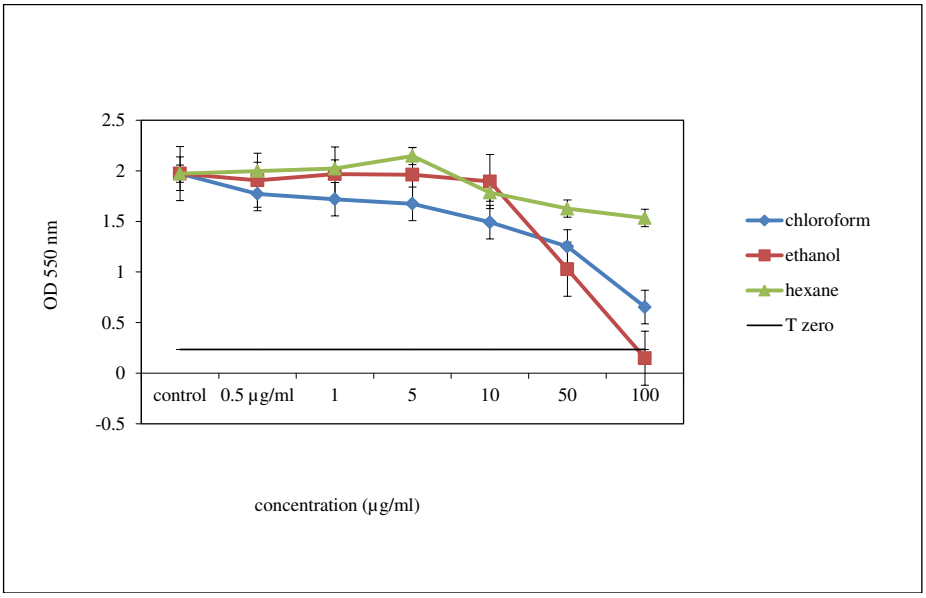


Figure 9.3b: Effects of extract of barks against HCT 116 growth

Natural products appeared to be a promising source for new types of compounds with antitumor activity [234].

9.5 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 [3]. Preliminary anticancer screening of six extracts of *Canarium patentinervium* Miq. showed that the chloroform and ethanol extract of the barks had promising effect on the cell viability of breast cancer cell line MDA 468. The results obtained suggest that further isolation studies can be performed on *Canarium patentinervium* Miq to identify the active constituents responsible for the anticancer activities.

CHAPTER 10

ISOLATION AND IDENTIFICATION OF COMPOUNDS

10.1 Introduction

10.1.1 Secondary metabolites

Medicinal plants have formed the basis of health care since earliest times of humanity and are still being widely used. The clinical, pharmaceutical and economic value continues to grow, varying between countries. Chemodiversity in plants has proven to be important in pharmacological research and drug development, not only for the isolation of bio-active compounds used directly as therapeutic agents, but also as leads to the synthesis of drugs or as models for pharmacologically active compounds [235]. The rapid identification of these bio-active compounds, however, is critical if this tool of drug discovery is to compete with developments in technology. Plant preparations are distinguished from chemical drugs due by their complexity of mixtures containing large numbers of bio-active compounds or secondary metabolites. This brings about the challenge of drug discovery from natural sources [236].

Plants produce primary and secondary metabolites which encompass a wide array of functions. Primary metabolites, which include amino acids, simple sugars, nucleic acids, and lipids, are compounds that are necessary for cellular processes [237]. Secondary metabolites include compounds produced in response to stress, such as the case when acting as a deterrent against herbivores. Plants can manufacture many

different types of secondary metabolites, which have been subsequently exploited by humans for their beneficial role in a diverse array of applications [237]. Although secondary metabolites from natural products have formed the basis of medicines, the presence of these compounds in the biochemistry of the plant is very often difficult to justify. It has been suggested that these compounds may have been synthesized by the plant as part of the defense system of the plant, e.g. plants are known to produce phytoalexins as a response to attack by bacteria and fungi [236]. The presence of highly toxic natural products has also been highlighted in some animals namely the Amazonian frogs so as to deter predation by other animals [236].

Secondary metabolites are organic molecules that are not involved in the normal growth and development of an organism. While primary metabolites have a key role in survival of the species, playing an active function in the photosynthesis and respiration, the absence of secondary metabolites does not result in immediate death but rather in long-term impairment of the organism's survivability, often playing an important role in plant defense [238]. Secondary metabolites are frequently produced at highest levels during a transition from active growth to stationary phase [238]. The producer organism can grow in the absence of their synthesis, suggesting that secondary metabolism is not essential, at least for short term survival. A second view proposes that the genes involved in secondary metabolism provide a ‘‘genetic playing field" that allows mutation and natural selection to fix new beneficial traits via evolution. A third view characterizes secondary metabolism as an integral part of cellular metabolism and biology; it relies on primary metabolism to supply the required enzymes, energy, substrates and cellular machinery and contributes to the long term survival of the producer [238].

Different classes of these compounds are often associated to a narrow set of species within a phylogenetic group and constitute the bioactive compound in several medicinal, aromatic, colorant, and spice plants and/or functional foods. These compounds are an extremely diverse group of natural products synthesized by plants, fungi, bacteria, algae, and animals [239]. A simple classification of secondary metabolites includes three main groups: terpenes (such as plant volatiles, cardiac glycosides, carotenoids and sterols) constituting about 25,000 types, phenolics (such as phenolic acids, coumarins, lignans, stilbenes, flavonoids, tannins and lignin) constituting about 8,000 types and nitrogen containing compounds (such as alkaloids and glucosinolates) constituting about 12,000 types [240]. Whatever the reasons for the presence of these compounds in nature, they provide an invaluable resource that have been used to find new drug molecules [241]. A number of traditional separation techniques with various solvent systems and spray reagents, have been described as having the ability to separate and identify secondary metabolites [48][238].

When an active extract has been identified, the first task is the identification of the bio-active phytocompounds. The coupling of preliminary bioassay screening on crude extracts and chromatographic methods such as column chromatography, preparative thin layer chromatography (PTLC), high performance liquid chromatography (HPLC), mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy, eventually leading to final bioassay screening, are important for the acquisition of biologically active compounds.. The successful isolation of compounds from plant materials largely depends on the type of solvent used for extraction [242]. The extract is separated into individual components and the biological activity of each fraction is determined until a pure active compound is obtained. The pure compound

is then identified using methods such as mass spectrometry and nuclear magnetic resonance spectroscopy.

Research has demonstrated that natural products represent an unparalleled reservoir of molecular diversity. The isolation and identification of bio-active phytocompounds in medicinal plant extracts generally used by local population to treat diseases would prove an immeasurable contribution to drug discovery and development [235]. This would not only validate the traditional use of herbal remedies but also provide leads in the search for new active principles. Investigations surrounding the constituents of the indigenous *Canarium patentinervium* Miq. in general, have not been the focus of any phytochemical study.

10.1.2 Isolation methods

Once the extract has been obtained, the activity within can be demonstrated by bioassay methods using both the crude extract or by using the fractionated extracts. Fractionation has the added advantage of getting to the biologically active material faster. One of the simplest separation methods is partitioning which is a widely used method as an initial extract purification step. A combination of solvents—miscible and immiscible ones are used to separate the phytochemicals making up the extract. This method relies on the ability or the components to be either soluble in water or in the organic phase [241]. This was then followed by various methods of chromatography to isolate the bioactive molecules.

10.1.3 Crude Fractionation

The objectives at this stage are to simplify the extract composition by dividing it into groups of compounds sharing similar physicochemical characteristics and/or to remove the bulk of unwanted materials and thus enrich the extract with respect to the target compounds. Procedures commonly employed involve solvent partitioning, defatting, and desalting. The procedure used in this study is a modification of the method developed by Kupchan [48]. It can be used for defatting and desalting as well. Most of fats will go with the n-hexane fraction, while inorganic salts will go with the aqueous one. The advantage of the method is total recovery of target compounds. Drawbacks are the problems of emulsion formation, time ineffectiveness, and use of large volumes of solvents.

10.1.4 Chromatography

'Chromatography' is the general term for a variety of physico-chemical separation techniques, all of which have in common the distribution of a component between a mobile phase and a stationary phase. The stationary phase is fixed in the system and the mobile phase which is a fluid, streams through the chromatographic system. The various chromatographic techniques are sub-divided according to the physical state of these two phases. The molecules of the analytes are distributed between the mobile and the stationary phases. When present in the stationary phase, they are retained and do not move through the system but when present in the mobile phase, they migrate with a velocity equivalent to that of the mobile phase. Due to the different distributions of the analytes, their mean residence time in the stationary phase differs,

resulting in a different 40 net migration velocity. This is the principle of the chromatographic system [243]. Chromatograhic techniques have been instrumental in the separation of natural products.

10.1.4.1 Thin Layer Chromatography (TLC)

One of the fastest and most widely used chromatographic techniques is Thin Layer Chromatography. TLC is an analytical technique that can be used to monitor reactions and for the qualitative analysis of complex mixtures and for the identification of unknown compounds. It is also important for determining the correct solvent system with which to run a column chromatography. TLC is composed of two phases, a mobile and a solid phase. The solid phase is a thin solid support that usually consists of alumina or silica [48].. The mobile phase is a solvent that moves through capillary action right through the solid phase. In general, the solid phase is usually polar while the mobile solvent is non polar relative to the solid phase. The choice of the solvent depends on the properties of compounds that you want to separate and the nature of the solid support you are using (ie, is it silica or alumina). If some compounds cannot be visualized with eyes UV light can be used and a variety of visualisation reagents can be used [48].

The behaviour of a compound on a TLC is usually described in terms of its relative mobility or retention factor (Rf) value. Rf is a unique value for each compound under the same conditions. The Rf for a compound is a constant from one experiment to the next only if the chromatography conditions (solvent system, adsorbent, thickness of adsorbent, amount of material spotted and temperature) are also constant [244]. Since

these factors are difficult to keep constant from experiment to experiment, relative Rf values are generally considered. “Relative Rf” means that the values are reported relative to a standard. Based on the Rf values, the identity and characteristics of the different compounds can be determined. More polar compounds will have smaller Rf values since they will have a stronger affinity for the polar solid phase. This would result in the compound not being carried very far along the TLC plate. Compounds with larger Rf values interact strongly with the less polar mobile phase and thus, tend to be non polar themselves [238].

PTLC is used for analytical separations of larger quantities of materials. Because sample mass loading capacity is proportional to the square root of the sorbent layer thickness, thicker layers are used (>250 μm up to 1 mm). When a binder is required, the softer inorganic binders (like calcium sulphate) are used so the sample bands can be easily removed. The compounds to be separated are often applied as long streaks, developed and then recovered by scraping the adsorbent from the plate and eluting with a strong solvent [244].

TLC has the advantage of being a highly cost-effective qualitative technique in as much as a large number or samples can be analysed or separated simultaneously. Although it is a very simple and convenient technique, one of its limitations is that it cannot tell the difference between enantiomers and some isomers. Another disadvantage of TLC is that in order to identify specific compounds, the Rf values for the compounds of interest must be known beforehand [245].

10.1.4.2 Liquid chromatography (column)

Column chromatography is the most common form of chromatography. Column chromatography is suitable for the physical separation of microgram to kilogram quantities of material. A solvent acts as the mobile phase while a finely divided solid surface acts as the stationary phase. The stationary phase will adsorb the components of the mixture to varying degrees. As the solution containing the mixture passes over the adsorbent, the components are distributed between the solvent and adsorbent surface. This process may be described by a three-way equilibrium between the sample, the solvent and the adsorbent [48]. The solvent and sample compete for positions on the solid adsorbent, the solvent displacing the sample reversibly and continuously in the direction of the solvent flow. Consequently, a weakly adsorbed compound will spend more time in the solvent, and will therefore be eluted first. The stationary phase is solid and packed in a column made of glass, stainless steel, or other inert material. The common solid adsorbents are alumina (aluminum oxide) and silica gel (silicon dioxide). The sample mixture is applied to the top of the column and the mobile phase passes through the column either by gravity, vacuum, or pressure [238].

Liquid-solid column chromatography is an effective separation technique when all appropriate parameters and equipment are used. This method is especially effective when the compounds within the mixture are colored, as this gives the scientist the ability to see the separation of the bands for the components in the sample solution. Even if the bands are not visible, certain components can be observed by other visualization methods. One method that may work for some compounds is irradiation

with ultraviolet light. This makes it relatively easy to collect samples one after another. However, if the components within the solution are not visible by any of these methods, it can be difficult to determine the efficacy of the separation that was performed. In this case, separate collections from the column are taken at specified time intervals. Since the human eye is the primary detector for this procedure, it is most effective when the bands of the distinct compounds are visible [246].

10.1.4.3 Size Exclusion Chromatography

Size exclusion chromatography separates molecules by their size. This is done by having the stationary phase be packed with small particles of silica or polymer to form uniform pores. The smaller molecules will get trapped in the silica particles and will elude from the column at a rate that is greater than that of larger molecules. Thus, the retention time depends on the size of the molecules. Larger molecules will be swept away in the mobile phase, therefore having a smaller retention time [245]. In this type of chromatography there isn't any interaction, being physical or chemical, between the analyte and the stationary phase. The most commonly used size inclusion sorbents are the dextran gels, particularly the lipophilic versions such as Sephadex LH-20, which are mostly used for the separation of small hydrophobic natural products from their larger ‘‘contaminants,’’ usually chlorophylls, fatty acids, and glycerides [48]. In organic solvents such as chloroform and methanol, these gels swell to form a matrix. As compounds migrate with the solvent through the gel, small molecules become included into the gel matrix, whereas larger ones are excluded and migrate at a greater rate [238].

One of the most extensively used gels in natural product separation, especially nonpolar or intermediate polarity compounds, is Sephadex LH-20, a hydroxypropylated form of Sephadex G-25. The derivatization offers lipophilicity to the gel, at the same time preserving its hydrophilicity. As a result of the added lipophilicity, LH-20 gel swells sufficiently in organic solvents and allows handling of natural products that are soluble in organic solvents [245]. The useful fractionation range of LH-20 is approx. 100–4000 amu, and is particularly ideal for removal of chlorophyll from plant extracts. It should be noted that separations on gels such as Sephadex LH-20 also involve the mechanisms of adsorption, partition, possibly ion exchange, and occasionally the trend of larger molecules eluting first and smaller ones eluting last may be reversed [245]. The gel filtration mode is operational when a single eluent is used. The partition mechanism comes into play when the eluent is a mixture of solvents (usually a mixture of polar and nonpolar solvents); the more polar of the solvents is taken up by the gel, resulting in a two-phase system with stationary and mobile phases of different compositions [238] .

This form of chromatography has found considerable use in the removal of ‘interfering’ plant pigments such as the chlorophylls that tend to be larger and more lipophilic than many plant natural products. Sephadex LH-20 has also been used for the separation of various avermectins and is the most common method for the preparative isolation of condensed and hydrolysable tannins [247]. This is a non-destructive method for the recovery of a high quantity or extract [48].

10.1.4.4 High performance liquid chromatography system

HPLC instrumentation includes a pump, injector, column, detector and recorder or data system. The heart of the system is the column where separation occurs. Since the stationary phase is composed of micrometer size porous particles, a high pressure pump is required to move the mobile phase through the column. The components (or analytes) are first dissolved in a solvent, and then forced to flow through a chromatographic column under a high pressure. The chromatographic process begins by injecting the solute onto the top of the column where the mixture is resolved into its components [243]. The amount of resolution is important and is dependant upon the extent of the interaction between the solute components and the stationary phase. The stationary phase is defined as the immobile packing material in the column. Separation of components occurs as the analytes and mobile phase are pumped through the column. Eventually each component elutes from the column as a narrow band (or peak) on the recorder [245]. The interaction of the solute with the mobile and stationary phases can be manipulated through different choices of both solvents and stationary phases. As a result, HPLC acquires a high degree of versatility not found in other chromatographic systems and it has the ability to easily separate a wide variety of chemical mixtures [243].

Modern HPLC uses a non-polar solid phase, like C-18, and a polar liquid phase, generally a mixture of water and another solvent. High pressures up to 400 bars are required to elute the analytes through the column before they pass through a diode-array detector. The detector measures the absorption spectra of the analytes to aid in their identification. HPLC is useful for compounds that cannot be vaporized or that

decompose under high temperature [243]. Initially, pressure was selected as the principle criterion of modern liquid chromatography and thus the name was 'high pressure liquid chromatography'. This was, however, an unfortunate term because it seems to indicate that the improved performance is primarily due to the high pressure. This is not true. In fact, high performance is the result of many factors: very small particles of narrow distribution range and uniform pore size and distribution, high pressure column slurry packing techniques, accurate low volume sample injectors, sensitive low volume detectors and good pumping systems [243].

There is a growing interest in the chemical composition of plants and the use of HPLC has several advantages over other methods. The technique is sensitive, rapid and does not require the preparation of derivatives before separation. A further advantage is that ultraviolet absorption detectors set in the aromatic region of the spectrum (e.g. 275 nm) may also be used. Several reports have also been published of the application of HPLC to various phenolic acids and aldehydes that occur in plants [248].

10.1.5 Structure elucidation

In most cases of extraction and isolation of natural products, the end point is the identification of the compound or the conclusive structure elucidation of the isolated compound. However, structure elucidation of compounds isolated from plants, fungi, bacteria, or other organisms is generally time consuming, and sometimes can be the ''bottleneck'' in natural product research [48]. There are many useful spectroscopic methods of getting information about chemical structures, but the interpretation of

these spectra normally requires specialists with detailed spectroscopic knowledge and wide experience in natural product chemistry. With the remarkable advances made in the area of artificial intelligence and computing, there are a number of excellent automated structure elucidation programs available that could be extremely useful [238].

If the target compound is known, it is often easy to compare preliminary spectroscopic data with literature data or to make direct comparison with the standard sample. However, if the target compound is an unknown and complex natural product, a comprehensive and systematic approach involving a variety of physical, chemical, and spectroscopic techniques is required [48]. Information on the chemistry of the genus or the family of plant or microbe under investigation could sometimes provide additional hints regarding the possible chemical class of the unknown compound [249]. Some of the spectroscopic methods used for structure elucidation include ultraviolet-visible spectroscopy (UV-vis), infrared spectroscopy (IR), mass spectrometry (MS) and nuclear magnetic resonance (NMR). In this study we have used NMR.

10.1.5.1 Nuclear magnetic resonance (NMR)

Nuclear magnetic resonance (NMR) is a spectroscopic technique involving a magnetic field in which a sample is placed. The sample is then subjected to radiofrequency radiation at the appropriate frequency, allowing for the absorption of energy depending on the type of nucleus, whether, for example, it is a ^{1}H or ^{13}C. The frequency also depends on chemical environment of the nucleus, whether methyl or

hydroxyl protons are present, molecular conformations, and dynamic processes. Both ^{1}H NMR and ^{13}C NMR are used, with the latter providing information on the carbon skeleton, and the former relating to the specifics of the hydrogen atoms, thus complementing each other [245].

10.2 Materials and methods

10.2.1 Isolation of compound 1-5 (crude fractionation)

The isolation scheme for compound 1-5 is shown in Figure 10.1. The ethanol extract of the leaves (LE-40 g) was dissolved in water. It is then partitioned with petroleum ether, chloroform, and water to yield the respective solvent extracts (LE-PE, LE-CL and LE-WA). Phytochemical analysis was repeated as in 3.2 (iv) to confirm the absence of tannins in LE-PE and LE-CL and the presence of tannins in LE-WA. The solvent partitioning thus concentrates all tannins in the water fraction.

10.2.2 Isolation of compound 1 & 2

10.2.2.1 Silica gel column chromatography

A glass column was clamped upright and packed with silica gel (size 0.063-0.200 mesh) mixed with the appropriate mobile phase and poured into the column (4 cm x 90 cm) as a compact even suspension. This constituted the stationary phase. The extract (LE-CL weighing 6 g) was then mixed with a small amount of chloroform

and introduced as a thin band to the silica gel. Once the extract was loaded onto the silica gel, the mobile phase was added at a constant flow rate. Gradient elution of increasing polarity was initiated with chloroform/methanol gradient elution (the ratio from 100:0 to 92:8). Thirteen column fractions were collected and analyzed by TLC (chloroform/methanol). Fractions with similar TLC pattern were combined to total of four fractions.

10.2.2.2 Preparative thin layer chromatography (PTLC)

Fraction 2 that was yielded from chloroform/ methanol ratio 96:4 was rechromatographed on a preparative TLC (silica gel plate with 2 mm thickness) with solvent system chloroform/ methanol (ratio of 1000:15) yielding total 7 bands. Band five was collected and rechromatographed on preparative TLC (0.5 mm thickness) with solvent system chloroform/ methanol (ratio of 89:11) to yield four bands, with band two and band three sent for NMR characterisation.

10.2.3 Isolation of compound 3, 4 & 5

10.2.3.1 Size-exclusion column chromatography

The principle governing this method is based on the molecular size differences of the compounds, which result in the separation of these compounds. The stationary phase comprised of a porous three-dimensional polymeric matrix, namely Sephadex^{TM} LH-20 (GE Healthcare). This matrix was initially saturated with ethanol in order to facilitate swelling before use. Sephadex^{TM} LH-20 in ethanol adsorbs tannins while it

desorbs tannins in acetone [247]. This is then useful for the separation of tannin and non-tannin containing fractions. Once swelling was achieved the matrix was then introduced into a glass column and the fraction (LE-WA) which was dissolved in ethanol was introduced as a thin band was then applied to the column. The mobile phase consisted ethanol. Sixteen fractions were collected analyzed by TLC. Fractions with similar TLC pattern were combined to total of four fractions. The adsorbed tannins were then flushed with a change of mobile phase to acetone:water (70:30) resulting in fraction WT. The phytochemical tannin test (section 3.2-iv) was again performed to confirm the presence of tannins in WT and absence in the other fractions. The matrix had irreversibly sorbed material that could be tannin [247].

Fraction WB was reintroduced to another column with Sephadex^{TM} LH-20 that was swelled in methanol. Total 3 fractions were collected and fraction WB2 was sent for nuclear magnetic resonance (NMR) characterisation. Fraction WD was also introduced to Sephadex^{TM} LH-20 that was swelled in methanol. Total five fractions were collected and fraction WD3 and WD4 were sent for nuclear magnetic resonance (NMR) characterisation.

10.2.4 Isolation of compound 6-8

10.2.4.1 Isolation of compound 6-8 (PTLC and solvent partioning)

The isolation scheme for compound 6-8 is shown in Figure 10.2. The chloroform extract of the barks were dissolved in dicloromethane:methanol (2:1). Mixture was chromatographed on a PTLC (silica gel plate with 2 mm thickness) with solvent

system ethyl acetate: methanol (10:1) yielding total three bands (Figure 10.3). First band (B1) was rechromatographed on PTLC (2 mm thickness) with solvent system ethyl acetate: methanol (6:1) yielding four bands. The third band upon reconstitution resulting in fraction B1-BA was subjected to HPLC (section 10.4.5). The second band (B2) from Figure 10.3 was rechromatographed on PTLC (2 mm thickness) with solvent system ethyl acetate: methanol (6:1) yielding three bands. The second band of this PTLC plate upon reconstitution (B2-BA) was subjected to HPLC (section 10.4.5). The third band (B3) from Fig 10.3 and all the other areas including origin was combined and partitioned between water and ethyl acetate resulting in aqueous (B3-AL) and organic layer (B3-OL). The B3-AL fraction was subjected to HPLC (section 10.4.5).

10.2.4.2 Isolation of compound 6-8 (HPLC)

Fraction B1-BA, B2-BA and B3-AL was concentrated in vacuum and then dissolved in methanol. The solution was then subjected to HPLC individually. The samples were then qualitatively analyzed by using Agilent 2690 semi-prep HPLC system (Luna C-18 column, 100 mm x 30 mm) equipped with a 996 photodiode-array detector (PDA). The injection of the sample was 5 µl and the flow rate was 2 ml/min.

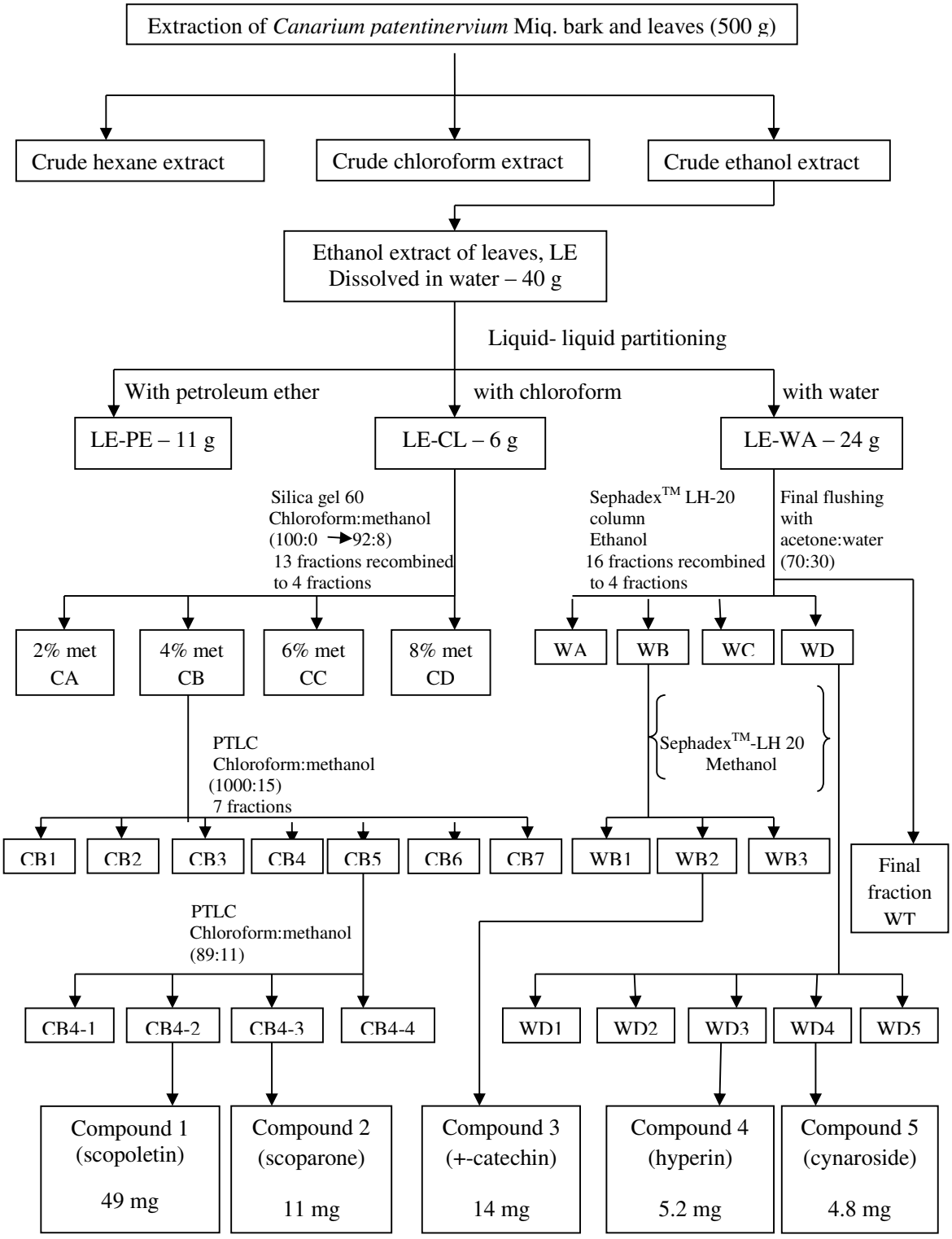


Figure 10.1: Schematic representation of the isolation and purification of compound 1 (scopoletin), compound 2 (scoparone), compound 3 (+-catechin), compound 4 (hyperin) and compound 5 (cynaroside) isolated from *Canarium patentinervium* Miq.

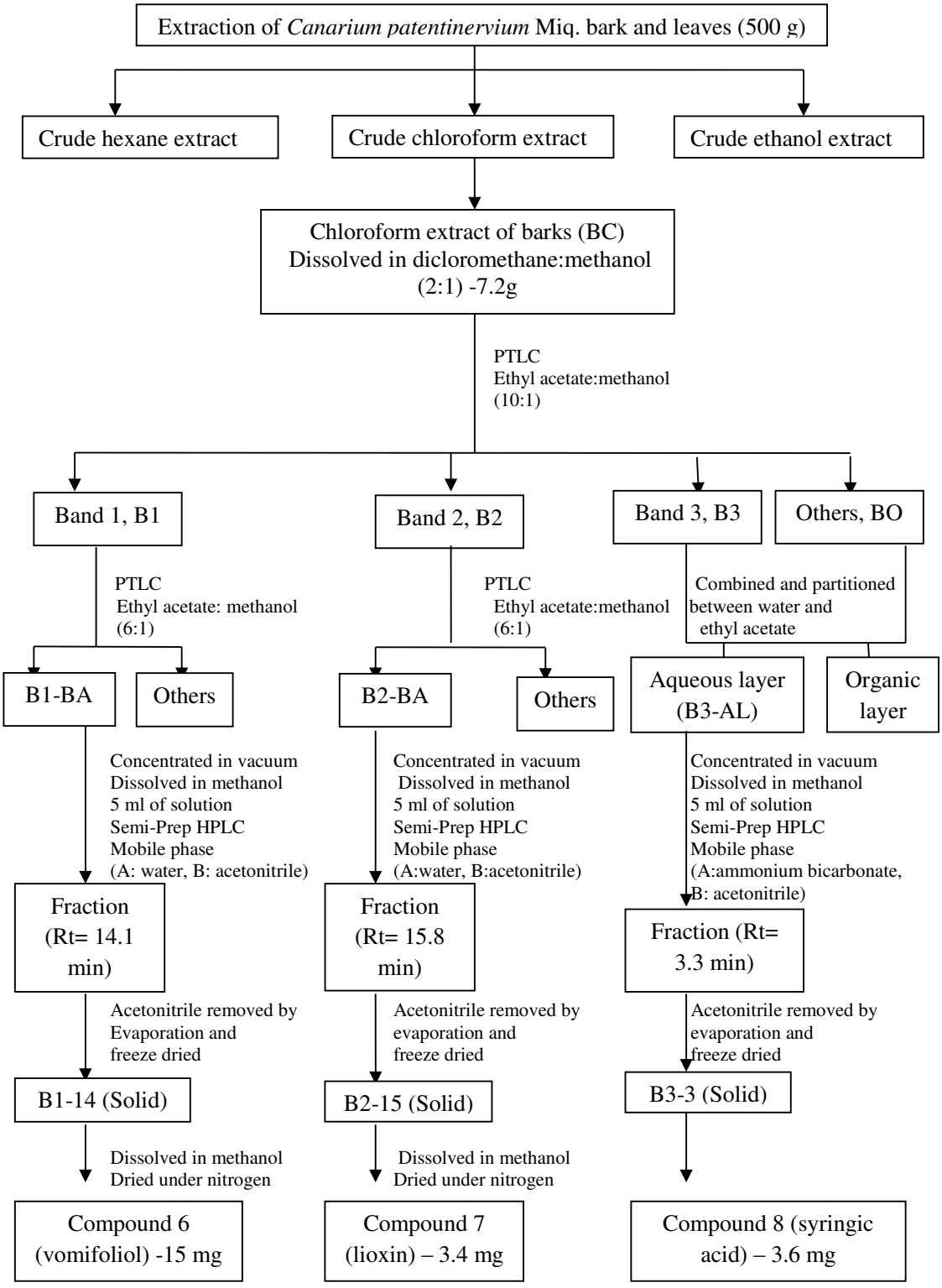


Figure 10.2: Schematic representation of the isolation and purification of compound 6 (vomifoliol), compound 7 (lioxin) and compound 8 syringic acid isolated from *Canarium patentinervium* Miq.

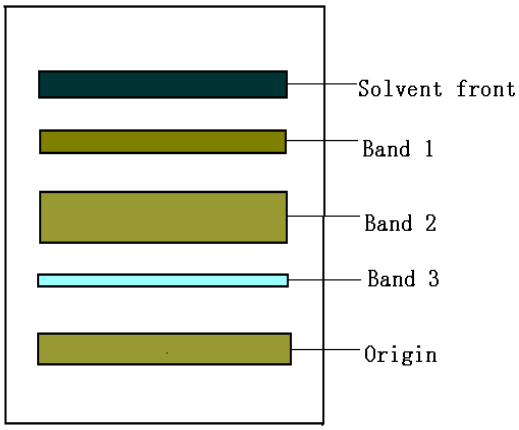


Diagram of TLC

Figure 10.3: The PTLC of chloroform extract of barks

Solution B1-BA: The mobile phase started off with 15 % acetonitrile and 85 % water. The solvent ratio was changed through a linear gradient to 20 % acetonitrile and 80 % water in 14 min. This ratio was maintained for 2 min and thereafter the solvent ratio was changed back to the initial starting conditions. At Rt (retention time) = 14.1 min the fraction eluted (B1-14) was collected and the acetonitrile was removed by evaporation and freeze-drying. The solid in the vial was then redissolved in methanol and dried under nitrogen. B1-14 was then sent for nuclear magnetic resonance (NMR) characterisation.

Solution B2-BA: The mobile phase started off with 15 % acetonitrile and 85 % water. The solvent ratio was changed through a linear gradient to 20 % acetonitrile and 80 % water in 14 min. This ratio was maintained for 4 min and thereafter the solvent ratio was changed back to the initial starting conditions. At Rt (retention time) = 15.8 min the fraction eluted (B2-15) was collected and the acetonitrile was removed by evaporation and freeze-drying. The solid in the vial was then redissolved in methanol

and dried under nitrogen. B2-15 was then sent for nuclear magnetic resonance (NMR) characterisation.

Solution B3-AL: The mobile phase started off with 5 % acetonitrile and 95 % water (containing 10 mM ammonium bicarbonate). The solvent ratio was changed through a linear gradient to 16% acetonitrile and 84 % water (containing 10 mM ammonium bicarbonate) at 1 min. This ratio was maintained for 5 min and thereafter the solvent ratio was changed back to the initial starting conditions. At Rt (retention time) = 3.3 min the fraction eluted (B3-3) was collected and the acetonitrile was removed by evaporation and freeze-drying. The solid in the vial was then redissolved in methanol and dried under nitrogen. B3-3 was then sent for nuclear magnetic resonance (NMR) characterisation.

10.2.5 Nuclear magnetic resonance (NMR)

Final chemical characterisation of the 8 isolated compounds was achieved by NMR, which was performed in collaboration with Assistant Professor Dr. Achyut Adhikari (HEJ Research Institute of Chemistry, University of Karachi, Pakistan). Nuclear magnetic resonance spectroscopy was performed on a Varian 500 MHz NMR spectrometer. Samples CB4-2 and CB4-3 were recorded at room temperature in deuterated chloroform ($CDCL_3$), samples B1-14,B2-15, WB-2, WD-3 and WD-4 were recorded at room temperature in deuteromethanol (CD_3OD) while sample B3-3 were recorded at room temperature in deuterated water (D_2O).

10.3 Results

Compound **1** was isolated as a pale yellow powder. On the basis of the spectral data obtained for ^{1}H NMR and ^{13}C NMR [Table 10.1 and Figure A1 & A2 (Appendix A)], and compared to that of relative references [250][251][252], compound 1 was identified as scopoletin (Figure 10.4). It has an empirical formula of $C_{10}H_8O_4$ with molecular weight of 192.16.

Table 10.1: ^{1}H and ^{13}C NMR spectral data ($CDCL_3$, 500 MHz) of scopoletin

Position	δ_C	δ_H	H's	Type	J (Hz)
2	161.6	-	-	-	-
3	111.6	6.3	1H	d	9.5
4	143.3	7.63	1H	d	9.5
5	103.2	6.87	1H	s	-
6	144	-	-	-	-
7	150.2	-	-	-	-
8	107.4	6.95	1H	s	-
9	149.7	-	-	-	-
10	113.5	-	-	-	-
6- OCH3	56.4	3.98	3H	s	-

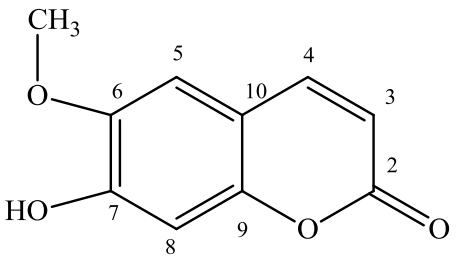


Figure 10.4: The chemical structure of compound 1 (scopoletin)

Compound **2** was isolated as a pale yellow powder. On the basis of the spectral data obtained for ^{1}H NMR and ^{13}C NMR [Table 10.2 and Figure A3 & A4 (Appendix A)], and compared to that of relative references [253][254], compound 2 was identified as scoparone (Figure 10.5). It has an empirical formula of $C_{11}H_{10}O_4$ with molecular weight of 206.19.

Table 10.2: ^{1}H and ^{13}C NMR spectral data ($CDCL_3$, 500 MHz) of scoparone

Position	**δ_C**	**δ_H**	**H's**	**Type**	**J (Hz)**
2	161.41	-	-	-	-
3	111.45	6.32	1H	d	9.6
4	150.06	7.64	1H	d	9.6
5	100.05	6.87	1H	s	
6	152.87	-	-	-	-
7	146.37	-	-	-	-
8	143.28	6.88	1H	s	
9	113.59	-	-	-	-
10	107.98	-	-	-	-
6-OCH3	56.38	3.95	3H	s	-
7-OCH3	56.4	3.98	3H	s	-

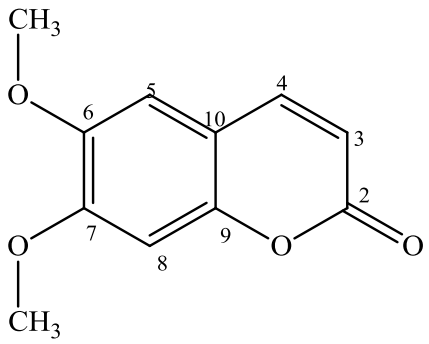


Figure 10.5: The chemical structure of compound 2 (scoparone)

Compound **3** was isolated as light yellow needles. On the basis of the spectral data obtained for ^{1}H NMR and ^{13}C NMR [Table 10.3 and Figure A5 & A6 (Appendix A)], and compared to that of relative references [255][256], compound 3 was identified as catechin. Catechin exists in nature as (+) and (-) enantiomers. The optical rotation of the compound in methanol was tested using Schmidt + Haensch Polartronic H532 Polarimeter. The experimental rotation was an average of +69.25 (n=4) concluding that this compound is (+)-catechin (Figure 10.6). Previous published data on (+)-catechin reports an optical rotation of +56.60 [257]. It has an empirical formula of $C_{15}H_{14}O_6$ with molecular weight of 290.26.

Table 10.3: ^{1}H and ^{13}C NMR spectral data (CD_3OD, 500 MHz) of (+)-catechin

Position	δ_C	δ_H	H's	Type	J (Hz)
2	81.46	4.01	1H	d	7.5
3	67.41	3.98	1H	m	5.5, 8.5
4	27.12				
4a	-	2.52	1H	dd	8.2, 16.1
4b	-	2.87	1H	dd	5.5, 16.1
5	156.19	-	-	-	-
6	94.86	5.94	1H	d	2.3
7	156.45	-	-	-	-
8	94.08	5.87	1H	d	2.3
9	155.52	-	-	-	-
10	99.40	2.52	1H	dd	-
OH	-	4.58	1H	d	-
1'	130.82	-	-	-	-
2'	113.84	6.85	1H	d	1.8
3'	144.83	-	-	-	-
4'	144.85	-	-	-	-
5'	114.66	6.78	1H	d	8.1
6'	118.62	6.74	1H	dd	1.8, 8.1

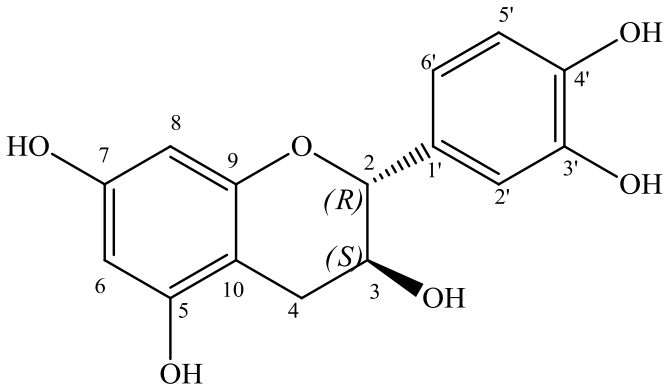


Figure 10.6: The chemical structure of compound 3 [(+)-catechin]

Compound **4** was isolated as a pale yellow powder. On the basis of the spectral data obtained for ^{1}H NMR and ^{13}C NMR [Table 10.4 and Figure A7 & A8 (Appendix A)], and compared to that of relative references [258][259], compound 4 was identified as hyperin (Figure 10.7). It has an empirical formula of $C_{21}H_{20}O_{12}$ with molecular weight of 460.37.

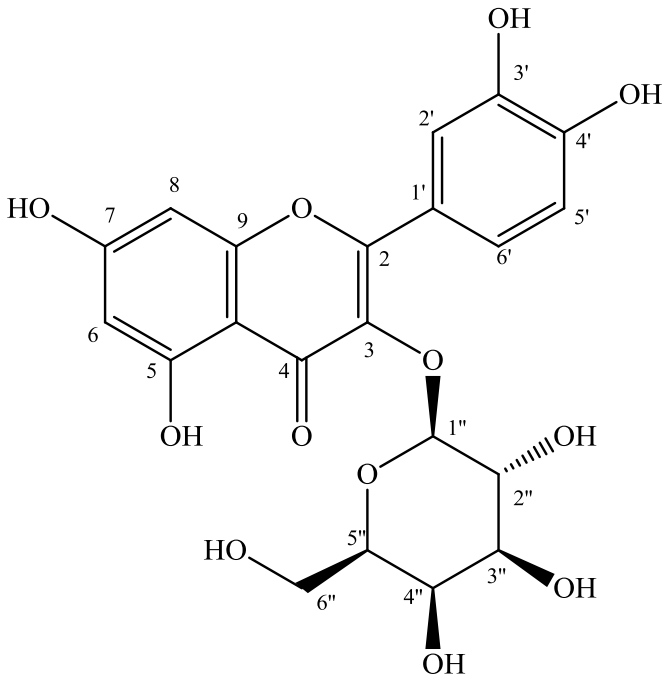


Figure 10.7: The chemical structure of compound 4 (hyperin)

Table 10.4: ^{1}H and ^{13}C NMR spectral data (CD_3OD, 500 MHz) of hyperin

Position	δ_C	δ_H	H's	Type	J (Hz)
2	157.37	-	-	-	-
3	134.36	-	-	-	-
4	178.13	-	-	-	-
5	161.63	-	-	-	-
6	98.55	6.23	1H	d	2.10
7	164.90	-	-	-	-
8	93.34	6.43	1H	d	2.10
9	157.08	-	-	-	-
10	104.17	-	-	-	-
1'	121.51	-	-	-	-
2'	114.67	7.86	1H	d	2.60
3'	144.43	-	-	-	-
4'	148.56	-	-	-	-
5'	116.36	6.88	1H	d	8.60
6'	121.51	7.60	1H	dd	2.6, 8.6
1''	103.99	5.19	1H	d	8.20
2''	71.77	3.84	1H	dd	8.2, 8.0
3''	75.80	3.60	1H	dd	8.0, 2.1
4''	68.62	3.87	1H	dd	3.2, 2.1
5''	77.88	3.45	1H	m	-
6''	60.53	-	-	-	-
6a''	-	3.67	1H	dd	11.0, 3.0
6b''	-	3.56	1H	dd	11.0, 5.8

Compound **5** was isolated as a light yellow powder. On the basis of the spectral data obtained for ^{1}H NMR and ^{13}C NMR [Table 10.5 and Figure A9 & A10 (Appendix A)], and compared to that of relative references [260][261], compound 5 was identified as cynaroside (Figure 10.8). It has an empirical formula of $C_{21}H_{20}O_{11}$ with molecular weight of 448.38.

Table 10.5: ^{1}H and ^{13}C NMR spectral data (CD_3OD, 500 MHz) of cynaroside

Position	δ_C	δ_H	H's	Type	J (Hz)
2	165.52	-	-	-	-
3	102.72	6.60	1H	s	
4	182.66	-	-	-	-
5	161.51	-	-	-	-
6	100.27	6.53	1H	d	2.00
7	163.43	-	-	-	-
8	94.65	6.83	1H	d	2.00
9	157.58	-	-	-	-
10	105.69	-	-	-	-
1'	121.98	-	-	-	-
2'	112.81	7.43	1H	d	2.20
3'	145.77	-	-	-	-
4'	149.80	-	-	-	-
5'	115.40	6.93	1H	d	8.40
6'	119.12	7.45	1H	dd	8.40, 2.20
1"	99.75	5.09	1H	d	7.30
2"	73.33	3.48	1H	t	9.20
3"	76.46	3.56	1H	t	9.20
4"	69.86	3.45	1H	t	9.20
5"	77.00	3.60	1H	m	-
6"	61.06	-	-	-	-
6a"	-	3.75	1H	dd	12.40, 5.80
6b"	-	3.95	1H	dd	12.40, 1.90

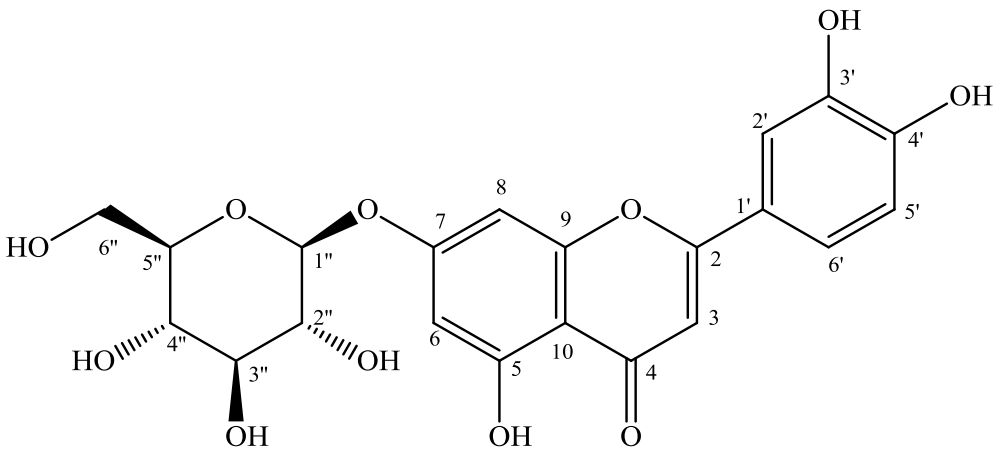


Figure 10.8: The chemical structure of compound 5 (cynaroside)

Compound **6** was isolated as white solid (HPLC chromatogram in Figure B1-Appendix B). On the basis of the spectral data obtained for ^{1}H NMR and ^{13}C NMR [Table 10.6 and Figure A11 & A12 (Appendix A)], and compared to that of relative references [262][263], compound 5 was identified as vomifoliol (Figure 10.9). It has an empirical formula of $C_{13}H_{20}O_3$ with molecular weight of 224.30.

Table 10.6: ^{1}H and ^{13}C NMR spectral data (CD_3OD, 500 MHz) of vomifoliol

Position	δ_C	δ_H	H's	Type	J (Hz)
1	78.54	-	-	-	-
2	41.03	-	-	-	-
3a	-	2.18	1H	d	15.00
3b	-	2.54	1H	d	15.00
4	199.85	-	-	-	-
5	125.69	5.90	1H	m	-
6	166.10	-	-	-	-
7	128.71	5.81	1H	m	-
8	135.49	5.82	1H	m	-
9	67.34	4.34	1H	m	-
10	22.41	1.26	3H	d	7.50
11	23.06	1.03	3H	s	-
12	22.05	1.06	3H	s	-
13	18.15	1.94	3H	s	-

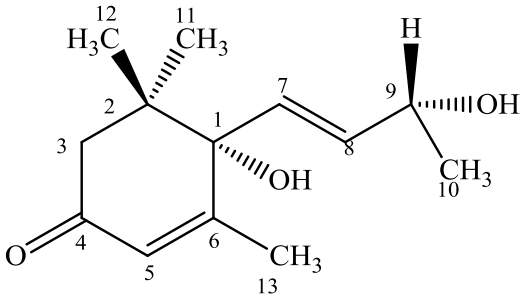


Figure 10.9: The chemical structure of compound 6 (vomifoliol)

Compound **7** was isolated as white powder (HPLC chromatogram in Figure B2-Appendix B). On the basis of the spectral data obtained for ^{1}H NMR and ^{13}C NMR [Table 10.7 and Figure A13 & A14 (Appendix A)], and compared to that of relative references [264][265], compound 7 was identified as lioxin (Figure 10.10). It has an empirical formula of $C_8H_8O_3$ with molecular weight of 152.15.

Table 10.7: ^{1}H and ^{13}C NMR spectral data (CD_3OD, 500 MHz) of lioxin

Position	δ_C	δ_H	H's	Type
1	127.72	-	-	-
2	110.07	7.30	1H	m
3	149.09	-	-	-
4	147.08	-	-	-
5	115.51	7.30	1H	d
6	125.64	7.31	1H	m
7	191.60	9.57	1H	s
OH	-	6.77	1H	s
10	54.80	3.80	3H	s

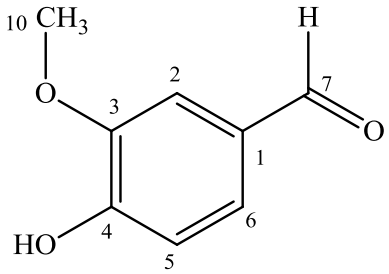


Figure 10.10: The chemical structure of compound 7 (lioxin)

Compound **8** was isolated as pale yellow powder (HPLC chromatogram in Figure B3-Appendix B). On the basis of the spectral data obtained for ^{1}H NMR and ^{13}C NMR [Table 10.8 and Figure A15 & A16 (Appendix A)], and compared to that of relative references [266][267], compound 8 was identified as syringic acid (Figure 10.11). It has an empirical formula of $C_9H_{10}O_5$ with molecular weight of 198.17.

Table 10.8: ^{1}H and ^{13}C NMR spectral data (D_2O, 500 MHz) of syringic acid

Position	δ_C	δ_H	H's	Type
1	121.08	-	-	-
2	106.92	7.11	1H	s
3	147.08	-	-	-
4	148.41	-	-	-
5	147.08	-	-	-
6	106.92	7.11	1H	s
2-OCH3, 6-OCH3	56.26	3.57	3H	s
COOH	174.29	-	-	-

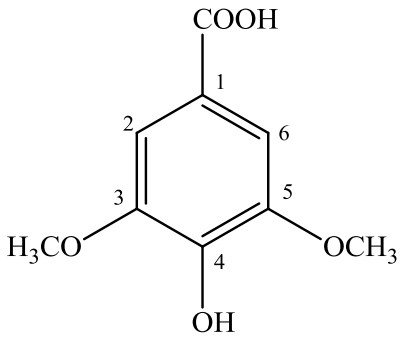


Figure 10.11: The chemical structure of compound 8 (syringic acid)

10.4 Discussion

All the compounds were isolated for the first time from *Canarium patentinervium* Miq. Seven phenolic compounds (compounds **1**-**6** and **8**) and one norsesquiterpene with cyclohexenone ring (compound **7**) was isolated.

The ^{1}H-NMR spectrum of compound **1** (Table 10.1) revealed the presence of two doublets at δ_H 7.63 (H-4, J = 9.5 Hz) and 6.30 (H-3, J = 9.5 Hz). The two singlet signals appeared at δ_H 6.87 and 6.95 were assigned for the two protons H-5 and H-8, respectively. The ^{1}H NMR spectra of compound **2** identified as scoparone (Table 10.2) showed two doublets with coupling constant of 9.60 Hz at δ 6.32 and δ 7.64, which were assigned as H-3 and H-4, respectively, characteristic for coumarins [253]. The ^{1}H NMR spectrum of scoparone was similar to that of scopoletin, except for an additional singlet at δ 3.98 ppm indicating its identity as scoparone [268].

Compound **3** were isolated as light yellow crystals. The structural identity of this compound was determined through NMR spectral analysis (Table 10.3) that was compared to literature [255][256]. Catechin possesses two benzene rings (called the A- and B-rings) and a dihydropyran heterocycle (the C-ring) with a hydroxyl group on carbon 3. The A ring is similar to a resorcinol moiety while the B ring is similar to a catechol moiety. There are two chiral centers on the molecule on carbons 2 and 3 resulting in 4 optical isomers (+) catechin, (-) catechin, (+) epicatechin and (-) epicatechin. Of these, (+) catechin and (-) epicatechin occur in nature [269]. From the experimental data of optical rotation, this compound was confirmed to be (+) catechin.

Compound **4** was isolated as pale yellow powders. In the ^{1}H-NMR spectrum, the typical proton signals of quercetin moiety, that is, those of an AMX system due to a 1',3',4'-trisubstitution of ring B [δ 7.86 (d, J = 2.6 Hz), 7.60 (dd, J = 2.6, 8.6 Hz) and δ 6.88 (d, J = 8.6 Hz)], and a typical meta-coupled pattern for H-8 and H-6 protons [δ 6.43 (d, J = 2.1 Hz) and 6.23 (d,J = 2.1 Hz)], were observed. The ^{13}C-NMR of compound 4 showed resonances for twenty one carbons and presence of a quercetin moiety with the exception of monosaccharide moiety (Table 10.4). An anomeric proton signals of compound 4 appeared at δ 5.19 (d, J = 8.2 Hz) and other oxygenated methines and methylene signals of sugar moiety at δ 3.87 (dd, J = 3.2, 2.1 Hz, H-4''), 3.84 (dd, J = 8.2, 8.0 Hz, H-2''), 3.67 (dd, J = 11.0, 3.0, H-6''a), 3.56 (dd, J = 11.0, 5.8 Hz, H-6''b), 3.60 (dd, J = 8.0, 2.1 Hz, H-3'') and 3.45 (m, H-5''). Also, the carbon signals of the sugar moiety were observed at δ 103.99 (C-1''), 77.88 (C-5''), 75.80 (C-3''), 71.77 (C-2''), 68.62 (C-4'') and 60.53 (C-6'') suggesting the presence of β-galactopyranoside units. Thus, the structure of compound 4 was identified as quercetin-3-O-β-D-galactopyranoside, hyperin [258].

The ^{1}H NMR spectrum of compound **5,** a light yellow powder, showed two *meta*–coupled doublets (*J*=2.0 Hz) at δ 6.83 and 6.53, each integrating for one proton, and were assigned to H-8 and H-6, respectively of ring A of 5,7-dihydroxyflavonoids [270]. The presence of ABX system at δ 7.45 (dd, *J* = 8.4, 2.2 Hz, H-6'), 7.43 (d, *J* = 2.2 Hz, H-2') and 6.93 (d, *J* = 8.3 Hz, H-5'), characteristic of 1, 2, 4-trisubstituted phenyl unit [270]. The only singlet at δ 6.60, integrating for one proton, was attributed C-3 to proton of flavonoids (35). These spectral data revealed the presence of luteolin skeleton [271]. In addition, the 1H NMR spectrum showed a series of signals between

δ 3.95 (dd, J = 12.40, 5.80 Hz, H-6"b), 3.75 (dd, J = 12.40, 1.90 Hz, H-6"a), 3.60 (t, H-5"), 3.56 (t, J = 9.2 Hz, H-3"), 3.48 (dd, J = 9.2, H-2") and 3.45 (t, J = 9.2 Hz, H-4") attributable to a sugar moiety. The coupling constant (*J* = 7.3 Hz) of the anomeric proton located at δ 5.09 (d, J = 7.3 Hz, H-1") and the 13C NMR (Figure 10.8) chemical shifts of the sugar carbons (δ 99.75, 77.00, 76.46, 73.33, 69.86 and 61.06) revealed the presence of β-*O*-glucoside unit in luteolin- 7-*O*-glucoside. The ^{13}C NMR data showed the presence of a ketone carbonyl at C-4 (δ 182.66), two olefinic carbons at C-2 and C-3 (δ 165.52 and 102.72), and four hydroxyl carbons at C-7, C-5, C-4' and C-3' (δ 163.43, 161.51, 149.80 and 145.77). On comparison with the literature on flavonoids of luteolin, it was revealed that the physical and spectral data of luteolin-7-*O*-glucoside isolated were in good agreement with those recorded for luteolin- 7-*O*-glucoside or cynaroside [271][260].

The comparison of the corresponding NMR data of compound **6**, a white solid (Table 10.6) with those reported in literature [262][263] led to its identification as vomifoliol. The cyclohexenone structure was confirmed by the observation in the HMBC (Figure A13-Appendix A) spectrum of correlations between the AB system H-3ab (δ 2.18, δ 2.54, *J* = 15.00 Hz) and C-4 (δ 199.85), C-1 (δ 78.54) and C-5 (δ 125.69). The location of the two high field methyl groups C-11 and C-12 at C-2 was ascertained by the detection of the H3ab-C11 and H3ab-C12 correlations. The position of the side chain on C-1 of the cyclohexenone ring is confirmed by the observation in the HMBC spectrum of the H7-C1 correlation. The COSY (Figure A14-Appendix A) spectrum revealing correlations between the olefinic signal H7 (δ 5.81) with the other olefinic proton H8 (δ 5.82) which in turn had a cross peak with H9 (δ 4.34) permitted us to

confirm the structure of this side chain. All these NMR data together suggested the structure of vomifoliol.

The ^{1}H NMR spectrum of compound **7** which was isolated as white powder exhibited the following signals (Table 10.7), δ 9.82 (CHO), multiplets at δ 7.30, 7.31 (2H) and δ doublet at δ 7.04 (1H) (aromatic protons), and 3.80 ppm (CH_3O). The ^{13}C NMR spectrum showed signals at δ 191.60 (CHO), 149.09, 147.08, 127.72, 125.64, 115.51, and 110.07 (aromatic carbons) and 54.80 ppm (CH_3O). The ^{1}H and ^{13}C spectra were identical to those of 4-hydroxy-3-methoxybenzaldehyde (Figure 10.10) (also known as vanillin or lioxin) [265].

Compound **8** eluted first to give a pale yellow powder on drying. The ^{13}C NMR showed two methoxyls, two olefinic methines and five quaternary carbons, including a carboxy, and three oxygenated olefinic carbons. The only signals appearing in the ^{1}H NMR were a 6H singlet for the two methoxyls (δ 3.57) and a 2H singlet for two aromatic protons (δ 7.11) (Table 10.8). Further analysis of the NMR data indicated a symmetric trisubstituted phenolic acid. Two of the substituents were methoxyl groups and the third a hydroxyl group. The data was found to fit well with that of 4-hydroxy-3,5-dimethoxybenzoic acid (syringic acid) [266][267]. Further evidence for the structure of 8 was provided by a 3-bond correlation between H-2/6 (δ 7.11) and the carboxy carbon (δ 174.29) (Figure 10.11). The possibility of 4-hydroxy-2,6-dimethoxybenzoic acid was thus eliminated.

10.5 Conclusion

Eight compounds were isolated for the first time from *Canarium patentinervium* Miq. using various isolation techniques such as TLC, CC, HPLC and identified with NMR method. Compounds isolated scopoletin, scoparone, (+)-catechin, hyperin, cynaroside, lioxin and syringic acid were phenolics while vomifoliol was a norsesquiterpene with a cyclohexenone ring. Lioxin, syringic acid and vomifoliol were isolated from this genus for the first time. These findings agree with the phytochemical analysis which was done in Chapter 2.

CHAPTER 11

BIOACTIVITY OF ISOLATED SECONDARY METABOLITES

11.1 Introduction

11.1.1 Terpenes and phenols

Terpenes are the most numerous and structurally diverse plant natural products. The term terpene usually refers to a hydrocarbon molecule while terpenoid refers to a terpene that has been modified, such as by the addition of oxygen. Terpenoids (sometimes called as isoprenoids) are hydrocarbon natural products based on 5-carbon (isoprene) units as their building blocks [237]. Terpenes are an enormous class of natural products spanning well over 30,000 members having been discovered to-date [272]. These compounds are classified as to the number of isoprene units, which include the hemiterpenes (1), monoterpenes (2), sesquiterpenes (3), diterpenes (4), sesterterpenes (5), tripterpenes (6), and polyterpenes (many units). Sometimes the prefix nor**-** is added to name a structural analog that can be derived from a parent compound by the removal of one carbon atom along with the accompanying hydrogen atoms (norsesquiterpene) [237].

Of these different major classes of terpenoids, the diterpenes are structurally most diversified, possessing at least 6 large structural groups. Biological active compounds are found in each class of the terpenoids, particularly among the sesquiterpenes, diterpenes and triterpenes [237]. Isopentyl diphosphate (IPP) and dimethyl allyl

diphosphate (DMAPP) are found to be the actual building blocks in terpene biosynthesis [273]. There are 2 biosynthetic pathways for the production of IPP and DMAPP, the mevalonate pathway and the more recently discovered deoxyxylulose pathway. All terpenes are formed through the reactions of IPP and DMAPP [237]. Rare terpenoids such as norisoprenoids, like the C_{13}-norisoprenoids 3-oxo-α-ionol are found to be present in Muscat of Alexandria leaves and 7,8-dihydroionone derivatives, and megastigmane-3,9-diol and 3-oxo-7,8-dihydro-α-ionol found in Shiraz leaves (both grapes in the species *Vitis vinifera*) or wine (responsible for some of the spice notes in Chardonnay) [274].

Polyphenols are the third most widespread class of metabolites in nature, and their distribution is almost ubiquitous [241]. It is estimated that 100,000 to 200,000 secondary metabolites exist and some 20% of the carbon fixed by photosynthesis is channeled into the phenylpropanoid pathway, thus generating the majority of the natural-occurring phenolics, such as flavonoids and stilbenes [275].. Although a large variety of plant phenols exists, most of these compounds arise from a common origin: the amino acids phenylalanine or tyrosine. These aminoacids are deaminated to cinnamic acids, which enter the phenylpropanoid pathway. As a general rule recently proposed by Quideau *et al.* [276], the term 'plant phenolics' should be strictly used to refer to secondary natural metabolites arising biogenetically from the shikimate/ phenylpropanoid pathway, which directly provides phenylpropanoids (Figure 11.1), or the 'polyketide' acetate/malonate pathway, which can produce simple phenols, or both of them [277]. A key step in this biosynthetic route is the introduction of one or more hydroxyl groups into the phenyl ring. As result, these compounds are derived from a common carbon skeleton building block: the C_6-C_3 phenylpropanoid unit [53].

Biosynthesis, according to this pathway, produces the large variety of plant phenols: cinnamic acids (C_6-C_3), benzoic acids (C_6- C_1), flavonoids (C_6-C_3-C_6), proanthocyanidins [(C_6-C_3-C_6)*n*], coumarins (C_6-C_3), stilbenes (C_6-C_2-C_6), lignans (C_6-C_3-C_3-C_6) and lignins [(C_6-C_3)*n*]. Main classes of phenolics include phenolic acid (benzoic acid derivatives and cinnamic acid derivatives), flavonoids (flavones, flavonols, flavanones, flavan-3-ols, anthocyanidins and isoflavones), coumarins, stilbenes and lignans (Figure 11.2) [275]. These pathways produce a bewildering array of monomeric and polymeric structures (the term 'polyphenols' defining those with more than one phenolic ring) that fulfill a very broad range of physiological roles in plants [277]. Although the bulk of these compounds play cell wall structural roles, plant tissues synthesize a vast array of non-structural constituents that have various roles in plant growth and survival. Thus, the expression "plant phenolics" embraces a highly diverse group with an extremely large structural diversity: tens of thousands of diverse structures have been identified, with the number continually increasing [276][278].

11.1.2 General antioxidant mechanisms of phenolics

Phenolics are able to act as antioxidants in a number of ways. Phenolic hydroxyl groups are good hydrogen donors: hydrogen-donating antioxidants can react with reactive oxygen and reactive nitrogen species in a termination reaction, which breaks the cycle of generation of new radicals [279]. Following interaction with the initial reactive species, a radical form of the antioxidant is produced, having a much greater chemical stability than the initial radical. The interaction of the hydroxyl groups of phenolics with the π-electrons of the benzene ring gives the molecules special

properties, most notably the ability to generate free radicals where the radical is stabilized by delocalization. The formation of these relatively long-lived radicals is able to modify radical-mediated oxidation processes [280]. The antioxidant capacity of phenolic compounds is also attributed to their ability to chelate metal ions involved in the production of free radicals [281]. However, phenolics can act as pro-oxidants by chelating metals in a manner that maintains or increases their catalytic activity or by reducing metals, thus increasing their ability to form free radicals [282]. Phenolic structures often have the potential to strongly interact with proteins, due to their hydrophobic benzenoid rings and hydrogen-bonding potential of the phenolic hydroxyl groups. This gives phenolics the ability to act as antioxidants also by virtue of their capacity to inhibit some enzymes involved in radical generation, such as various cytochrome P_{450} isoforms, lipoxygenases, cyclooxygenase and xanthine oxidase [280][283].

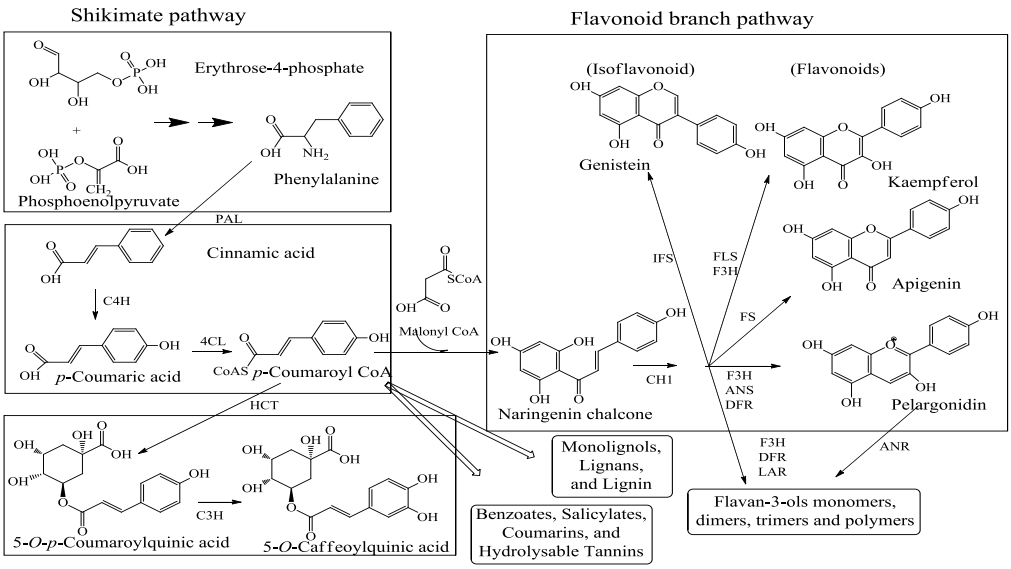


Figure 11.1: Schematic of the major branch pathways of (poly) phenol biosynthesis. PAL: phenylalanine ammonia-lyase, C4H: cinnamate-4-hydrolase, 4CL: 4-coumaroyl:CoA-ligase, HCT: hydroxycinnamoyl transferase, C3H: *p*-coumarate-3-hyroxylase, CHS: chalcone synthase, CHI: chalcone isomerase, ANS: anthocyanidinstnthase, DFR: dihydroflavonol reductase, FS: flavone synthase, F3H: Flavanone 3-hydroxylase, IFS: isoflavone synthase, ANR: anthocyanidin reductase, LAR: leucoanthocyanidin reductase [277].

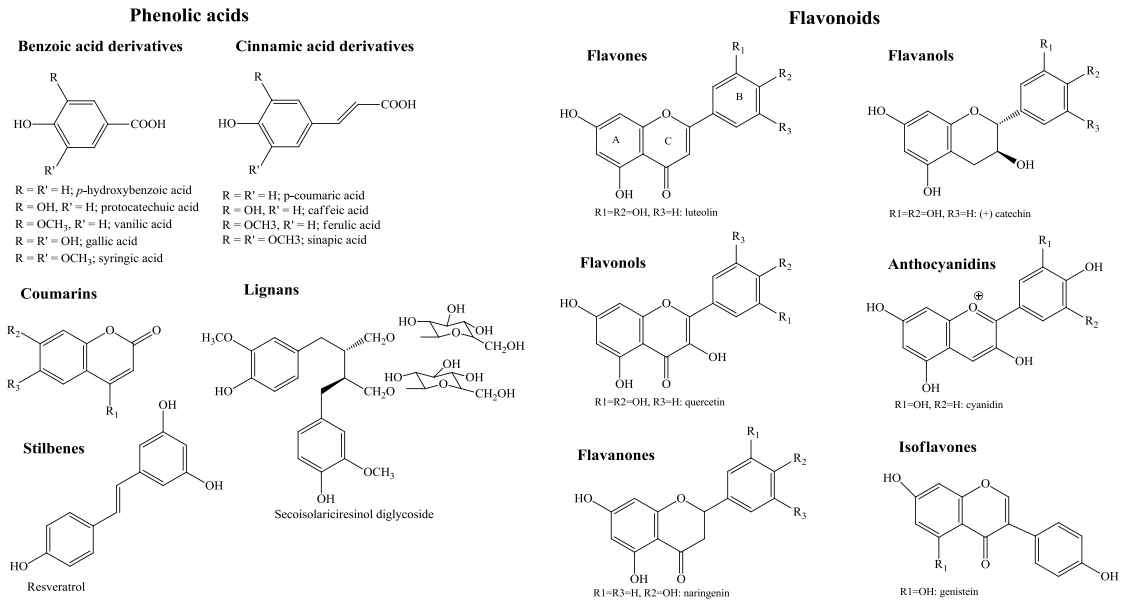


Figure 11.2: Main classes of phenolics [275]

11.1.3 Flavonoids

Approximately 9000 different flavonoids have been reported from plant sources, and with almost certainty many more are still to be discovered, as they continue to capture the interests of scientists from numerous disciplines. Based on the 10-carbon skeleton of flavonoids, they can be substituted by a range of different groups, viz. hydroxyl, methoxyl, methyl, isoprenyl and benzyl substituents [284]. Flavonoids are characterized by a phenylbenzopyran chemical structure. The general structure includes a C_{15} (C_6-C_3-C_6) skeleton joined to a chroman ring (benzopyran moiety). The heterocyclic benzopyran ring is known as the C ring, the fused aromatic ring as the A ring, and the phenyl constituent as the B ring (Figure 11.2). Commonly flavonoids are further modified by the addition of substituent groups such as methyl groups, aromatic acyl groups, and/or sugar moieties [284]. Increasingly, flavonoids are becoming the subject of medical research. Flavonoids play a role in disease prevention in humans. They have been reported to possess many useful properties, including anti-inflammatory, oestrogenic, enzyme inhibition, antimicrobial, antiallergic, vascular and cytotoxic antitumour activity [285], but the antioxidant activity is, without a doubt, the most studied one attributed to flavonoids. This well established antioxidant activity of flavonoids is also responsible for other biological activities in which the prevention of oxidative stress is beneficial [286].

The anti-oxidants activity of the flavonoids which neutralize damaging chemicals in the body such as free radicals results in reduction of oxidative stress, which are thought to contribute to a number of diseases, including atherosclerosis, degenerative brain diseases like Alzheimer's disease as well as premature ageing [52]. Flavonoids

have also been found to help the body fight viruses and decrease allergic reactions. There is abundant evidence to support the role of flavonoids in disease prevention a high dietary intake of quercetin is related to a reduced risk of heart disease [287]. Rutin has been found to help prevent stomach ulcers by protecting the stomach lining and it also protects blood vessels. Flavonoids have also been found to reduce the 'stickiness' of blood platelets and thus reduce the formation of blood clots, which reduce the risks of certain cardiovascular diseases [288]. Flavan-3-ols are thought to interfere in the pathogenesis of cardiovascular disease *via* several mechanisms: antioxidative, antithrombogenic, and antiinflammatory. In particular, proanthocyanidins and flavan-3-ol monomers aid in lowering plasma cholesterol levels, inhibit LDL oxidation, and activate endothelial nitric oxide synthase to prevent platelet adhesion and aggregation that contribute to blood clot formation [289][290].

The anticancer activity of some flavonoid compounds is due to their ability to scavenge free radicals, thus avoiding the early stages of cancer promotion. Besides this mechanism, flavonoids have also been reported to act as anticancer agents *via* regulation of signal transduction pathways of cell growth and proliferation, suppression of oncogenes and tumor formation, induction of apoptosis, modulation of enzyme activity related to detoxification, oxidation and reduction, stimulation of the immune system and DNA repair, and regulation of hormone metabolism [289]. In the past few years we have witnessed the establishment of other flavonoid classes as potent molecules for the treatment of other pathologies that do not involve these compounds' antioxidant properties. This is the case of some isoflavones, whose estrogen-like capacity is now well established. The activity of these compounds is related with their similarity to estradiol estrogen. Genistein and daidzein have

demonstrated to be promising molecules for the treatment of conditions in which the agonist effect in estrogen receptors is beneficial, such as menopause conditions. In fact, several preparations containing these compounds, mainly soya-derived, are now used in therapeutics [291].

11.1.4 Phenolic acids

Phenolic acids have a carboxyl group attached or linked to benzene ring (Figure 11.2). Two classes of phenolic acids can be distinguished depending on their structure: benzoic acid derivatives (*i.e.* hydroxybenzoic acids, C_6-C_1) and cinnamic acid derivatives (*i.e.* hydroxycinnamic acids, C_6-C_3) (Figure 11.2) [292]. Hydroxycinnamic acid compounds (p-coumaric, caffeic acid, ferulic acid) occur most frequently as simple esters with hydroxy carboxylic acids or glucose, while the hydroxybenzoic acid compounds (p-hydroxybenzoic, gallic acid, ellagic acid) are present mainly in the form of glucosides. Furthermore, phenolic acids may occur in food plants as esters or glycosides conjugated with other natural compounds such as flavonoids, alcohols, hydroxyfatty acids, sterols, and glucosides [293].

They have been the subject of a great number of chemical, biological, agricultural, and medical studies. Among the wide diversity of naturally occurring phenolic acids, at least 30 hydroxy- and polyhydroxybenzoic acids have been reported in the last 10 years to have biological activities [294]. They have antioxidant, antimutagenic and even leaf movement regulating agents that protect the organism that produces them from the oxidative stress created by metabolism and their physical environment [294]. They also have antiviral, antibacterial (bactericidal, bacteriostatic), algicidal, plant

growth regulating, phytotoxic, antifungal, antiprotozoal, nematicidal, insecticidal, antifeedant, and mammalian estrogenic, keratolytic, platelet aggregation inhibiting, hypoglycemic, cytotoxic, and neurotoxic activities that may serve to protect the organism that biosynthesizes them from competing, pathogenic, and herbivorous organisms in their biological environment [295][296]. The diverse biological functions of these phenolic acids suggest potential pharmacological activities.

11.1.5 Coumarins

Coumarins owe their class name to 'coumarou', the vernacular name of the tonka bean (*Dipteryx odorata* Willd., Fabaceae), from which coumarin itself was isolated in 1820 [297]. The primary site of synthesis of coumarins is suggested to be the young, actively growing leaves, with stems and roots playing a comparatively minor role. Coumarins belong to a group of compounds known as the benzopyrones, all of which consist of a benzene ring joined to a pyrone (Figure 11.2). To this days, around 1,300 coumarins are known, with all of them being derivatives of 5,6-benzo-2- pirone (α-chromone) (with OH, OCH_3 or CH_3 substituents on the benzoic ring.). Coumarins may also be found in nature in combination with sugars, as glycosides. As derivatives of simple coumarins, other compounds are known, such as furanocoumarins, which include a furanic ring, linear pyranocoumarins, angular pyranocoumarins, dimeric coumarins, of which dicoumarol is an example and also furanochromones [298].

Historically, the ability of dicoumarol to inhibit blood clotting, that later led to the development of the anticoagulant drug warfarin, was the first call to this class of compounds' biological properties. Coumarin is the parent molecule of warfarin,

which acts as a vitamin K antagonist. Warfarin is a clinically useful anticoagulant and widely employed rodenticide. Several biological activities have been reported in natural-occurring coumarins, from photo sensitizers to vasodilatation. Recently, the interest has been given to synthetic derivatives of coumarins, such as fluorinated and 1-azo coumarins, which displayed moderate analgesia properties, and excellent anti-inflammatory and anti-microbial activities [299].

Coumarins have a variety of bioactivities including anticoagulant, estrogenic, dermal, photosensitizing, antimicrobial, vasodilator, molluscidal, antihelmintic, sedative, hypnotic, analgesic and hypothermic activity [300]. Various coumarins have been reported to possess anti-inflammatory activity as shown in carrageenan-induced inflammation and cotton pellet granuloma tests [301][302]. There have been reports on efficacies of pure coumarins as well as extracts containing them against Gram-positive and Gram-negative bacteria as well as fungi [303]. The free OH group at C-6 in the coumarins nucleus has been found to be important for antifungal activity, while the free hydroxyl group at C-7 is important for antibacterial activity [304]. The bioactivities of phototoxic psoralens and dicoumaroul derivatives are well known and several of these compounds are used in antipsoriatic and anticoagulant therapy [305]. Other than psoriasis, skin disease like cutaneous T-cell lymphoma, atopic dermatitis, urticaria pigmentosa and lichen planus [306] are treated with the photochemotherapy of linear furanocoumarins (psoralens). The most widely used compound is xanthotoxin [307]. Bergapten is considered a valuable alternative for chemotherapy of psoriasis, since its clinical efficacy is comparable to that of xanthotoxin, although bergapten requires higher cumulative UVA doses.

The inherent fluorescent properties of many coumarins are a key factor in many applications. Areas where coumarins are widely used include estimation of enzymatic activity [308], labeling of proteins, antibodies, DNA and lipids, derivatising agents in chromatography, etc. Coumarins have a wide variety of uses in industry, mainly due to their strong fragrant odour [308]. Uses include that of a sweetener and fixative of perfumes (e.g. 3,4-dihydrocoumarin), an enhancer of natural oils such as lavender, a food additive in combination with vanillin, a flavour/odour stabilizer in tobaccos and an odour masker in paints and rubbers. 6-Methylcoumarin is mainly used as a flavour enhancer, and 7- hydroxycoumarin is mainly used in sunscreens.

11.2 Material and methods

Eight compounds that have been isolated in Chapter 11 were subjected to various biological assays.

11.2.1 Antioxidant capacity assays

Samples were dissolved in dimethyl sulfoxide (DMSO, R&M) prior to assay at a stock concentration of 1 mg/ml, and serial dilution was done accordingly to obtain a good EC_{50} curve. Trolox (6-hydroxy-2,5,7, 8-tetramethylchromon-2-carboxylic acid, Sigma-Aldrich), vitamin C (l-ascorbic acid), and quercetin were used as positive control at a stock concentration of 1 mg/ml. Assay was performed using Thermo Scientific Varioskan Flash microtiter plate reader, linked to a computer equipped with (SkanIt Software 2.4.3). EC_{50} values were determined using Prism 5.00 software. At

least three independent tests were performed for each sample. Method is as explained in Chapter 4, DPPH assay (section 4.2.1.2), ABTS assay (section 4.2.2.2), FRAP assay (section 4.2.3.2), β-carotene bleaching assay (section 4.2.4.2) and SOD assay (section 4.2.5.2)

11.2.2 Anti-inflammatory determination assays

Test samples and positive controls were dissolved in dimethyl sulfoxide (DMSO, R&M) prior to assay at a stock concentration of 1 mg/ml, and serial dilution was done according to the assay to obtain a good EC_{50} curve. Absorbance was recorded using Thermo Scientific Varioskan Flash microtiter plate reader, linked to a computer equipped with (SkanIt Software 2.4.3). Percentage inhibition of enzyme was determined by comparison of rates of reaction of samples relative to blank sample using the formula $(E - S)/ E \times 100$, where E is the activity of enzyme without test sample and S is the activity of enzyme with test sample. The experiments were done in triplicate. Method has been explained in Chapter 5, 5-LOX inhibition assay (section 5.4.2.2) and the peroxidase endpoint assay for COX-1 and COX-2 (section 5.5.3.2)

11.2.3 Anti-acetylcholinesterase determination assays

Test samples and galanthamine which was used as positive control was dissolved in dimethyl sulfoxide (DMSO, R&M) prior to assay at a stock concentration of 1 mg/ml, and serial dilution was done accordingly to obtain a good EC_{50} curve. The reaction was monitored utilizing a 96-well microplate Thermo Scientific Varioskan Flash microtiter plate reader, linked to a computer equipped with (SkanIt Software 2.4.3).

Percentage inhibition of AChE was determined by comparison of rates of reaction of samples relative to blank sample (ethanol in phosphate buffer pH = 8) using the formula (E – S)/ E × 100, where E is the activity of enzyme without test sample and S is the activity of enzyme with test sample. The experiments were done in triplicate. Method is explained in Chapter 6 (section 6.4.6.2).

11.2.4 Anticancer assay

All samples except syringic acid (insufficient yield) were tested for this activity against human derived cell lines MDA 468 (ER-) breast carcinoma]. Samples were prepared as 1 mg/ml stock solutions, dissolved in DMSO, and stored at 4 ^{0}C, protected from light for a maximum period of 4 weeks. Serial drug dilutions were prepared in medium immediately prior to each assay. Due to good activity exhibited, scopoletin was also tested against HT-29, MCF-7 and HCT 116 cell lines. Method is as explained in Chapter 9 section 9.3.2.

11.2.5 Antibacterial assay

Due to insufficient yield of compounds isolated, all samples were tested for the antimicrobial effect only against S. aureus ATCC 11632. Samples was dissolved in dimethyl sulfoxide (DMSO, R&M) prior to assay at a stock concentration of 1 mg/ml. Method used is as explained previously, MIC assay (section 7.2.2.2), MBC assay (section 7.2.3.2) and time-kill assay (section 7.2.4.2).

11.2.6 Antiparasitic assay

Due to insufficient yield of compounds isolated, scopoletin was tested for both assays and only scoparone, (+)-catechin, lioxin and vomifoliol was tested for the antileshmanial assay. Method is as explained in chapter 8, for antiparasitic assay against *Giardia intestinalis* and *Entamoeba histolytica* (section 8.3.2.2.1) and antileishmanial activity against *Leishmania donovani* promastigotes (section 8.3.2.2.2).

11.2.7 Formulation of scopoletin

Due to potent antioxidant, antibacterial and anti-inflammatory activity (to be discussed in 11.8), scopoletin was formulated as a gel. Scopoletin (25 mg) was prepared as a 0.5 % w/w preparation. Scopoletin was dissolved in few drops of carrier solvent DMSO. Then orange oil is added to increase the penetration via skin. Pure aloe vera gel was then added in sufficent amount to make up a total of 5 g of gel.

11.2.8 Statistical analysis

For section 11.2.1-11.2.3, 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. All data were expressed as

mean ± standard deviation. SD was computed but values were not shown in the graphs via this software. For section 11.2.4-6 growth curve of bacteria/parasite/cell were analysed using MS-Excel and data were reported as mean and SD values obtained from a minimum of three determinations. All data were expressed as mean ± standard deviation. Data were analyzed using one way Anova followed by Tukey test. A significant difference was considered at the level of $P < 0.01$.

11.3 Results

11.3.1 Antioxidant capacity assays

The antioxidant capacity of the isolated compounds is shown in Table 11.1. In the DPPH assay, the highest antioxidative action was exhibited by (+)-catechin (EC_{50} 4.77±0.01 µg/ml) similar with positive control trolox (EC_{50} 4.77±0.01 µg/ml), followed by hyperin (EC_{50} 4.89±0.02 µg/ml), cynaroside (EC_{50} 7.89±0.03 µg/ml), syringic acid (EC_{50} 15.43±0.02 µg/ml) and scopoletin (EC_{50} 36.80±0.01 µg/ml). Both lioxin and vomifoliol had EC_{50} >100 µg/ml.

In the ABTS assay, the highest antioxidative action was exhibited by cynaroside (EC_{50} 0.26±0.02 µg/ml) followed by scopoletin (EC_{50} 1.08±0.03 µg/ml), (+)-catechin (EC_{50} 5.03±0.06 µg/ml), syringic acid (EC_{50} 7.38±0.02 µg/ml) hyperin (EC_{50} 8.19±0.04 µg/ml), vomifoliol (EC_{50} 34.89±0.04 µg/ml) and lioxin (EC_{50} 83.22±0.03 µg/ml).

In the FRAP assay, antioxidant activity was calculated as Ferrous Equivalents, the concentration of samples which produced an absorbance value equal to that of 1mM $FeSO_4$. The compounds absorbance equivalent to 1 mM FeSO4 was calculated from equation of linearity of scopoletin (y=0.0203x+0.5259, r^2=0.9837), scoparone (y=0.0009x+0.1708, r^2=0.5026), (+)-catechin (y=0.0141x+0.1442, r^2=0.7415), hyperin (y=0.0140x+0.2189, r^2=0.8319), cynaroside (y=0.0156x+0.3277, r^2=0.6009), vomifoliol (y=0.0062x+0.0549, r^2=0.6990), lioxin (y=0.0126x+0.0471, r^2=0.8546), syringic acid (y=0.0172x+0.2963, r^2=0.6901). Total FRAP value was determined from the absorbance value above using the standard Fe (II) calibration curve equation (y=0.0105x+0.0136, r^2=0.9817). Both scopoletin and (+)-catechin displayed significantly lower FRAP value (49.00±0.64 µg/ml and 75.59±0.42 µg/ml respectively) than positive control ascorbic acid and quercetin (347.00±0.23 µg/ml and 86.00±0.24 µg/ml respectively).

In the β-carotene decolourisation assay, the most potent activity was displayed by vomifoliol (EC_{50} 6.85±0.37 µg/ml), followed by lioxin (EC_{50} 20.13±0.47 µg/ml), hyperin (EC_{50} 32.21±0.35 µg/ml), syringic acid (EC_{50} 53.69±0.36 µg/ml), cynaroside (EC_{50} 56.38±0.38 µg/ml), (+)-catechin (EC_{50} 83.22±0.56 µg/ml) and scopoletin (EC_{50} 124.50±0.07 µg/ml). Only scoparone had EC_{50} of >100 µg/ml.

In the SOD assay, both hyperin and (+)-catechin has IC_{50} values significantly lower (IC_{50} 0.75±0.03 µg/ml and IC_{50} 0.94±0.27 µg/ml respectively) than positive control SOD enzyme (IC_{50} 1.60±0.06 µg/ml). Cynaroside (IC_{50} 1.87±0.03 µg/ml) had activity similar to SOD, followed by scoparone (IC_{50} 48.72±0.02 µg/ml) and lioxin (IC_{50} 53.69±0.38 µg/ml). Scopoletin, vomifoliol and syringic acid had IC_{50} of >100 µg/ml.

11.3.2 Anti-inflammatory determination assays

The results are shown in Table 11.2 and Figure 11.3-11.5. In the 5-LOX inhibition assay, all isolated compounds displayed significantly lower IC_{50} compared to positive control NDGA. Both scoparone and scopoletin (IC_{50} 0.20±0.01 µg/ml and 0.34±0.01 µg/ml respectively) had the most potent 5-LOX enzyme inhibition (Figure 11.3) which was more than eightyfold compared to NDGA (IC_{50} 29.21±0.05 µg/ml). This was followed by syringic acid (IC_{50} 1.38±0.03 µg/ml), hyperin (IC_{50} 3.02±0.02 µg/ml), vomifoliol (IC_{50} 8.72±0.02 µg/ml), (+)-catechin (IC_{50} 16.10±0.03 µg/ml), lioxin (IC_{50} 17.79±0.03 µg/ml) and cynaroside (IC_{50} 18.12±0.04 µg/ml) (Figure 11.4).

In the COX enzyme inhibition assay, only (+)-catechin and syringic acid showed good enzyme inhibition while no enzyme inhibition was noticed with lioxin and all other compounds had IC50 values >100 µg/ml. Compound (+)-catechin displayed COX-1 selective activity with COX-1/COX-2 ratio of 0.14 with COX-1 enzyme IC_{50} of 12.08±0.02 µg/ml and COX-2 enzyme IC_{50} 83.89±0.03 µg/ml (Figure 11.5). Syringic acid had COX-1 enzyme inhibition at IC_{50} 34.89±0.02 µg/ml with COX-2 inhibition at IC_{50} >100 µg/ml.

11.3.3 Anti-acetylcholinesterase determination assays

The anti-acetylcholinesterase values for isolated compounds are shown in Table 11.3 and Figure 11.6. Only 4 compounds showed moderate enzyme inhibition namely syringic acid (IC_{50} 29.53±0.19 µg/ml), scopoletin (IC_{50} 51.00±0.02 µg/ml), scoparone (IC_{50} 86.58±0.05 µg/ml) and vomifoliol (IC_{50} 96.64±0.09 µg/ml) as shown in Figure

Table 11.1: Antioxidant values of isolated compounds from *Canarium patentinervium* Miq.

Compounds	**ABTS assay, EC_{50} (µg/ml)**	**DPPH assay, EC_{50} (µg/ml)**	**FRAP assay, FRAP value (µg/ml)**	**β-carotene bleaching assay, EC_{50} (µg/ml)**	**Superoxide dismutase assay, IC_{50} (µg/ml)**
scopoletin	1.08±0.03	36.80±0.01.	49±0.64	124.5±0.07	>100
scoparone	>100	>100	11492±0.16	>100	48.72±0.02
(+)-catechin	5.03±0.06	$4.77±0.01^{A}$	75.59±0.42	83.22±0.56	$0.94±0.27^{C}$
hyperin	8.19±0.04	4.89±0.02	791.96±0.52	32.21±0.35	$1.87±0.03^{D}$
cynaroside	0.26±0.02	7.79±0.03	694.63±0.32	56.38±0.38	$0.75±0.03^{C}$
vomifoliol	34.89±0.04	>100	1686.89±0.28	6.85±0.37	>100
lioxin	83.22±0.03	>100	830.67±0.53	20.13±0.47	53.69±0.38
syringic acid	7.38±0.02	15.43±0.02	594.03±0.41	53.69±0.36	>100
AA	1.57±0.06	1.83±0.01	345.15±0.23	NA	NA
QC	0.98±0.03	$2.78±0.02^{A}$	88.16±0.24	$1.70±0.03^{B}$	NA
TRO	0.65±0.02	$4.77±0.01^{A}$	35.00±0.54	$1.69±0.03^{B}$	NA
SOD	NA	NA	NA	NA	$1.60±0.06^{D}$

AA: ascorbic acid, QC:quercetin, TRO:trolox and SOD: superoxide dismutase
Data were obtained from three independent experiments, each performed in triplicates (n=9) and represented as mean ± SD.
Values with the same capital letter are not significantly different ($P<0.05$) according to Tukey multiple comparison test.

Table 11.2: Anti-inflammatory values of the isolated compound from *Canarium patentinervium* Miq.

Compounds	**Anti-inflammatory assay 5-LOX, IC_{50} (µg/ml)**	**COX-1 inhibition assay IC_{50}(µg/ml)**	**COX-2 inhibition assay IC_{50}(µg/ml)**	**COX-1/COX-2 ratio**
scopoletin	0.34±0.01	>100	>100	
scoparone	0.20±0.01	>100	>100	
(+)-catechin	16.1±0.03	12.08±0.02	83.89±0.03	0.14
hyperin	3.02±0.02	>100	>100	
cynaroside	18.12±0.04	>100	>100	
vomifoliol	8.72±0.02	>100	>100	
lioxin	17.79±0.03	NO INH	NO INH	
syringic acid	1.38±0.03	34.89±0.02	>100	
NDGA	29.21±0.05	NA	NA	NA
INDO	NA	0.30±0.04	0.25±0.03	1.20

NDGA: nordihydroguairetic acid, INDO: indomethacin, NO INH: no inhibition noted
Data were obtained from three independent experiments, each performed in triplicates (n=9) and represented as mean ± SD.
Values with the same letter are not significantly different ($P<0.01$) according to Tukey multiple comparison test.

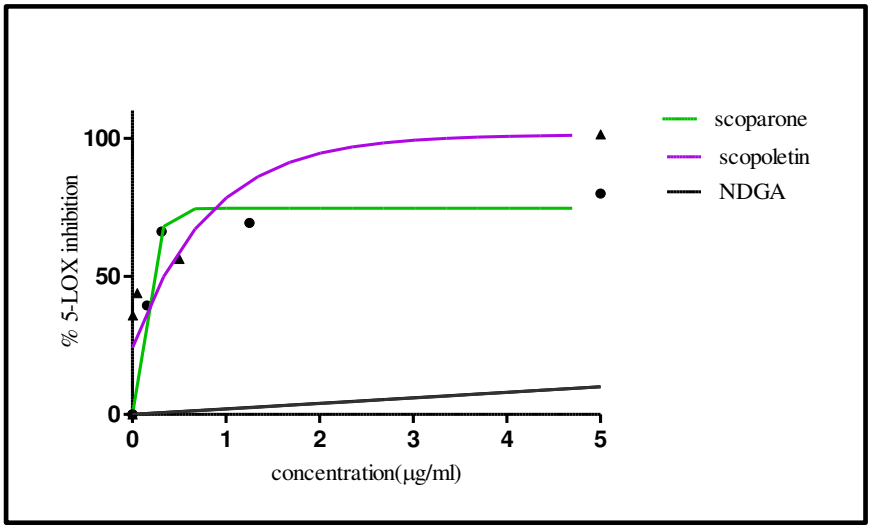


Figure 11.3: 5-LOX inhibition by scopoletin and scoparone isolated from *Canarium patentinervium* Miq

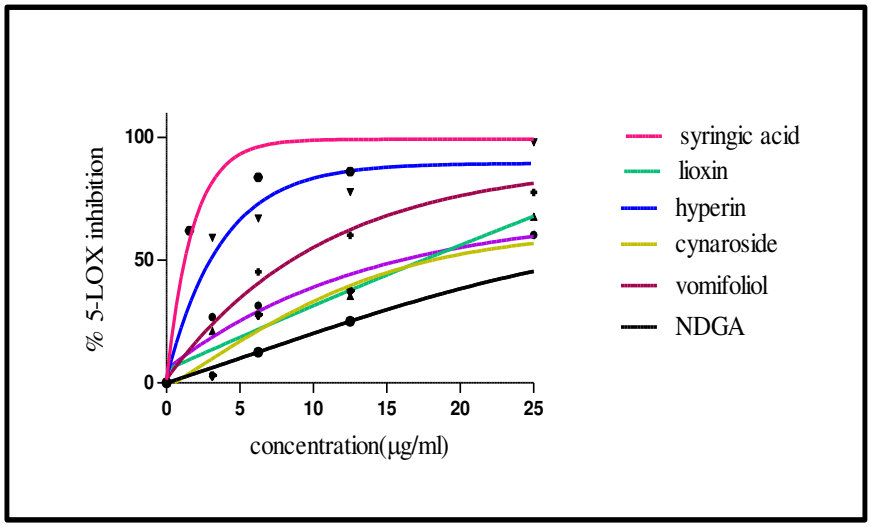


Figure 11.4: 5-LOX inhibition by compounds isolated from *Canarium patentinervium* Miq

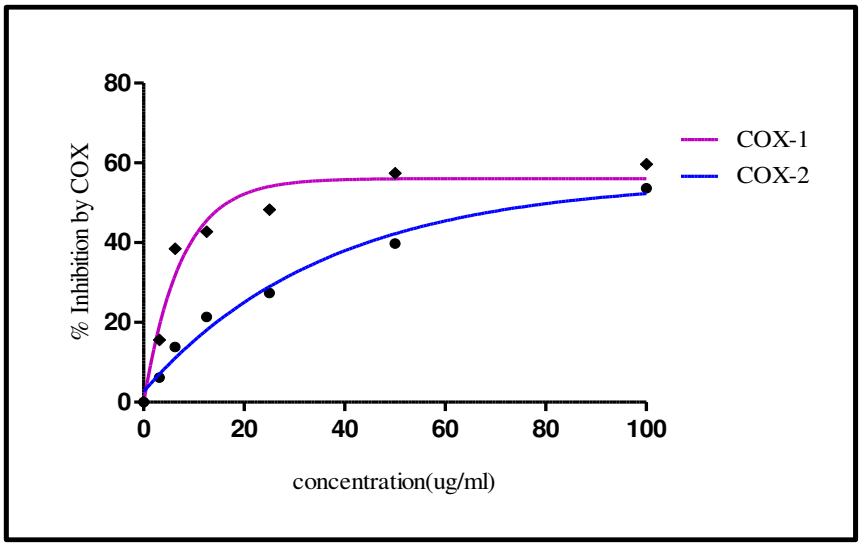


Figure 11.5: COX inhibition by (+)-catechin isolated from *Canarium patentinervium* Miq.

11.6. Rest of the compounds showed poor enzyme inhibition with IC_{50} values >100 µg/ml.

11.3.4 Anticancer assay

The anticancer activity of the compounds that were tested are shown in Table 11.4 and Figure 11.7a and 11.7b. Isolated compound with the highest yield scopoletin was tested against four cell lines, two human breast cancer cell lines, MCF-7 (ER+) and MDA 468 (ER-), and two colon cancer cell line, HCT 116 and HT-29. Scopoletin exhibited potent growth inhibition of MDA 468 (GI_{50} 0.09±0.25 µg/ml) (Figure 11.7a) and HT-29 (GI_{50} 0.17±0.05 µg/ml) (Figure 11.7b) with significantly lower GI_{50} compared to positive control doxorubicin (GI_{50} 0.69±0.06 µg/ml) for the HT-29 cell line. Cell line MCF-7 and HCT 116 were less sensitive to scopoletin with GI_{50} values >2 µg/ml. Scoparone, (+)-catechin, hyperin, cynaroside, vomifoliol and lioxin were tested only against MDA 468 cell line and all having GI_{50} values >2 µg/ml.

11.3.5 Antibacterial assay

The antibacterial assay results are shown in Table 11.5 and Figure 11.8. *Staphylococcus aureus* ATCC 11632 was sensitive against all compounds tested. Scopoletin had the best activity with MIC 25.00±0.00 µg/ml and MBC of 50.00±0.00 µg/ml with complete bacterial kill at 50.00±0.00 µg/ml (Figure 11.8). Scoparone, Hyperin, cynaroside and syringic acid had bactericidal activity at 100.00±0.00 µg/ml.

Table 11.3: Anti- acetylcholinesterase values for isolated compounds from *Canarium patentinervium* Miq.

Compounds	**Anti-acetylcholinesterase assay, IC_{50}(µg/ml)**
scopoletin	51.00±0.02
scoparone	86.58±0.05
(+)-catechin	>100
hyperin	>100
cynaroside	>100
vomifoliol	96.64±0.09
lioxin	>100
syringic acid	29.53±0.19
galantamine	0.77±0.09

Data were obtained from three independent experiments, each performed in triplicates (n=9) and represented as mean ± SD. Values with the same letter are not significantly different (P<0.01) according to Tukey multiple comparison test.

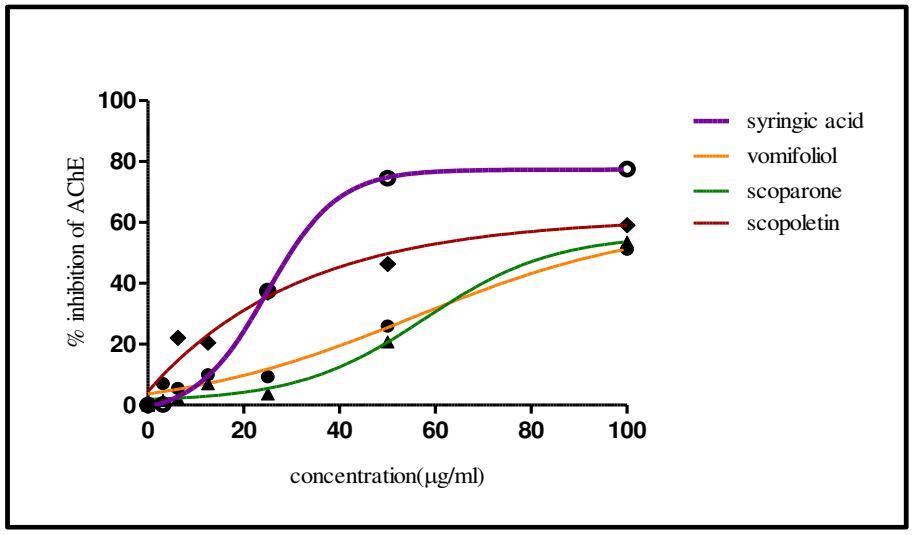


Figure 11.6: AChE inhibition by isolated compounds from *Canarium patentinervium* Miq.

Table 11.4: Growth inhibition of human cancer cell lines by isolated compounds from *Canarium patentinervium* Miq.

Compounds	**Cell line/ GI_{50} (µg/ml)**			
	MDA 468	**HT-29**	**MCF-7**	**HCT 116**
scopoletin	0.09±0.25	0.17±0.05	>2	>2
scoparone	>2	nt	nt	nt
(+)-catechin	>2	nt	nt	nt
hyperin	>2	nt	nt	nt
cynaroside	>2	nt	nt	nt
vomifoliol	>2	nt	nt	nt
lioxin	>2	nt	nt	nt
doxorubicin	0.04±0.12	0.69±0.06	0.38±0.28	0.65±0.18

Nt = not tested, Data were obtained from three independent experiments, each performed in triplicates (n=9) and represented as mean ± SD. Values with the same letter are not significantly different (P<0.01) according to Tukey multiple comparison test.

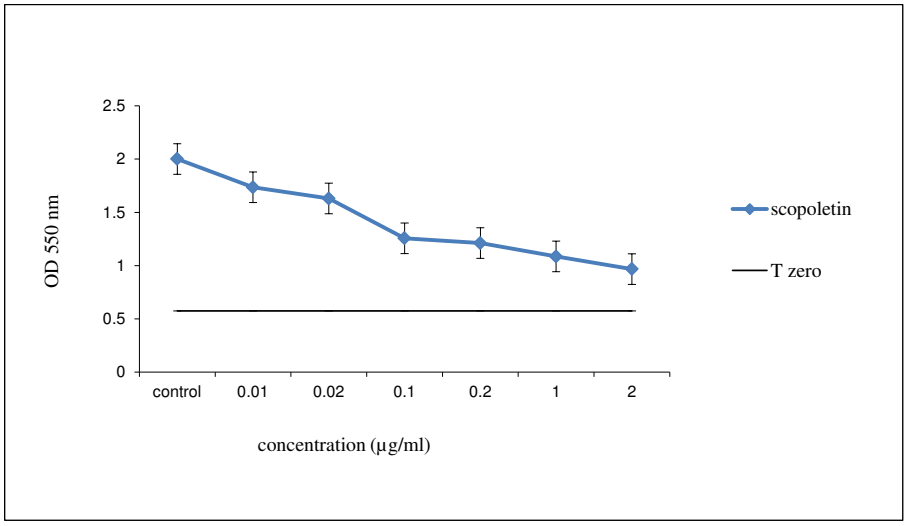


Figure 11.7a: Effect of scopoletin isolated compounds from *Canarium patentinervium* Miq. against growth of breast cancer cell line MDA 468 (GI_{50} = 0.09 µg/ml)

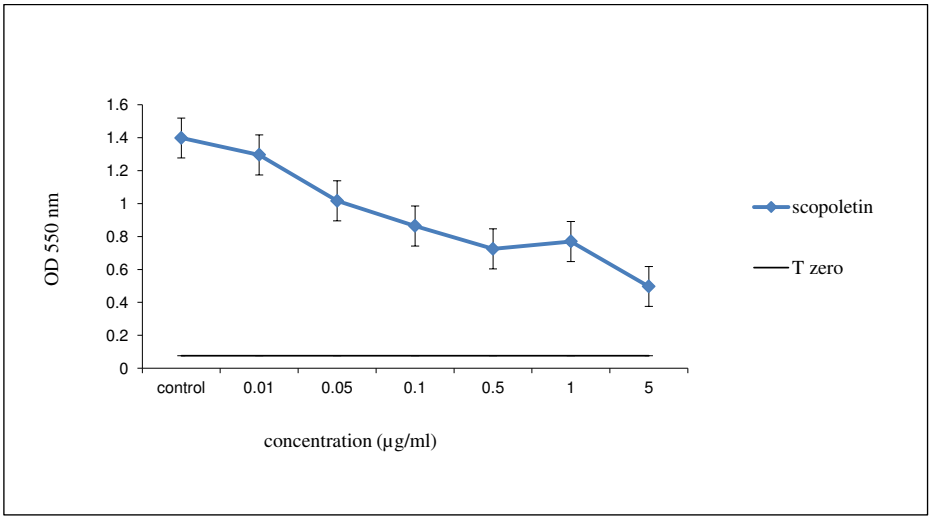


Figure 11.7b: Effect of scopoletin isolated compounds from *Canarium patentinervium* Miq. against growth of colon carcinoma cell line HT-29 (GI_{50} = 0.17 µg/ml)

11.3.6 Antiparasitic assay

The antiparasitic activity of the compounds tested is shown in Table 11.6. Compound scopoletin was tested against all three parasite amd it was more potent against *Leishmania donovani* (IC_{50} 163.30±0.32 µg/ml) and MIC of >200 µg/ml for both *Giardia intestinalis* and *Entamoeba histolytica*. Sensitivity of *Leishmania donovani* is then followed by with the presence of lioxin (IC_{50} 211.48±0.32 µg/ml), vomifoliol (IC_{50} 302.80±0.33 µg/ml), scoparone (IC_{50} 329.90±0.32 µg/ml) and (+)-catechin (IC_{50} 478.93±0.28 µg/ml).

11.4 Discussion

Of the eight isolated compounds, five were phenolics (hyperin, cynaroside, (+)-catechin, lioxin and syringic acid), two were coumarins (scopoletin and scoparone) and one was a norsesquiterpene with a cyclohexenone ring (vomifoliol). Among the phenolics, (+)-catechin is a flavanol, hyperin is a flavonol with a glucosidic moiety and cynaroside is a flavone with a glycosidic moiety and two were benzoic acid derivatives. The basic skeleton of these groups are shown in Figure 11.2.

Scopoletin possesses interesting activities, in particular, vasorelaxant [309], antioxidant [310],antimicrobial [311], anti-inflammatory [312], anti-pyretic [313], anti-platelet aggregation [314] and anti-diabetes mellitus properties [252]. In addition,

Table 11.5: MIC, MBC and MBC/MIC ratio for isolated compounds from *Canarium patentinervium* Miq. against *Staphylococcus aureus* ATCC 11632

Compounds	Concentration (µg/ml)		MBC/MIC ratio
	MIC	MBC	
scopoletin	25.00±0.00	50.00±0.00	2 (+)
scoparone	50.00±0.00	100.00±0.00	2 (+)
(+)-catechin	50.00±0.00	>100	nd
hyperin	50.00±0.00	100.00±0.00	2 (+)
cynaroside	50.00±0.00	100.00±0.00	2 (+)
vomifoliol	100.00±0.00	>100	nd
lioxin	100.00±0.00	>100	nd
syringic acid	50.00±0.00	100.00±0.00	2 (+)

ND: not determined

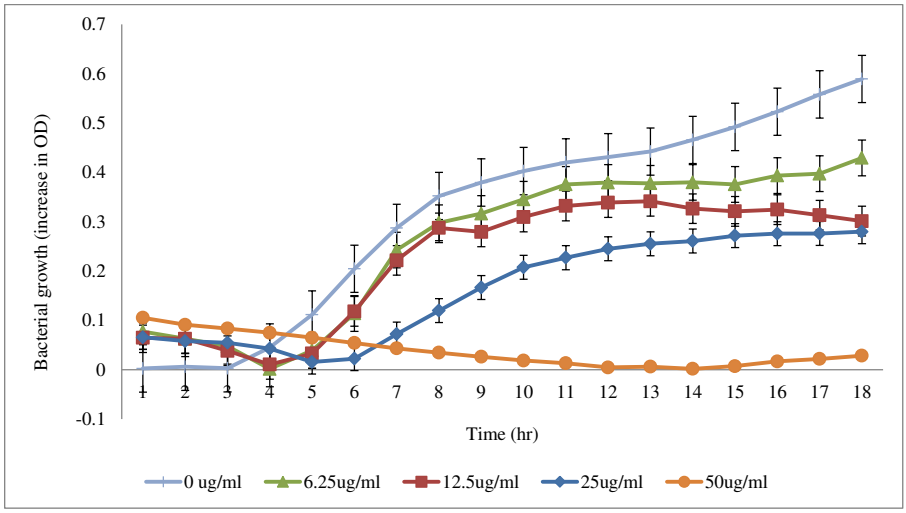


Figure 11.8: Time-kill plot for MSSA ATCC 11632 in presence of scopoletin isolated from *Canarium patentinervium* Miq.

Table 11.6: Antiparasitic values of isolated compounds from *Canarium patentinervium* Miq. against *Leishmania donovani* (AG 83) promastigotes and MIC values against *Giardia intestinalis* and *Entamoeba histolytica* growing *in vitro*.

	IC_{50} ((µg/ml)	MIC (µg/ml)	
Compounds	***Leishmania donovani***	***Giardia intestinalis***	***Entamoeba histolytica***
scopoletin	163.30±0.32	>200	>200
scoparone	329.90±0.32	nt	nt
(+)-catechin	478.93±0.28	nt	nt
vomifoliol	302.80±0.33	nt	nt
lioxin	211.48±0.32	nt	nt

Data were obtained from three independent experiments, each performed in triplicates (n=9) and represented as mean ± SD. Values with the same letter are not significantly different (P<0.01) according to Tukey multiple comparison test. nt: not tested

it exerted neuroprotective [315] and hypotensive [316] activities in addition to applications in cardiovascular disease [252], antitumor [317], anti-proliferation and antithyroid [318] treatment. Scoparone has been studied for its potential pharmacological properties including immunosuppression [319] and vasorelaxation [320]. Compound (+)-catechin is reported to induce longevity in the nematode worm *Caenorhabditis elegans* [321], reduces atherosclerotic lesion development in mice [322], have opposite effects on glycogen metabolism in isolated rat hepatocytes [323], inhibits intestinal tumor formation in mice [324], inhibits the oxidation of low density lipoprotein [325] and as an antioxidant [326]. Cynaroside has been reported for moderate inhibition of enzymes for the synthesis of thromboxane B_2 and leukotriene B_4 as well as hydrogen peroxide scavenging activity, scavenge reactive oxygen and nitrogen species [327] to chelate transition metals, antibacterial [328], antiviral [329] and antifungal [330] activity.

Hyperin has been found to have antioxidant activity [331], inhibition of lipid peroxidation in rat liver microsomes [332] and protective effects to PC_{12} cells against cytotoxicity induced by hydrogen peroxide and *tert*-butyl hydroperoxide [333]. Lioxin had been reported to exert strong antioxidant (oral protectant) [334], powerful wound healing properties [335], protective effects against DNA damage [336] as well as antimutagenic [337] and immunostimulating [338] properties. Syringic acid has shown to have antioxidant, antibacterial [339] and hepatoprotective [340] activities. Vomifoliol have been reported to have weak DPPH activity [341] and anti-inflammatory activity by inhibition of TNF-α [341] and plays an important role as an endogenous regulator of the stomatal aperture [262].

11.4.1 Antioxidant activity

The antioxidant mechanisms of flavonoids comprise free radical scavenging, but also enzyme inhibition/induction, metal chelation, interaction with receptors and modulation of gene expression [342]. The direct scavenging of radicals is believed to occur in cells, but, to avoid the interference of other mechanisms, this work was developed using a cell-free system. The past few decades of structure-activity relationships research have generated several consistent lines of evidence supporting the role of specific structural components as requisites for antioxidant activity. The antioxidant activity of flavonoids and their metabolites *in vitro* depends upon the arrangement of functional groups about the nuclear structure, namely ring A, B and C [343]. Consistent with most polyphenolic antioxidants, both the configuration and total number of hydroxyl groups substantially influence several mechanisms of antioxidant activity. The B-ring hydroxyl configuration is the most significant determinant of scavenging of ROS and RNS. A-ring substitution correlates little with antioxidant activity [344].

Some characteristics of flavonoids are important to the antioxidant capacity according to the famous three Bors criteria [344] (Figure 11.9): (i) *o*-dihydroxy structure in the B ring ie a 3'4'-catechol structure, which confers higher stability to the radical form and participates in electron delocalization ; (ii) C_2-C_3 double bond in conjugation with a 4-oxo function in the C ring, responsible for electron delocalization of the aromatic nucleus; (iii) 3- and 5-OH groups with 4-oxo function in the A and C rings, required for maximum radical scavenging potential. Moreover, an additional criterion could be added: (iv) In the absence of *o*-dihydroxy structure in the B ring, hydroxyl

substituents in a catechol structure on the A ring are able to compensate and become a larger determinant of flavonoid antiradical activity.

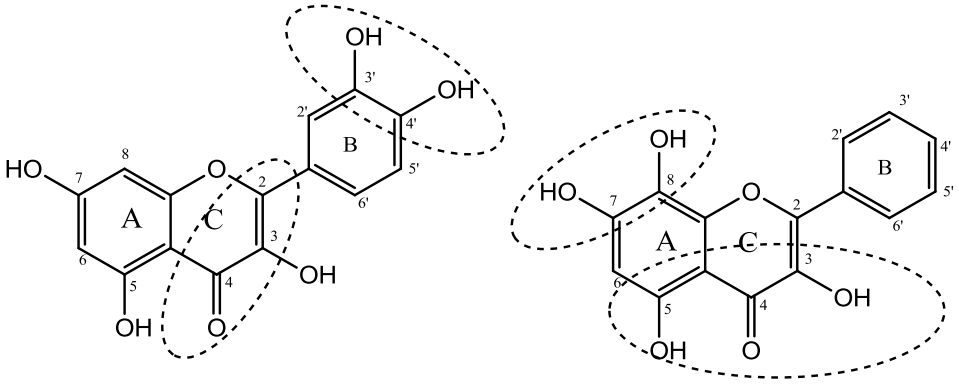


Figure 11.9: Structural features of flavonoids with high antioxidant activity.

Of the three flavonoids, (+)-catechin and hyperin exhibited the most consistent free radical scavenging capability across the five antioxidant assay (Table 11.1). Good correlation was found between FRAP values and hydroxyl groups and especially the catechol moiety and 3-OH [345], explaning the best activity shown by (+)-catechin (FRAP value 75.59±0.42 µg/ml). Results in the ABTS and DPPH assay were relatively similar as they have the same mechanism of action [53]. Sawai *et al.* [346] have identified the antioxidant mechanism using NMR analytical approaches to identify the antioxidative molecular mechanism in DPPH assay and found that the B ring of (+)-catechin has been changed to a quinone structure as shown in Figure 11.10.

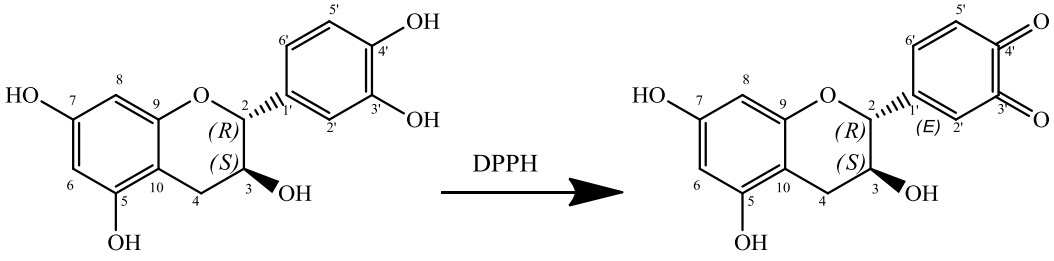


Figure 11.10: (+)-catechin and the corresponding fully oxidized *ortho*-quinone product

Flavanols, namely here (+)-catechin and flavonol (hyperin) are more effective free radical scavengers than flavones (cynaroside) [343], which may be ascribed to the greater number of hydroxyl groups and 3-OH to the former two. Plumb and co-workers [347] reported that the antioxidant properties of flavonol glycosides from tea decreased as the number of glycosidic moieties increased. In addition, glycosylation of flavonoids mostly decreases their antioxidant activity. Multiple A-ring methoxy groups as seen in cynaroside also reverse the positive effect of a B-ring catechol [344].

However in the β-carotene bleaching assay, which involves lipid peroxidation via hydrogen atom transfer (HAT method- section 4.5), hyperin and cynaroside outweighed activity of (+)-catechin. Flavonoids with a C_2–C_3 double bond in conjugation with a 4-carbonyl group has been known to exhibit lower EC_{50} values (indicative of stronger antioxidant activity) in a microsomal system compared to those with saturated heterocycles [344] explaining the lower EC_{50} values of cynaroside (56.38±0.38 µg/ml) and hyperin (32.21±0.35 µg/ml) in the β-carotene bleaching assay. However, hyperin has a lower EC_{50} than cynaroside in this assay as the C-3

position is an excellent choice for substitution to give the flavonoid an optimum lipophilicity [348].

Between both glycosides, hyperin was superior to cynaroside in all five assays (Table 11.1) as C-glycosylation in the A-ring (seen in A ring of cynaroside) results in greater diminution of activity than 3-glycosylation (seen in hyperin) in the heterocycle [349]. Studies have also shown that *O*-glycosylation at carbon 7, but not carbon 3, weakens the antioxidant effect of flavonoids in rat mitochondria [349]. *O*-glycosylation interferes with the coplanarity of the B-ring with the rest of the flavonoid and the ability to delocalize electrons [350]. Though glycosides are usually weaker antioxidants than aglycones, bioavailability is sometimes enhanced by a glucose moiety [349]. The SOD assay involves flavonoids having SOD-like activity and inhibition of XOD that produces the free radicals. Hyperin and (+)-catechin have significantly lower IC_{50} (0.75±0.03 µg/ml and 0.94±0.27 µg/ml respectively) compared to SOD enzyme. This is due to the structure arrangement of hyperin as the planar flavonoid core (double bond between C-2 and C-3) and the presence of free hydroxyl groups at C-5 and C-7 are important prerequisites for this activity [344]. (+)-catechin has slightly less activity compared to hyperin as it lacks the double bond between C-2 and C-3).

Phenolic acids have been investigated for the radical scavenging activity via ABTS assay by Rice- Evans [351]. Structure-activity comparisons suggest that antioxidant activity of phenolic acids depends on the number and orientation of hydroxyl groups relative to the electron-withdrawing CO_2H, CH_2CO_2H, or $(CH)_2CO_2CH$ functional

group [39]. It can be seen that syringic acid has more electron withdrawing groups compared to lioxin confirming the lower EC_{50} in ABTS assay (7.38±0.02 µg/ml).

Between both coumarins, scopoletin exhibited potent antioxidant activity compared to scoparone. It had significantly lower EC_{50} values in ABTS (EC_{50} 1.08±0.03 µg/ml) compared to ascorbic acid (EC_{50} 1.57±0.06 µg/ml) and lower values in FRAP assay (FRAP value 49.00±0.64 µg/ml) than quercetin (FRAP value 88.16±0.24 µg/ml) and ascorbic acid (FRAP value 345.00±0.23 µg/ml). The result is consistent with a previous report that the scavenging capacity was higher for coumarin bearing OH groups than the methoxy-substituted derivatives [352] as in scoparone. In the SOD assay, the SOD-like activity and inhibition of XOD that produces the free radicals seemed to be better with scoparone (IC_{50} 48.72±0.02 µg/ml) than scopoletin (IC_{50} >100 µg/ml). Reports have stated that a methoxy substituted for 6- OH diminishes inhibition potency against XOD [352] as in the result of both coumarins here. This further enhances the hypothesis that the H atom of the 6-hydroxyl plays a more significant role than the O atom. It also can be observed that perhaps the 7-methoxy group in scoparone increases the inhibition activity.

Not much biological data is available on vomifoliol except weak DPPH activity and anti-inflammatory activity by inhibition of TNF-α [341]. However in this study, though consistent low DPPH activity is seen (EC_{50} >100 µg/ml), moderate activity in noted in ABTS (EC_{50} 34.89±0.04 µg/ml) and potent β-carotene bleaching activity was noted (EC_{50} 6.85±0.37 µg/ml).

11.4.2 Anti-inflammatory activity

All isolated compounds exhibited significantly lower 5-LOX inhibition than positive NDGA (IC_{50} 29.21±0.05 µg/ml). Among the five phenolics, syringic acid and hyperin had the most potent 5-LOX inhibitory activity (IC_{50} 1.38±0.03 µg/ml and 3.02±0.02 µg/ml respectively). Hydroxylations at 5, 7, and 4' have also shown to enhance overall anti-inflammatory activity in flavonoids [102]. Flavonols were found to be stronger inhibitors of 5-LOX than flavones [353], as established in this study with hyperin (IC_{50} 3.02±0.02 µg/ml) being superior to cynaroside (IC_{50} 18.12±0.04 µg/ml). Although it is difficult to establish structural-activity relationships due to their varieties of chemical structures, these inhibitory activities against 5-LOX could explain, at least in part, the anti-inflammatory/ antiallergic activities of flavonoids. No clinical data showing the relation of flavonoid intake and incidence (severity) of inflammatory disorders such as RA and AD was available, although several studies demonstrated some inverse correlation of flavonoid intake and incident rate of cardiovascular failure [354].

Lipoxygenases (LOXs) also function as prooxidant enzymes that catalyze enzymatic lipid peroxidation. Additionally, they are a source of free radicals initiating nonenzymatic lipid peroxidation and other oxidative processes involved in the formation of atherosclerotic lesions [354]. Consequently, inhibition of LOXs may contribute to the universal antioxidant activities of flavonoids. Planar flavonoid structure with delocalized electrons achieved by the C_2-C_3 double bond seems to be a prerequisite for inhibitory activity. The following structural features were found to enhance the inhibitory potency: (i) presence of a catechol moiety in the B or A ring,

(ii) 4-carbonyl group in the C ring [344], thus confirming the results of this study with hyperin having more 5-LOX inhibition than (+)-catechin.

However (+)-catechin had a more comprehensive anti-inflammatory activity with dual inhibition of 5-LOX (IC_{50} 16.10±0.03 µg/ml) and COX (COX-1; IC_{50} 12.08±0.02 µg/ml, COX-2; IC_{50} 83.89±0.03 µg/ml). (+)-catechin displayed COX-1 selective activity with the COX-1/COX-2 ratio <1 and this confirms previous findings [107]. Benzoic acid derivatives, syringic acid exhibited potent 5-LOX inhibition (IC_{50} 1.38±0.03 µg/ml) and moderate COX-1 inhibition (IC_{50} 34.89±0.02 µg/ml) compared to lioxin that had good 5-LOX inhibition (IC_{50} 17.79±0.03 µg/ml) but no COX inhibition. Consideration of the structure-activity relationship of phenolic acids on the anti-inflammatory action revealed that both C-4 hydroxy and C-3 methoxy radicals of the benzoic acid derivatives play an important role in anti-inflammatory activities [355]. However the presence of extra methoxy group at C-5 seemed to drastically increase the activity as seen in syringic acid. One study reported potent COX-2 inhibition of syringic acid (IC_{50} 0.40 µg/ml) using cell based study [356] but we demonstrated poor COX-2 inhibition in this study.

Both coumarins scopoletin and scoparone were potent inhibitors of 5-LOX recording lowest IC_{50} values (IC_{50} 0.34±0.01 µg/ml and 0.20±0.01 µg/ml respectively). Scoparone has slightly lower IC_{50} than scopoletin as the SAR findings has reported that the chloro and methoxy substitution in coumarin ring showed increase anti 5-LOX activity [357]. Cyclohexenone derivatives are well known lead molecules for the treatment of inflammation and autoimmune diseases [358]. The anti-inflamatory activity previously reported for vomifoliol were by inhibition of TNF-α [341]. In this

study, vomifoliol has exhibited potent 5-LOX inhibition (IC50 8.72±0.02 µg/ml) and poor COX inhibition.

11.4.3 Anti-acetylcholinesterase activity

Only the scopoletin, scoparone, vomifoliol and syringic acid showed AChE inhibition at IC_{50} <100 µg/ml. The studies have also shown that naturally occurring as well as the chemically synthesised coumarin analogs exhibit potent AChE inhibitory activity [359]. Coumarin ring seems to be essential for the optimal activity and its replacement with related structural moiety such as chromone is associated with loss of AChE inhibitory activity. The substituents at coumarin moiety particularly at 6th and 7th position also influence the activity in a significant manner. The presence of electron-donating groups such as –OCH3, – OH, and –NH2 increase the activity and it has been generally attributed to increase in lipophilicity of compounds [360]. This might explain good activity of scopoletin (IC_{50} 51.00±0.02 µg/ml) and scoparone (IC_{50} 86.58±0.05 µg/ml) as both have electron donating groups at position 6th and 7th. The presence of bulkier substituents at 6th and 7th positions of the coumarin is associated with significant loss in AChE inhibitory activity indicating the critical role of electronic as well as steric effects in influencing the AChE inhibitory activity [360], this explains why scoparone has higher IC_{50} due to it having bulkier two methoxy groups.

Up to date, quite a lot of studies have reported affirmative effects of phenolics in neurodegenerative diseases depending upon their antioxidative properties [361]. However, there has been a small number of data on AChE inhibitory activities of

phenolic compounds. Among the phenolics isolated in this study only syringic acid exhibited good AChE inhibition (IC_{50} 29.53±0.19 µg/ml), lowest of all compounds tested. The literature concerning the role of phenolic acids and their derivatives in the neuroprotection of the CNS is, however, still incomplete. Thus the actual mechanism of inhibition needs to be investigated. Cyclohexenone derivatives, vomifoliol had moderate AChE inhibition (IC_{50} 96.64±0.09 µg/ml), which is to the best of our knowledge the first to be reported.

11.4.4 Antiparasitic activity

Only scopoletin exhibited moderate antileishmanial activity (IC_{50} 163.30±0.32 µg/ml) followed by lioxin, vomifoliol, scoparone and (+)-catechin. Poor activity of scopoletin was noted against *Giardia intestinalis* and *Entamoeba histolytica* while the others were not tested against them. Comparing the anti AChE and antiparasitic activity, it can be deduced that same compounds seemed active in both assays. Choline is the precursor of phosphatidylcholine (PC), a main component of *Leishmania* promastigote membranes [362]. Therefore, inhibition of choline formation may decrease *Leishmania* survival. This hypothesis can be tested by using inhibitors of the acetylcholinesterase enzyme (AChE), which catalyzes the hydrolysis of acetylcholine to choline and acetic acid, as leishmanicidal compounds. This may identify another mechanism of action for leishmanicidal activity [363].

The lactone groups present in coumarins are also present in the structures of *Annonaceous* acetogenins that show leishmanicidal activity [364]. The AChE inhibitory activity of a previous study on scoparone indicates a possible action mechanism

by disrupting the viability of leishmania's cell membranes. There has been a hypothesis that the coumarin and scoparone have mechanism of action acting on the same pathway as above compounds, resulting in a net negative effects on choline uptake by the parasite [363]. Only flavonoid (+)-catechin was tested in this study. Flavonoids however, are able to inhibit acetylcholine hydrolysis and interfere in the PC due to their low concentration of choline precursor from the host. Nevertheless, the leishmanicidal activity of these compounds may be related to their ability to chelate iron (Fe), depriving this essential nutrient from the intracellular forms [365].

11.4.5 Anticancer activity

In this study, scopoletin displayed potent anticancer effect against breast cancer cell line MDA 468 (GI_{50} 0.09±0.25 µg/ml) and colorectal cancer cell line HT-29 (GI_{50} 0.17±0.05 µg/ml), the latter being more significant that positive control doxorubicin (GI_{50} 0.69±0.06 µg/ml). Previously studies on scopoletin have shown it to inhibit the cell proliferation by inducing cell cycle arrest and the increase of apoptosis in human prostate tumor cells [366], and it induced apoptosis of HL-60 cells [367]. The ability of scopoletin have been said to induce programmed cell death in leukemic cells representing a novel and important aspect of the discussion of the anti-tumoral effects of various plants containing scopoletin [367]. In this study, scopoletin has shown potent growth inhibition of these two cell lines warranting further study.

11.4.6 Antibacterial activity

All isolated compounds tested against *Staphylococcus aureus* ATCC 11632 showed bacterial growth inhibition. Scopoletin, scoparone, hyperin, cynaroside and syringic acid had bactericidal effect <100 µg/ml. Only scopoletin had bactericidal effect and complete kill at MBC 50.00 µg/ml. The antibacterial properties of coumarins were first recognised in 1945 when an investigation with dicoumarol was found to inhibit the growth of several strains of bacteria. All the methods reported in the study of the antibacterial activity of coumarins were disc diffusion methods [368]. It has then been suggested that coumarins possess antibacterial activity act selectively against Gram-positive microorganisms [368]. Free hydroxyl group at position 7 has been shown to be important for antibacterial activity [298] as confirming the activity of scopoletin that has free 7-OH.

11.4.7 Formulation of scopoletin as a gel

Previously study by Seon *et al*. [369] have demonstrated scopoletin to inhibit tyrosinase kinase with an IC_{50} of 10.2 µg/ml suggecting its possible use in skin aging and preventive activities. Biosynthesis of melanin in animals is normally initiated through the oxidation of tyrosine into DOPA by tyrosinase which is the rate limiting enzyme in this pathway. The production of abnormal pigmentation such as melasma, freckles, senile lentigines, and other forms of melanin hyperpigmentation could be a serious aesthetic problem [369]. In this study scopoletin was found to exhibit potent antioxidant, anti-inflammatory and antibacterial properties. Scopoletin has been studied to have good pharmacokinetics data with good absorption in the stomach and

colon of rats [370]. Reported physical properties of scopoletin which passes the Lipinsky rule (mass < 500, log*P* < 5, donor count < 5, and acceptor count < 10), for possible lead compound in drug discovery [371] and, in agreement with its potent antioxidant power, good anti-inflammatory and antibacterial activity enables it to be considered for a formulation for further test and development.

Transdermal drug delivery offers the following advantages over oral administration: (1) peak and valley levels in the serum are avoided; (2) first-pass metabolism is avoided and the skin metabolism is relatively low; (3) less frequent dosing regimens is needed due to the maintenance and longer sustainability of zero-order drug delivery and (4) less inter-subject variability occurs [372]. Other advantages listed include aspects such as the accessibility of the skin; a relatively large surface area for absorption and the fact that it is non-invasive make it more patient compliant [373]. Thus, an external formulation suitable for cosmetic use (skin hyperpigmentation, acne and wrinkle protection) and for medicinal use as an anti-inflammatory gel was derived. The formulation was prepared with aloe vera gel as a base due to the well known skin and medicinal properties of aloe [374]. Orange oil was used as studies have shown the limonene (moneterpene) content has a high ability to enhance *in vitro* percutaneous transport greater than conventional lipophilic penetration enhancing compounds (e.g., Span® 20, α-bisabolol, oleic acid) and hydrophilic penetration enhancing compounds (e.g., ethanol, polyethylene glycol 600, 1,8-cineole) [375]. Further studies on the efficacy of the gel and penetration test are required.

11.5 Conclusion

For the first time, eight compounds (scopoletin, scoparone, (+)-catechin, lioxin, cynaroside, hyperin, vomifoliol and syringic acid) were isolated from *Canarium patentinervium* Miq. Three of these compounds (vomifoliol, lioxin and syringic acid) were isolated for the first time from the genus *Canarium*. These compounds were then subjected to a range of biological assays, all reported for the first time from this plant.

Canarium patentinervium Miq. is a rare plant from the family of Burseraceae and genus *Canarium* found in Asia Pacific region previously recorded for its usage in wound healing by the indigenous people of Malaysia. One of the objectives of this study was to ascertain the pharmacological and phytochemical aspect for the ethnopharmacological usage. Wound healing involves manifold inflammatory processes of which notably the massive release of leukotrienes from arachidonic acid via the 5-lipoxygenase pathway (5-LOX) and the generation of nitric oxide (NO) from inducible nitric oxide synthase (iNOS). Of note, nitric oxide (NO) is a free radical, and the generation of cytokines involves a reactive oxygen species (ROS) outburst. Therefore, agents able to block the enzymatic activity of 5-LOX and to scavenge free radical are of immense interest against inflammatory conditions which englobe not only epidermal insults but also neurodegeneration and obesity.

Cells produce superoxide anion (O_2^-), peroxide anion (HO_2^-), and hydroxyl ion (HO^-) as part of the physiological aerobic metabolism which are quickly scavenged by cytoplasmic antioxidant defense system. However, in the event of ageing or pathologies, the antioxidant defense system is overwhelmed, and cells suffer massive

oxidative stress leading eventually to carcinogenesis or apoptosis. In fact, oxidative stress is the main causative factor for cholinergic and dopaminergic neurons apoptosis hence AD and Parkinson's disease (PD). In addition, there is a growing body of evidence that point to the fact that 5-LOX inhibitors are of immense therapeutic values. The various isolated secondary metabolites clearly demonstrate various potent biological activities that substantiate the ethnopharmacological claim.

Given the aforementioned evidence it is tempting to speculate that *Canarium patentinervium* Miq. represents an exciting scaffold from which to develop leads for treatment of neurodegenerative diseases.

CHAPTER 12

GENERAL CONCLUSIONS

The objectives of the study were to:

i. Extraction of leaves and barks of plant with solvents of different polarity to obtain crude extract with solvents of different polarity.

ii. Investigation of the possible secondary metabolite types present in the crude extracts of leaves and barks with qualitative phytochemical analysis.

iii. Isolation and identification of the chemical compounds responsible for the antibacterial and antioxidant activity in the most active extract through bioassay-guided fractionation using liquid-liquid extraction or solvent partioning, column chromatography, thin layer chromatography (TLC) and nuclear magnetic resonance (NMR).

iv. Investigation of the antioxidant activity of the crude extracts of both leaves and barks using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, ferric reducing ability of plasma (FRAP) assay, 2,2'-Azino-*bis*(3-ethyl-14 benzthiazoline-6-sulfonic acid) (ABTS)assay, β-carotene bleaching assay and superoxide dismutase assay.

v. Investigation of the anti-inflammatory potential of the crude extracts against 5-lipoxygenase and cycloxygenase systems *in vitro.*

vi. Investigation of the anti AChE potential of the crude extracts *in vitro.*

vii. Investigation of the *in vitro* antibacterial activity of the crude extracts of leaves and barks using the disc diffusion assay and determination of minimum inhibitory concentration, minimum bactericidal concentration and death kill rate against four ATCC bacterial strains namely Gram-positive bacteria *Staphylococcus aureus*, *Bacillus cereus*, methicillin-resistant *Staphylococcus aureus* and Gram-negative *Pseudomonas aeruginosa* and ten clinical strain bacteria.

viii. Investigation of the *in vitro* antifungal activity of the crude extracts of leaves and barks using the disc diffusion assay against three clinical strain fungi namely, *Candida parapsilosis, Candida glabrata* and *Candida albicans.*

ix. Investigation of the anticancer activity of the crude extracts of the leaves and barks against human derived cell lines [HCT 116, colon cancer cell line and MCF-7 (ER+), MDA 468 (ER-) breast carcinoma] using MTT assay.

x. Investigation of the *in vitro* anti-parasitic activity of the crude extracts of leaves and barks against *Leishmania. donovani* promastigotes, *Entamoeba histolytica* and *Giardia intestinalis.*

xi. Bioactivity screening of all isolated compounds using the same bioassay as mentioned in iii-ix.

The following appropriate conclusions were drawn 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 leaves were 1.25 %, 1.11 % and 6.45 % respectively. The yield for the hexane, chloroform and ethanol extract of barks was 1.04 %, 0.4 % and 2.61 %. Crude extracts were kept at -20^{o}C until further use.

ii. Phytochemical analysis

Phytochemical analysis of *Canarium patentinervium* Miq. revealed presence of tannins and flavonoids in the ethanol extract of leaves and barks. Steroids were detected in the ethanol extract of leaves and all tested bark extracts. Results from our phytochemical analysis revealed that the ethanol extract of leaves and barks of *Canarium patentinervium* Miq. accumulate substantial amounts of flavonoids and tannins which could be possibly result in antioxidant or antibacterial activity.

iii. Isolation of biologically active compounds

Eight compounds were isolated for the first time from *Canarium patentinervium* Miq. using various isolation techniques such as TLC, CC, HPLC and identified with NMR method. Compounds isolated scopoletin,

scoparone, (+)-catechin, hyperin, cynaroside, lioxin and syringic acid were phenolics while vomifoliol was a norsesquiterpene with a cyclohexenone ring. Lioxin, syringic acid and vomifoliol were isolated from this genus *Canarium* for the first time.

iv. Antioxidant activity

The ethanol extract of leaves and barks displayed superior antioxidant capacities. The EC_{50} values of the samples were consistently low in SET methods (ABTS, DPPH and FRAP assay) superior to standard as opposed to HAT method (β-carotene bleaching assay). The present study demonstrates that *Canarium patentinervium* Miq. is a potent source of antioxidants that exhibits its antioxidant activity predominantly via the SET method.

Compounds (+)-catechin and hyperin exhibited the most consistent free radical scavenging capability across the five antioxidant assay. Flavanols, namely here (+)-catechin and flavonol (hyperin) are more effective free radical scavengers than flavones (cynaroside). Hyperin and (+)-catechin have significantly lower IC_{50} (0.75±0.03 µg/ml and 0.94±0.27 µg/ml respectively) compared to SOD enzyme. Between both coumarins, scopoletin exhibited potent antioxidant activity compared to scoparone. It had significantly lower IC_{50} values in ABTS (IC_{50} 1.08±0.03 µg/ml) compared to ascorbic acid (IC_{50} 1.54±0.03 µg/ml) and lower values in FRAP assay (IC_{50} 49.00±0.64 µg/ml) than quercetin (IC_{50} 86.00±0.24 µg/ml) and ascorbic acid (IC_{50} 347.00±0.23 µg/ml). Vomifoliol displayed

low DPPH activity (IC_{50} >100 µg/ml), moderate activity is noted in ABTS (IC_{50} 34.89±0.04 µg/ml) and potent β-carotene bleaching activity was noted (IC_{50} 6.85±0.37 µg/ml).

v. Anti-inflammatory activity

In view of dual inhibition of 5-LOX and COX activity, the ethanol extract of leaves seemed to have moderate inhibition against 5-LOX and good inhibition against COX enzymes (selective COX-2). Chloroform extract of the bark though had good 5-LOX inhibition but did not have activity against COX enzymes below 100 µg/ml.

In the 5-LOX inhibition assay, all isolated compounds displayed significantly lower IC_{50} compared to positive control NDGA. Both scoparone and scopoletin (IC_{50} 0.20±0.01 µg/ml and 0.34±0.01 µg/ml respectively) had the most potent 5-LOX enzyme inhibition (Figure 11.2) which was more than eightyfold compared to NDGA (IC_{50} 29.19±0.02 µg/ml). This was followed by syringic acid (IC_{50} 1.38±0.03 µg/ml), hyperin (IC_{50} 3.02±0.02 µg/ml), vomifoliol (IC_{50} 8.72±0.02 µg/ml), (+)-catechin (IC_{50} 16.10±0.03 µg/ml), lioxin (IC_{50} 17.79±0.03 µg/ml) and cynaroside (IC_{50} 18.12±0.04 µg/ml).

vi. Anti AChE activity

Chloroform extract of the barks displayed the best activity (IC_{50} = 88.59±0.14 µg/ml) as opposed to galanthamine (IC_{50} = 0.74±0.06 µg/ml).

The ethanol extract of barks and leaves follow through with IC_{50} = 186.00±0.15 µg/ml and IC_{50} = 201.24±0.15 µg/ml respectively). Hexane extracts of bark and leaves and the chloroform extract of leaves had the lowest enzyme inhibition activity (IC_{50} = 570.00±0.08 µg/ml, IC50 = 842.00±0.25 µg/ml and IC_{50} = 1780.00±0.24 µg/ml respectively). The potency of activity against AChE was BC > BE > LE > BH >LH > LC.

Only 4 compounds showed good to moderate enzyme inhibition namely syringic acid (IC_{50} 29.53±0.19 µg/ml), scopoletin (IC_{50} 51.00±0.02 µg/ml), scoparone (IC_{50} 86.58±0.05 µg/ml) and vomifoliol (IC_{50} 96.64±0.09 µg/ml). Rest of the compounds showed poor enzyme inhibition with IC_{50} values >100 µg/ml.

vii. Antimicrobial activity

All crude extracts of *Canarium patentinervium* Miq. under investigation exhibited exceptional concentration-dependent antimicrobial activity against both Gram-positive and Gram-negative bacteria. The most promising activity was displayed against Gram-positive bacteria *Staphylococcus aureus*, MRSA, *Bacillus cereus* and Gram-negative bacteria and *Pseudomonas aeruginosa, Klebsiella* sp, *Klebsiella pneumoniae* ESBL and yeast *Candida parapsilopsis*. The antimicrobial activity exhibited by the bark extracts and ethanol extract of leaves against MSSA and MRSA indicates exceptional antimicrobial activity. All the tested bacterias were susceptible to either bark or leaf extracts.

All isolated compounds tested against *Staphylococcus aureus* ATCC 11632 showed bacterial growth inhibition. Scopoletin, scoparone, hyperin, cynaroside and syringic acid had bactericidal effect <100 µg/ml. Only scopoletin had bactericidal effect and complete kill at MBC 50.00 µg/ml.

viii. Antifungal activity

The most promising activity was displayed against yeast *Candida parasilopsis.* The ethanol extracts and hexane extract of barks also showed inhibition against *Candida parasilopsis* with a cidal and static action respectively.

ix. Anticancer activity

The chloroform and ethanol extract of barks showed good antitumor activities with GI_{50} values of 23.44±0.05 µg/ml and 34.40±0.21 µg/ml. The most susceptible cell lines were found to be the breast cancer cell line, MDA 468.

Scopoletin displayed potent anticancer effect against breast cancer cell line MDA 468 (GI_{50} 0.09±0.25 µg/ml) and colorectal cancer cell line HT-29 (GI_{50} 0.17±0.05 µg/ml), the latter being more significant that positive control doxorubicin (GI_{50} 0.66±0.60 µg/ml).

x. Anti parasitic activity

The hexane extract of leaves showed moderate antileishmanial activity with IC_{50} values of 257.40±0.30 µg/ml. This could be due to essential oils

present in the hexane extracts as shown previously in the family of Burseraceae [216]. Only ethanol extracts showed activity against *Giardia intestinalis* and *Entamoeba histolytica* at concentration of 500 µg/ml. Other extracts were inactive against these two intestinal parasites.

Scopoletin was tested against all three parasite amd it was more potent against *Leishmania donovani* (IC_{50} 163.30±0.32 µg/ml) and MIC of >200 µg/ml for both *Giardia intestinalis* and *Entamoeba histolytica*. Sensitivity of *Leishmania donovani* is then followed by with the presence of lioxin (IC_{50} 211.48±0.32 µg/ml), vomifoliol (IC_{50} 302.80±0.33 µg/ml), scoparone (IC_{50} 329.90±0.32 µg/ml) and (+)-catechin (IC_{50} 478.93±0.28 µg/ml).

In Chinese folk medicine, *Canarium album* (Lour.) Raeusch has been used for treatment of various diseases ranging from cardiovascular, gastrointestinal and toxicities while *Canarium schweinfurthii* Engl. has been used by traditional healers as a remedy for diabetes mellitus in southern Senegal while in Congo and Central African Republic the plant is used in fever, as stimulant, emollient, in post-partum pain, constipation, malaria, diarrhoea, sexual infections and rheumatism (Chapter 1) to name a few. The fact only 12 % of 75 species of *Canarium* have been studied for their pharmacological activities promises an unopened crypt of various secondary metabolites as lead compounds and biological effects that needs to be uncovered and investigated. Thus, by using an ethnopharmacological approach, the investigation of *Canarium patentinervium* Miq. a plant from Malaysia that has been used by the orang asli (indigenous people) for healing wounds was done. Crude extracts and isolated

secondary metabolites were investigated for their various activities.. Three out of eight compounds were isolated for the first time from this genus of 75 species (*Canarium*). Six kinds of major biological effects were evident in the crude and compounds namely, antioxidant, antibacterial, anti-inflammatory, anti AChE, anti parasitic, and anticancer all of which were reported for the first time from this plant. This study is therefore a small leap in the field of an important endeavor for the discovery of potent biologically active molecules for the treatment of various diseases. This discovery also opens forth a pathway where biologically active molecules isolated from biosynthesis of plants can now pose as lead molecules in the field of synthetic chemistry for drug discovery.

As modern cultures and scientific advances spread around the world, the depth of the knowledge store of traditional use still remains crucial. The full significance of the indigenous knowledge forfeited may not be realised. It is thus important that the knowledge be documented and the traditional use be given some credence through modern scientific studies. *Canarium patentinervium* Miq. is such an example.

CHAPTER 13

RECOMMENDATIONS FOR FUTURE WORK

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

i**. Antioxidant activity**

The isolated compounds should be tested for synergistic or antagonistic effect by the construction of an isobologram.

ii. Antimicrobial activity

• Isolation and structural elucidation of the compound/s from the tannin fraction responsible for the antimicrobial activity of *Canarium patentinervium* Miq. should be investigated.

• Determination of synergistic, antagonistic or additive effects should be investigated.

iii. Anti-inflammatory Activity

• The actual efficacy of an anti-inflammatory activity needs to be validated by testing the isolated bioactive compounds in a cell-based environment (*in vitro*) and in a biological system (*in vivo*).

• The effects of these active compounds on the suppression of the COX-2 gene expression, requires investigation and clarification. The effectiveness of interaction of these extracts and isolated compounds, including the flavonoid derivatives, with other pro-inflammatory biochemical pathways may be assessed, and the possible structure-activity relationships determined. This will provide a better understanding of the possible mode of action.

iv. Anti AChE activity

• More secondary metabolites should be isolated and tested in vitro as it may lead to the development of novel treatments in the global struggle against neurodegenerative diseases.

• All the compounds should also be subjected to cell based study to investigate the mechanism of actions.

v. Anticancer

• This study has, without a doubt, proven the existence of other compound or compounds (besides scopoletin) with potential *in vitro* anticancer activity in different species and extracts *of Canarium patentinervium* Miq. Activity-guided fractionation, isolation and identification of these compounds is imperative and may lead to the development of novel treatments in the global struggle against cancer and cancer-related ailments.

vi. Antiparasitic activity

• More secondary metabolites should be isolated and tested for the efficacy against parasitic kill and it should be tested against various clinical strains of parasites.

vii. Toxicity

• While indigenous *Canarium patentinervium* Miq. has edible fruit, it is important to note that in order to establish a toxicity profile, other cell lines should be investigated such as the liver HepG2 cells, as well as *in vivo* studies.

viii. Phytochemical investigation

• Research on the non-volatile compounds warrant further study. Further analysis must be conducted using different detectors and chromatographic techniques such as liquid chromatography – mass spectrometry, to provide a greater insight of the phytochemical composition of this species.

• Plants produce a wide range of secondary metabolites, which may prove to be invaluable in development of drugs, flavours, fragrances, dyes, anti-oxidants and insecticides. It is thus important to locate and determine the role of these secondary metabolites in plants and unravel their biosynthesis.

Finding new leads for drug development, and determining the biosynthesis of such products, thus requires a different approach which researchers are developing. Metabolomics investigates the end products of cellular functions. The levels of these metabolites are viewed as a response of the biological systems to environmental or genetic manipulation. The use of metabolomics in plant studies will enable the characterisation and differentiation of genotypes and phenotypes based on the levels of metabolites, and also aid in the rapid screening of multiple extracts giving an exceptionally broad overview of the chemistry. This may also provide a means of improving the production of certain metabolites in plants through genetic engineering.

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APPENDIX A: NMR DATA

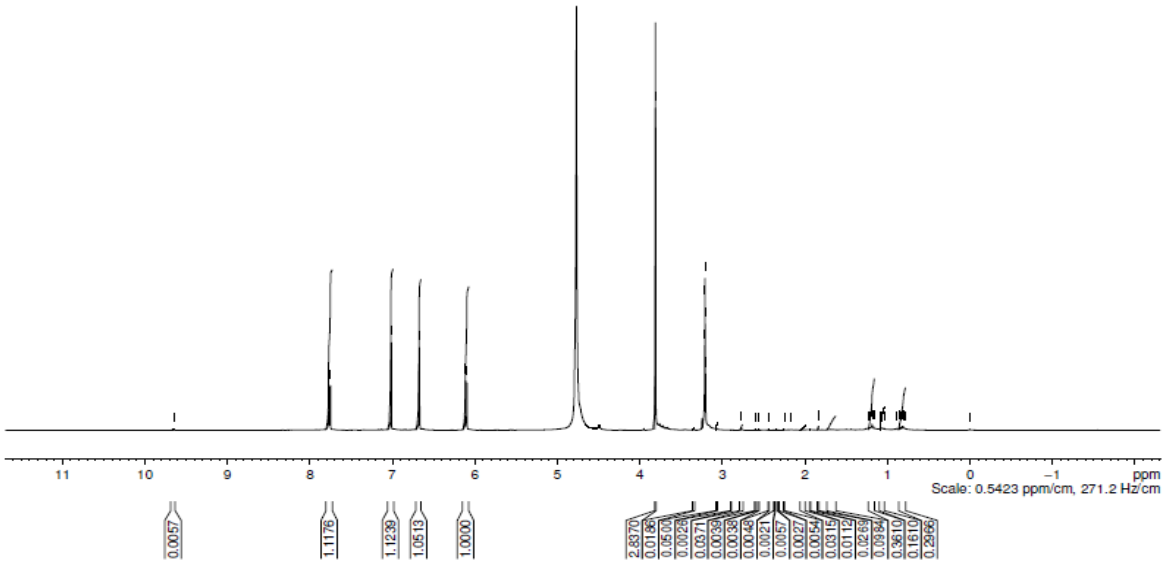


Figure A1: ^{1}H NMR spectrum of compound 1

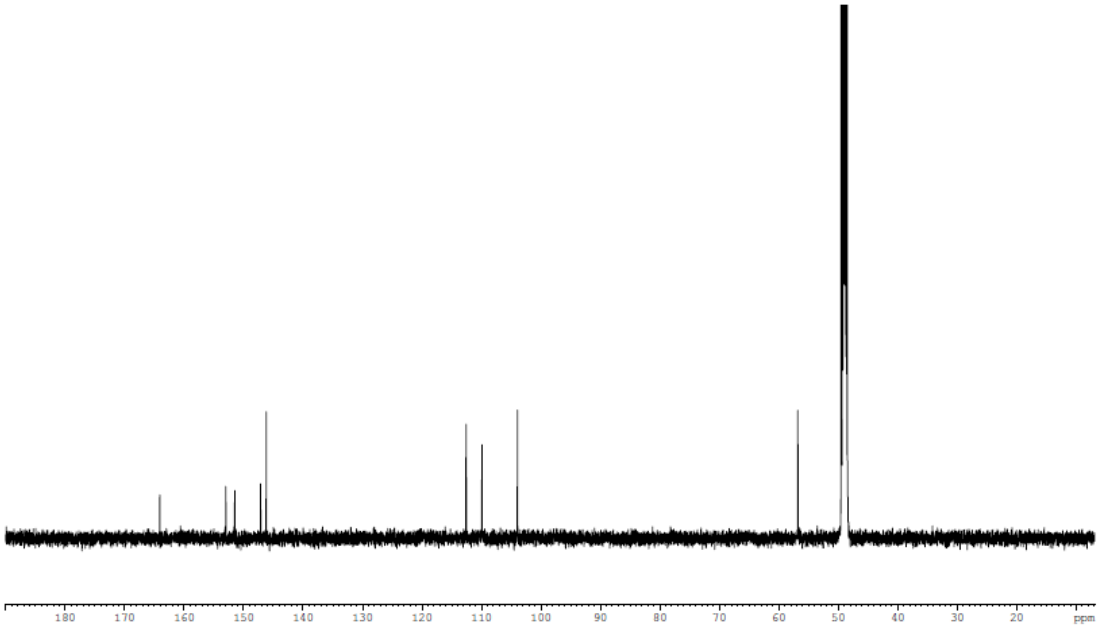


Figure A2: ^{13}C NMR spectrum of compound 1

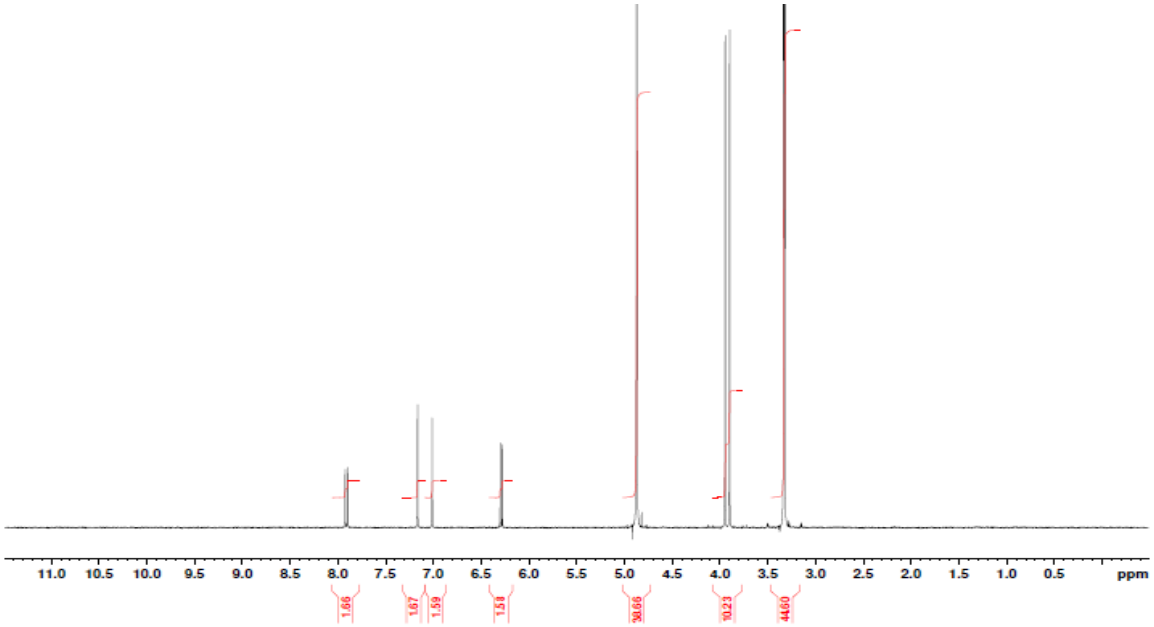
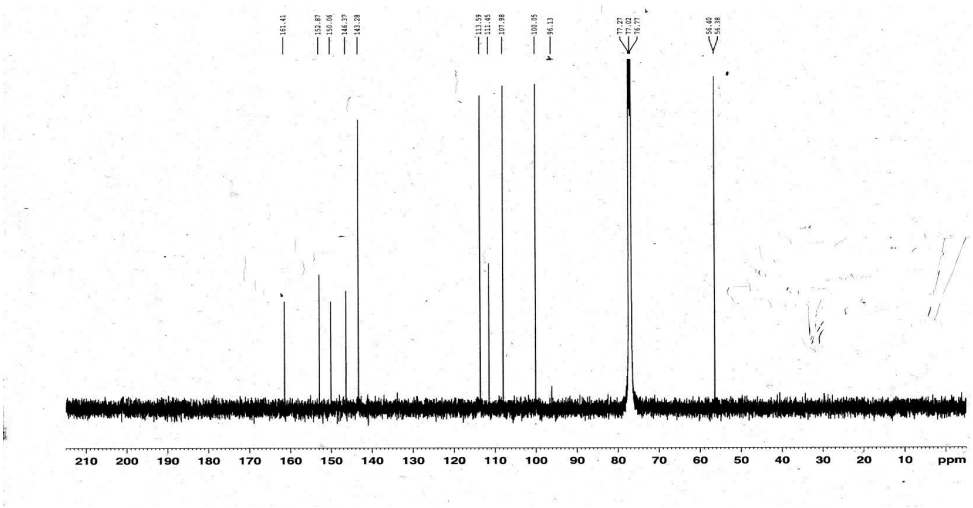


Figure A3: ^{1}H NMR spectrum of compound 2



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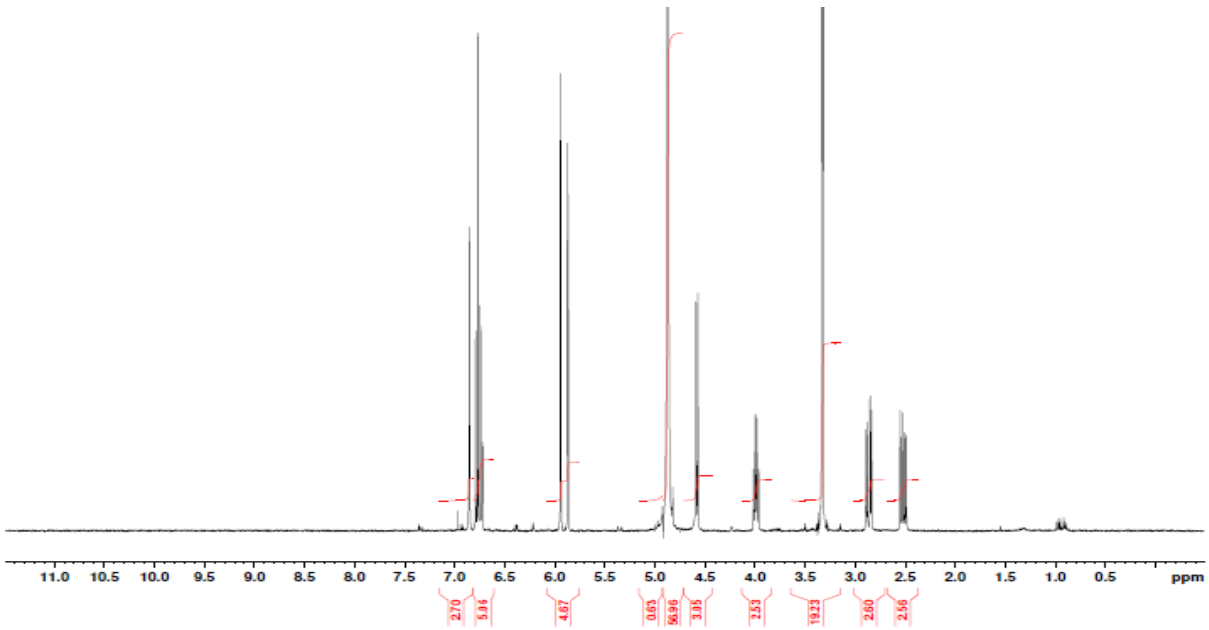


Figure A5: ^{1}H NMR spectrum of compound 3

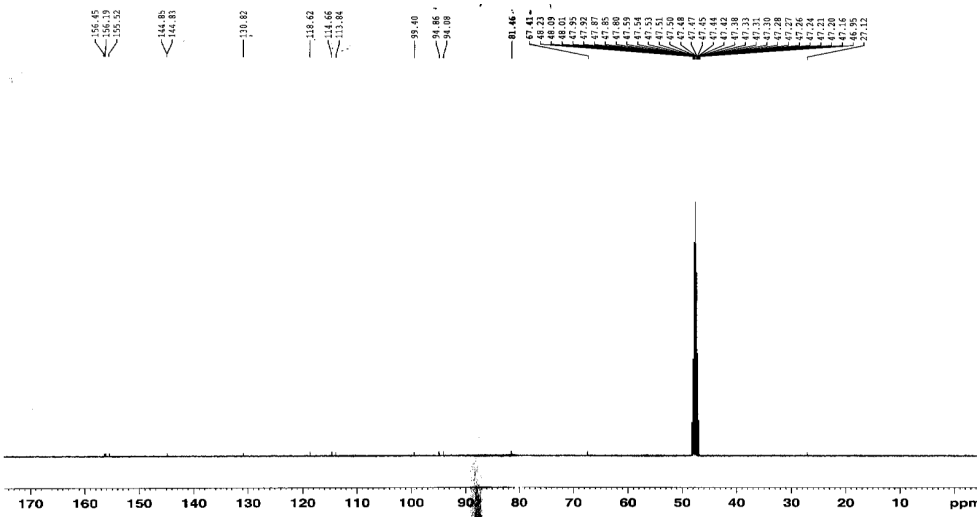


Figure A6: ^{13}C NMR spectrum of compound 3

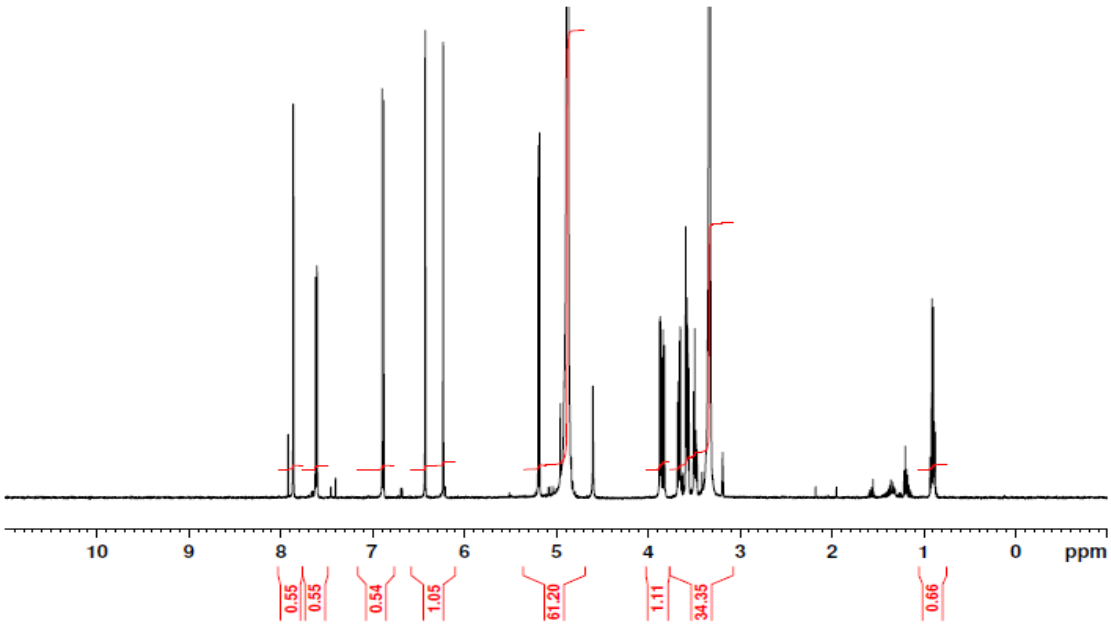


Figure A7: ^{1}H NMR spectrum of compound 4

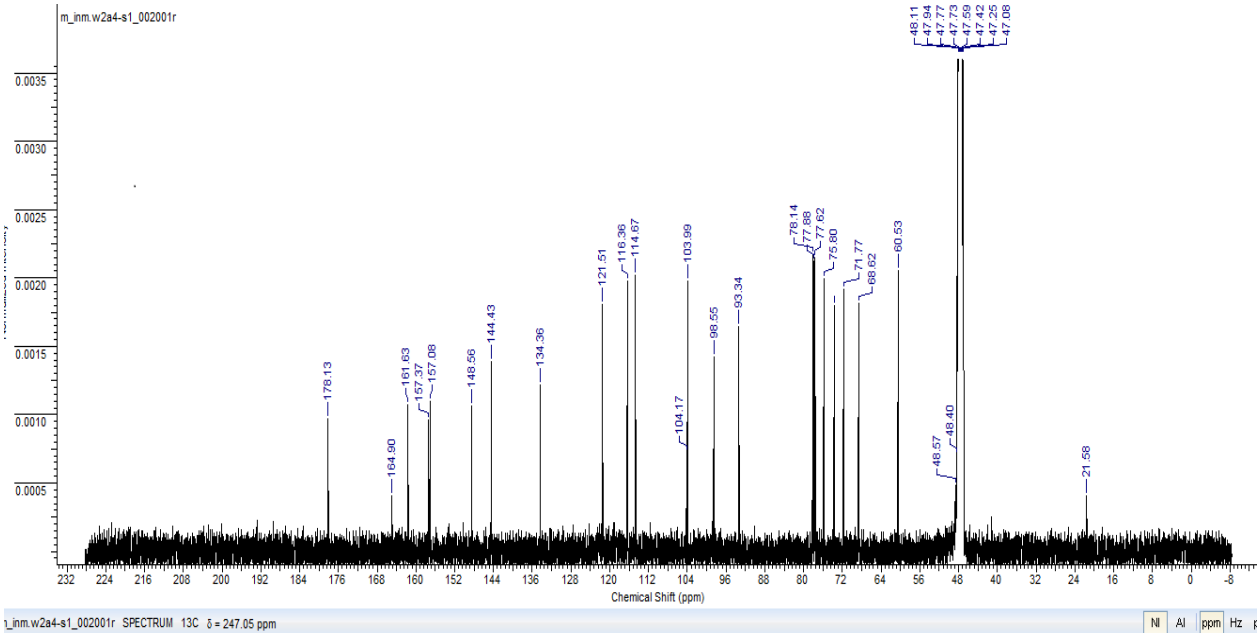


Figure A8: ^{13}C NMR spectrum of compound 4

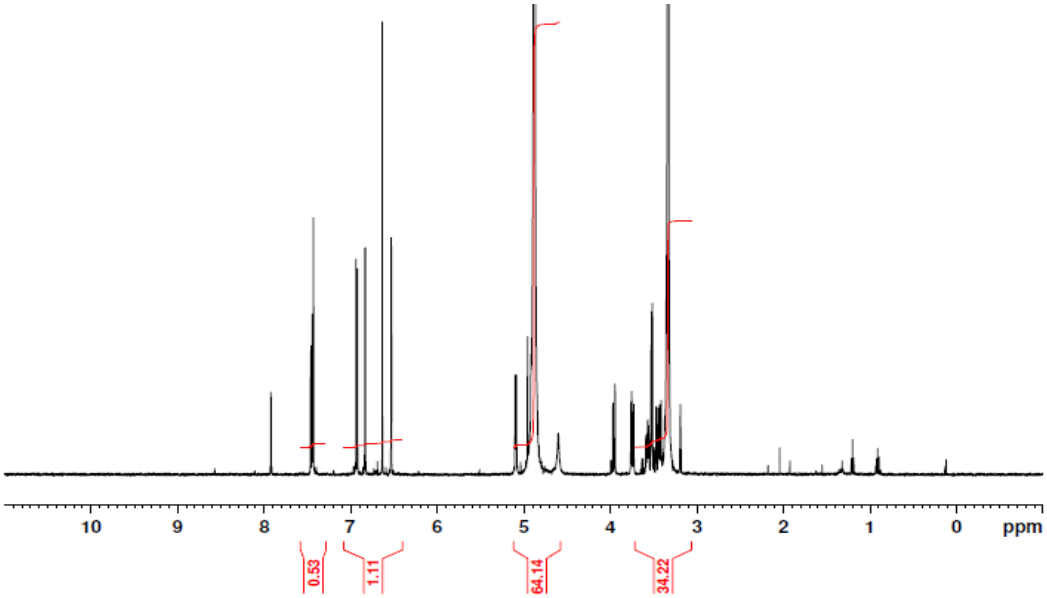


Figure A9: ^{1}H NMR spectrum of compound 5

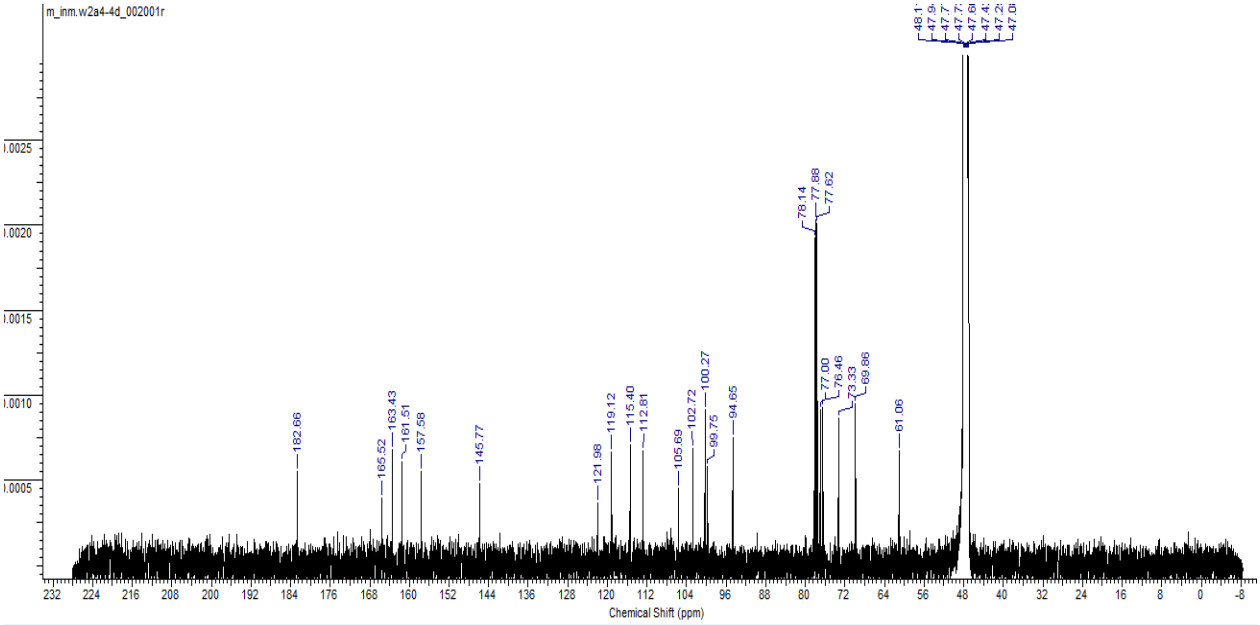


Figure A10: ^{13}C NMR spectrum of compound 5

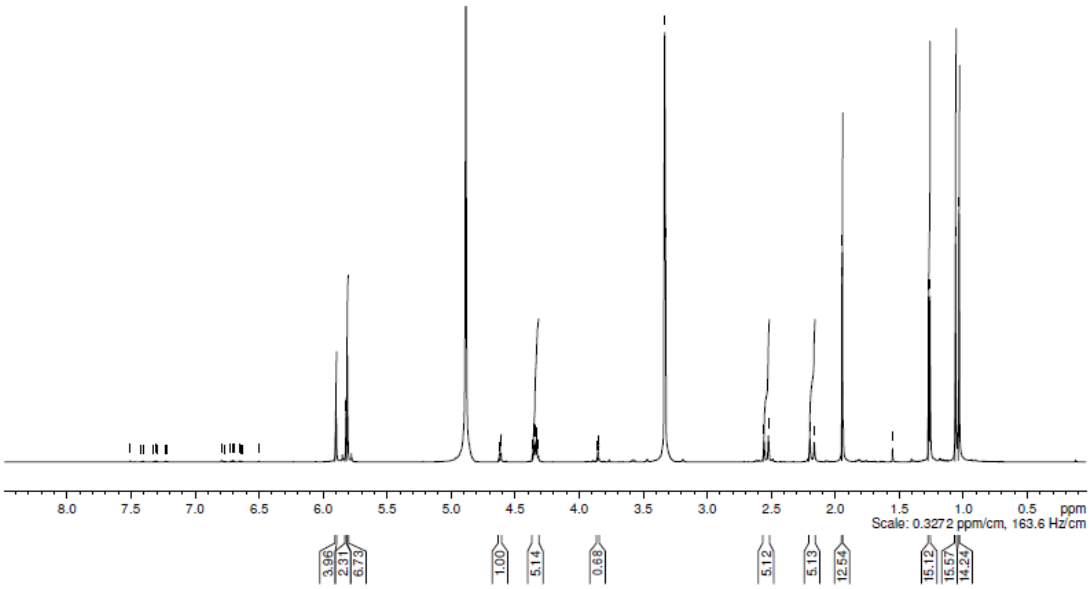
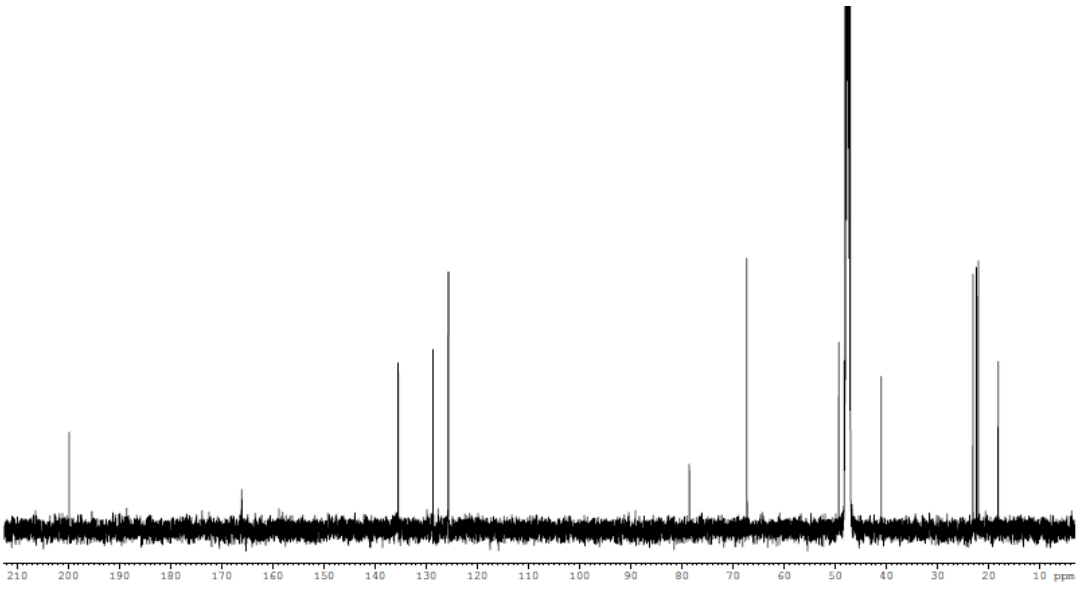


Figure A11: ^{1}H NMR spectrum of compound 6



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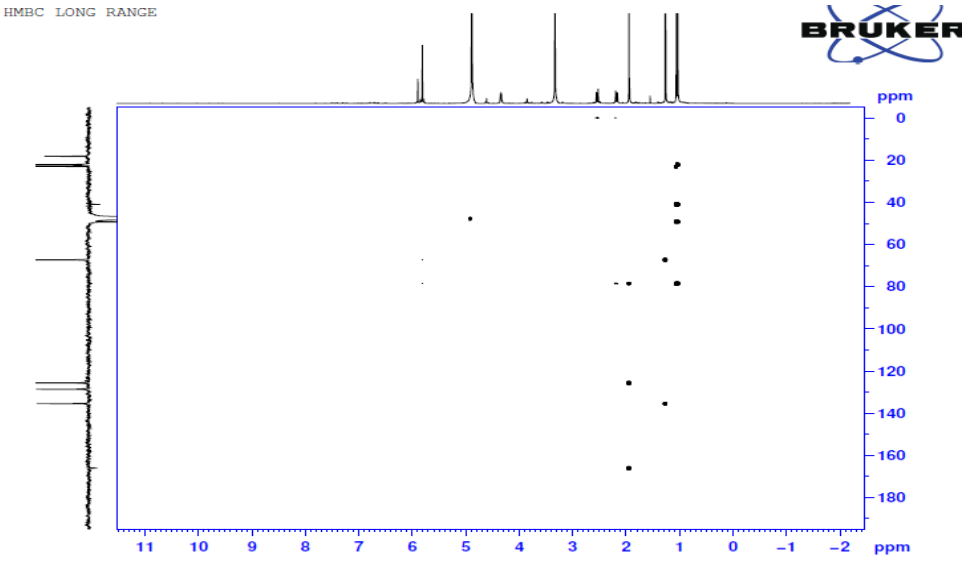


Figure A13: HMBC spectrum of compound 7

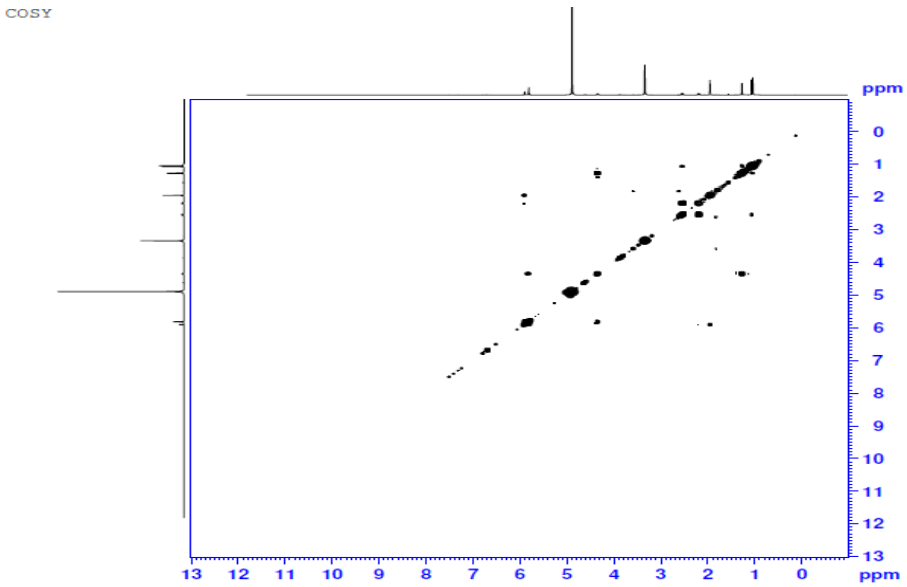


Figure A14: COSY spectrum of compound 7

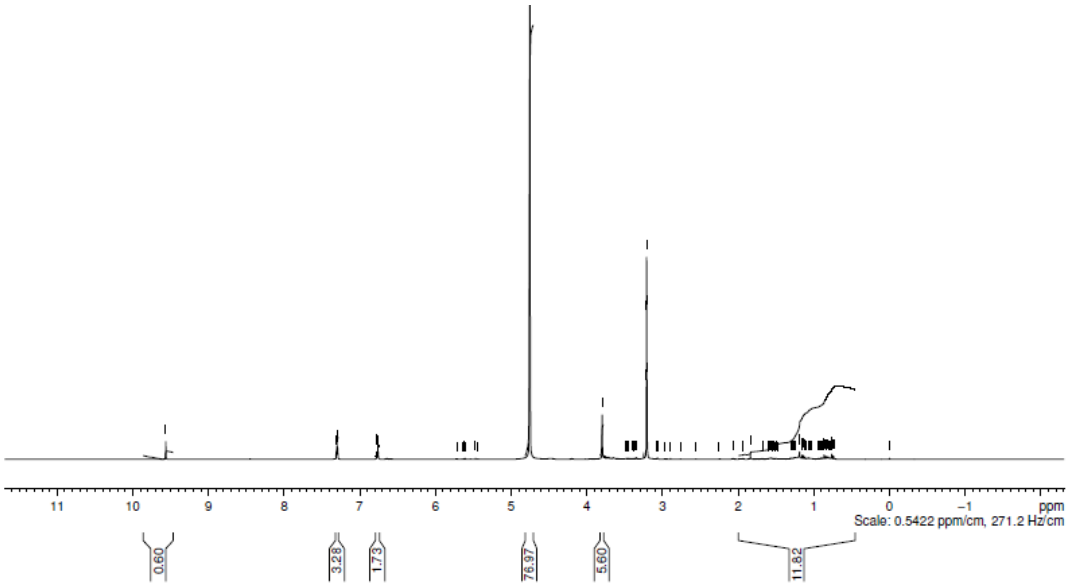


Figure A15: ^{1}H NMR spectrum of compound 7

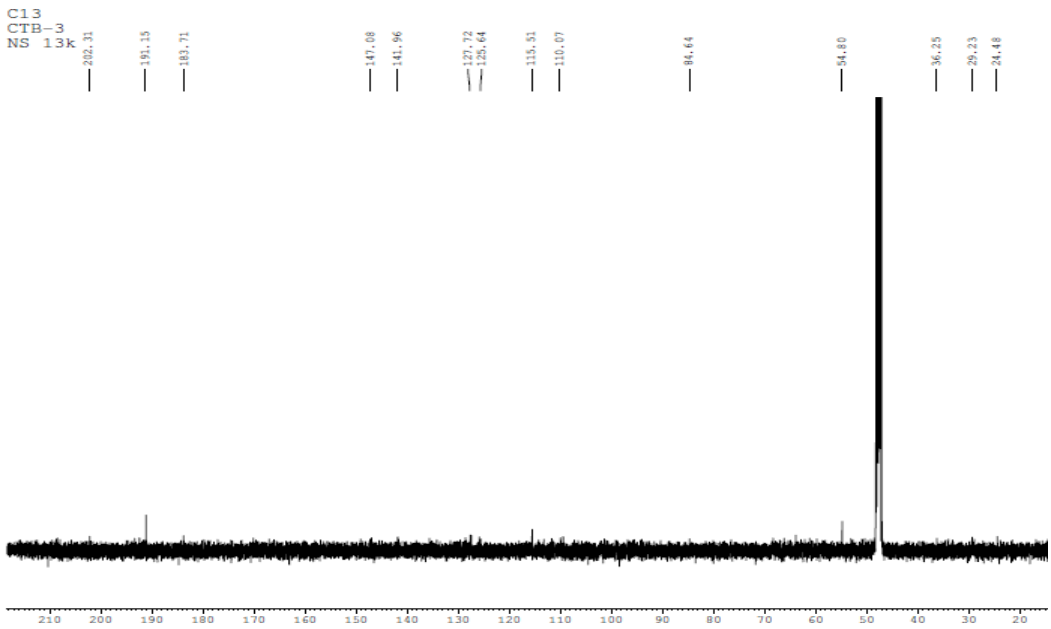


Figure A16: ^{13}C NMR spectrum of compound 7

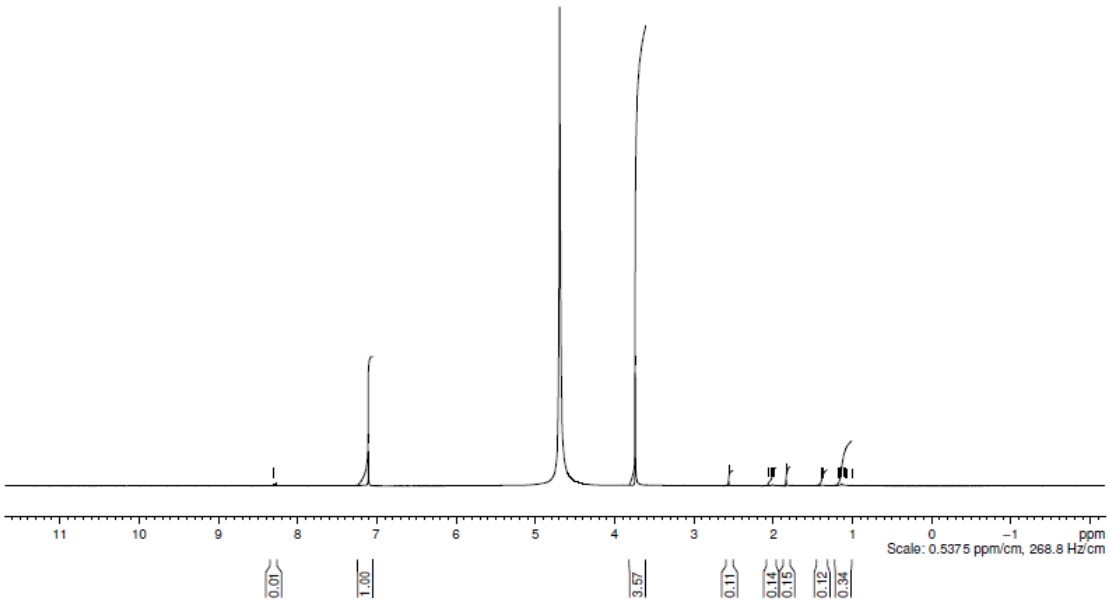
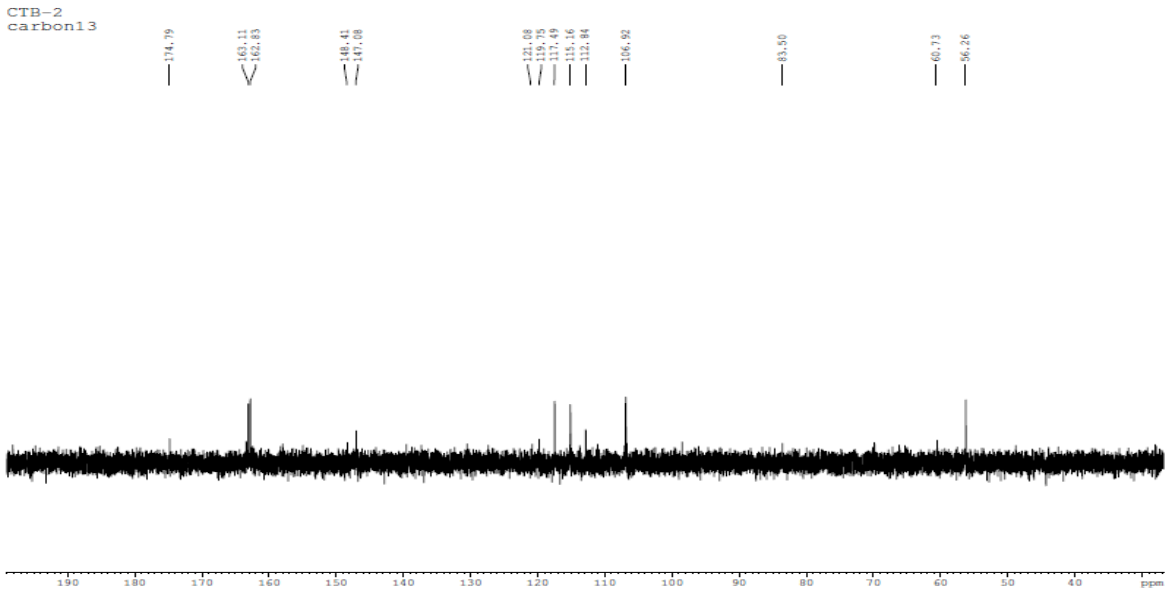


Figure A17: ^{1}H NMR spectrum of compound 8



Fiigure A18: ^{13}C NMR spectrum of compound 8

APPENDIX B: HPLC CHROMATOGRAMS

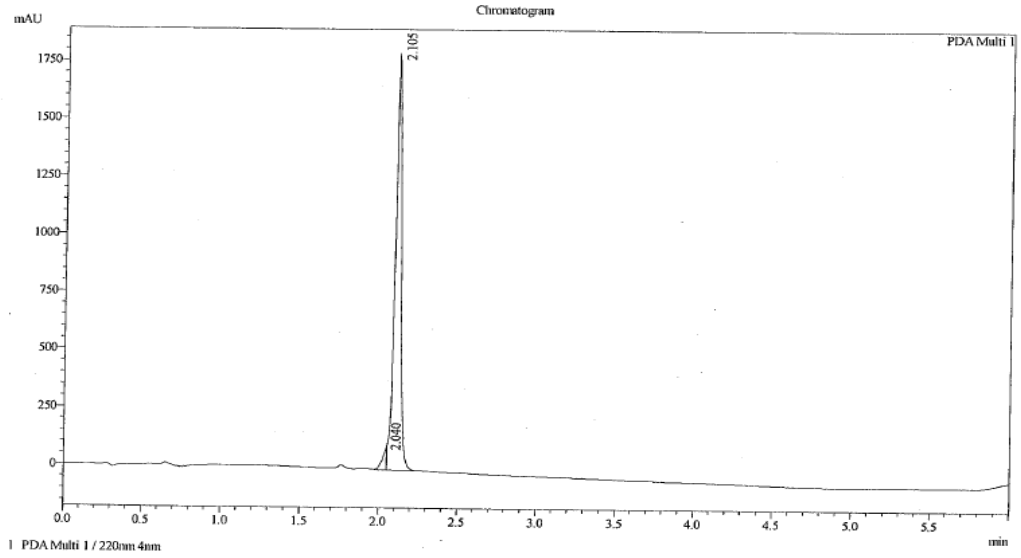


Figure B1: HPLC chromatogram for compound 6

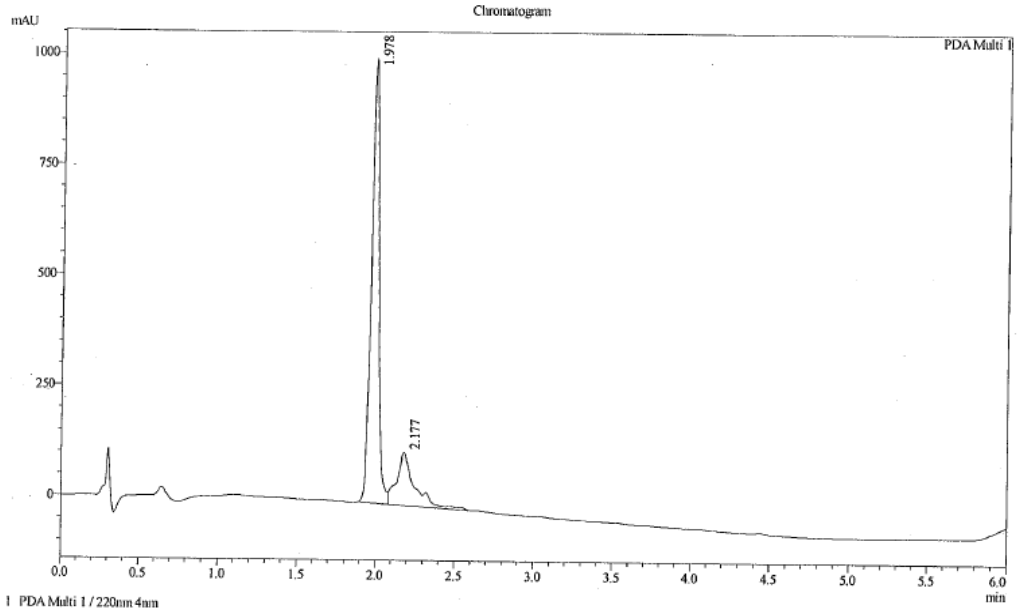


Figure B2: HPLC chromatogram for compound 7

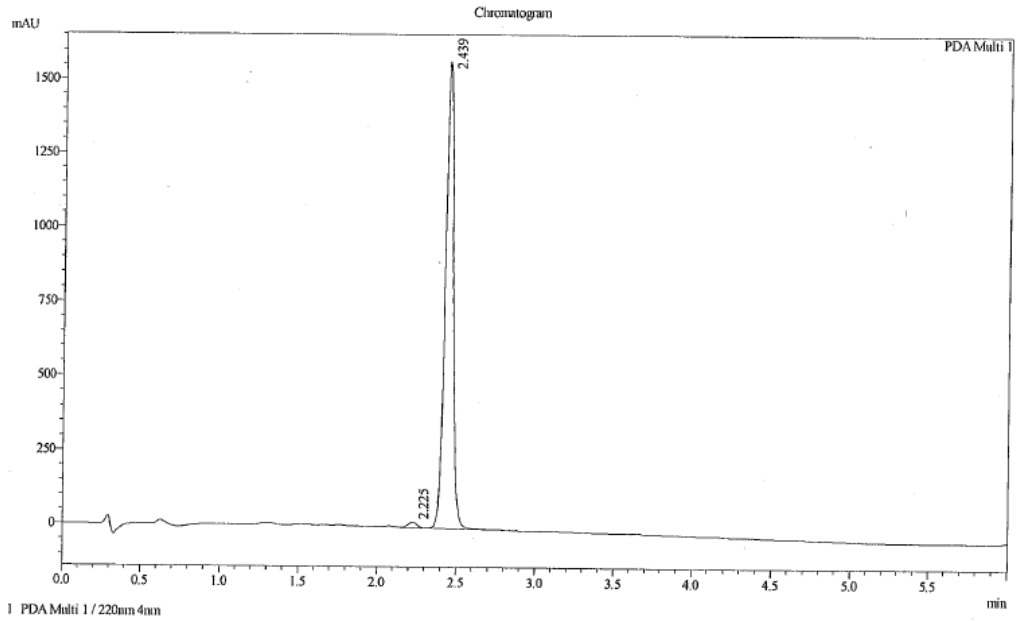


Figure B3: HPLC chromatogram for compound 8

APPENDIX C: PUBLICATIONS

SAGE-Hindawi Access to Research
Biotechnology Research International
Volume 2011, Article ID 768673, 5 pages
doi:10.4061/2011/768673

Research Article

In Vitro Antimicrobial, Antioxidant Activities and Phytochemical Analysis of Canarium patentinervium Miq. fromMalaysia

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Academic Editor: Goetz Laible

Six different extracts of *Canarium patentinervium* Miq. (Burseraceae) leaves and barks were screened for their phytochemical composition, and antimicrobial and free radical scavenging activities. Among the different extracts tested, the ethanol extract of leaves showed significant antimicrobial and radical scavenging activities. The most susceptible micro-organisms were found to be Gram-positive bacteria (*Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* or MRSA) and Gram-negative bacteria (*Pseudomonas aeruginosa*). Phytochemical analysis of the extracts revealed that the antimicrobial and the radical scavenging activities are mainly due to the presence of tannins and flavonoids. The results obtained suggest that *Canarium patentinervium*
Miq. could be exploited in the management of various infectious diseases.

Academic Journal of Cancer Research 4 (1): 1-4, 2011
ISSN 1995-8943

In Vitro Antitumor Potential of *Canarium patentinervium* Miq

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Abstract: In the present study, six different extracts of *Canarium patentinervium* Miq. (Burseraceae) leaves and barks were screened for their *in vitro* antitumor activities, among the different extracts tested, the ethanol and chloroform extract of barks showed significant antitumor activities with GI_{50} values of 23.44 μg/ml and 34.40 μg/ml. The most susceptible cell lines were found to be the breast cancer cell line, MDA 468. The results obtained suggest that further isolation studies can be performed on *Canarium patentinervium* Miq to identify the active constituents responsible for the antitumor activities.

Key words: Antitumor · *Canarium patentinervium* Miq. · MDA468 · Breast Cancer

Review Article
ISSN: 0974-6943

Available online through
www.jpronline.info

Canarium L. : A Phytochemical and Pharmacological Review

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Received on: 17-05-2011; Revised on: 12-06-2011; Accepted on:16-07-2011

ABSTRACT

The genus *Canarium* L. consists of 75 species of aromatic trees which are found in the rainforests of tropical Asia, Africa and the Pacific. The medicinal uses, botany, chemical constituents and pharmacological activities are now reviewed. Various compounds are tabulated according to their classes their structures are given. Traditionally *Canarium* L. species have been used to treat a broad array of illnesses. Pharmacological actions for *Canarium* L. as discussed in this review include antimicrobial, antioxidant, anti-inflammatory, hepatoprotective and antitumor activity.

Keywords: *Canarium* L., Burseraceae, antibacterial, antioxidant, pharmacology, secondary metabolites

Planta Med 2011; 77 - PL9
DOI: 10.1055/s-0031-1282658

The first antibacterial activity report of three selected Malaysian rainforest medicinal plants

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Despite increasing resistance among clinically important gram-negative and gram-positive pathogens to many common antibacterial agents, many large pharmaceutical companies are showing decreased interest in this product area that is so critical to public health. This downturn in antibacterial discovery and development, in turn, is leaving us vulnerable to emerging resistance, particularly to recently arrived vancomycin-resistant *Staphylococcus aureus* and multiply resistant, gram-negative bacilli for which we do not have adequate antimicrobial therapy in the future. Rainforest plants possess therapeutic potential, including antimicrobial activity [1,2]. Therefore, this study is a screening program of several extracts from three endemic medicinal plants in Malaysian rainforest which have not been fully discovered and investigated for their antimicrobial properties against several Gram-positive and Gram-negative bacteria strains. These plants included families namely Annonaceae, Ebenaceae and Burseraceae. The antibacterial activity of hexane, chloroform and ethanol fractions of some parts of *Uvaria grandiflora* Roxb. (Annonaceae), *Diospyros wallichii* King & Gamble (Ebenaceae) and *Canarium patentinervium* Miq. (Burseraceae) was determined against Gram-positive bacteria *Bacillus cereus* ATCC10876, Staphylococcus aureus ATCC11632, Methicilin resistant Staphylococcus aureus ATCC43300 and Gram- negative bacteria *Pseudomonas aeruginosa* ATCC10145 and *Escherichia coli* ATCC10536 using the disk diffusion method. Results showed that the bark ethanol fraction of *U. grandiflora*, the fruit hexane fraction of *D. wallichii* and the leaf ethanol fraction of *C.patentinervium* are active (Table 1). The results indicate that these medicinal herbs can be used as active and potent ingredients in the formulation of natural antibacterial products.

References:

1- Wiart C (2006) Medicinal Plants of the Asia-Pacific: Drugs from the Future, World Scientific, Singapore.

2. Harvey AL (1999) Trends Pharmacol Sci 20: 196-198.

Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2013, Article ID 734824, 7 pages
http://dx.doi.org/10.1155/2013/734824

Research Article

Anti-Inflammatory, Anticholinesterase, and Antioxidant Potential of Scopoletin Isolated from *Canarium patentinervium* Miq. (Burseraceae Kunth)

R. Mogana, K. Teng-Jin, and C.Wiart

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Received 13 March 2013; Accepted 14 June 2013

Academic Editor: Mohd Roslan Sulaiman

Bioassay guided fractionation of an ethanol extract of leaves of *Canarium patentinervium* Miq. (Burseraceae Kunth.) led to the isolation of scopoletin. The structure of this coumarin was elucidated based on spectroscopic methods including nuclear magnetic resonance (NMR-1D and 2D) and mass spectrometry. Scopoletin inhibited the enzymatic activity of 5-lipoxygenase and acetyl cholinesterase with an IC50 equal to 1.76 ± 0.01 μM and 0.27 ± 0.02mM, respectively, and confronted oxidation in the ABTS, DPPH, FRAP, and β-carotene bleaching assay with EC50 values equal to 5.62 ± 0.03 μM, 0.19 ± 0.01 mM, 0.25 ± 0.03mM and 0.65 ± 0.07 mM, respectively. Given the aforementioned evidence, it is tempting to speculate that scopoletin represents an exciting scaffold from which to develop leads for treatment of neurodegenerative diseases.

Hindawi Publishing Corporation
ISRN Biotechnology
Volume 2013, Article ID 986361, 8 pages
http://dx.doi.org/10.5402/2013/986361

Research Article

The Medicinal Timber *Canarium patentinervium* Miq. (Burseraceae Kunth.) Is an Anti-Inflammatory Bioresource of Dual Inhibitors of Cyclooxygenase (COX) and 5-Lipoxygenase(5-LOX)

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Received 10 July 2013; Accepted 19 August 2013

Academic Editors: A. O. Ballesteros, A. Maggio, and D. Pant

The barks and leaves extracts of *Canarium patentinervium*Miq. (Burseraceae Kunth.) were investigated for cyclooxygenase (COX) and 5-lipoxygenase (LOX) inhibition via *in vitro* models.The corresponding antioxidative power of the plant extract was also tested via nonenzyme and enzyme *in vitro* assays.The ethanolic extract of leaves inhibited the enzymatic activity of 5-LOX, COX-1, and COX-2 with IC50 equal to 49.66±0.02 μg/mL, 0.60±0.01 μg/mL, and 1.07±0.01 μg/mL, respectively, with selective COX-2 activity noted in ethanolic extract of barks with COX-1/COX-2 ratio of 1.22.The ethanol extract of barks confronted oxidation in the ABTS, DPPH, and FRAP assay with EC50 values equal to 0.93 ± 0.01 μg/mL, 2.33 ± 0.02 μg/mL, and 67.00 ± 0.32 μg/mL, respectively, while the ethanol extract of leaves confronted oxidation in β-carotene bleaching assay and superoxide dismutase (SOD) assay with EC50 value of 6.04 ± 0.02 μg/mL and IC50 value of 3.05 ± 0.01 μg/mL. The ethanol extract acts as a dual inhibitor of LOX and COXenzymes with potent antioxidant capacity.The clinical significance of these data is quite clear that they support a role for *Canarium patentinervium* Miq. (Burseraceae Kunth.) as a source of lead compounds in the management of inflammatory diseases.

APPENDIX D: CONTINUOUS EDUCATION

- Optimizing Your Laboratory's Productivity via improved Sample Preparation Technique and Advanced Column Technology, Monash University, 12th Oct 2010.
- Project Management for Research, Graduate School Training Course, Nottingham University Malaysia Campus, 4th Oct 2010
- Getting Going on Your Thesis, Graduate School Training Course, Nottingham University Malaysia Campus, 5th Oct 2010
- Lab Demonstrations (Science) , Graduate School Training Course, Nottingham University Malaysia Campus, 17th Jan 2011.
- Back To Nature symposium, Prof Iqbal Choudhary, UITM Puncak Alam, Jan 2011.
- NMR Theory & Applications: Natural products, UITM Puncak Alam, 17-18th Feb 2011.
- HPLC column determination, UITM Puncak Alam, April 2011.
- Workshop 2011: NMR for Intermediates and Metabolomics, UITM Puncak Alam, 17-19th Nov 2011.
- Technical Writing and Getting Published, Graduate School Training Course, Nottingham University Malaysia Campus, 14th March 2012.
- Finishing Your Thesis, Graduate School Training Course, Nottingham University Malaysia Campus, 30th Nov 2012.
- NMR Spectroscopy Workshop, UiTM Puncak Alam, 1-3rd March 2013.