ANTIPARASITIC, ANTIBACTERIAL, ANTIOXIDANT, AND CYTOTOXIC ACTIVITIES OF SIX MALAYSIAN MEDICINAL PLANTS AND IDENTIFICATION OF THEIR ACTIVE CONSTITUENTS

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BSc. (Hons)

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

NOVEMBER 2019

Table of contents

Contents **Page**

Declaration

I, Nadiah Syafiqah Nor Azman, 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 Campus, Malaysia. It has not been submitted before for any degree or examination at this or any other University.

Signature:

Date: 3rd October 2019

Dedication

Specially dedicated to:

My beloved parents (Nor Azman & Samidah), husband (Muhammad Asyeqal), son (Ahmad Naqeeb), mom-in-law (Arbai'yah), siblings, siblings-in law, niece and nephew.

Acknowledgements

Alhamdulillah. Finally, I reached the final stage of my Ph.D. study after facing a lot of struggle, hardship, challenges, and tests. First and foremost, I would like to express my greatest gratitude to my supervisor, Assoc. Prof. Dr. Christophe Wiart for his continuous support, motivation, wonderful guidance, high enthusiasm, and immense knowledge during my Ph.D. study. Besides supervisor, I would like to thank the rest of my co-supervisors: Assoc. Prof. Dr. Veeranoot Nissapatorn, Assoc. Prof. Dr. Khoo Teng Jin, and Dr. Chandramathi Samudi Raju for giving me their unerring eminent advice, sharing experiences and etching me with invaluable guidance, constructive suggestions, and brilliant ideas for resolution throughout my Ph.D. years.

In fact, I would like to give credits to the following sponsorship for their kindness funding throughout my entire Ph.D. research: (i) Fundamental Research Grant Scheme (FRGS), Ministry of Education Malaysia, (ii) MyBrain15 Programme Scholarship, Ministry of Education Malaysia, Malaysia, (iii) School of Pharmacy- Ph.D. Fee Waiver, University of Nottingham and (iv) School of Pharmacy-Fund for Research and Conference, University of Nottingham.

I would like to show appreciation to the university staff and lab assistants who involve throughout my research study in the following departments: (i) Faculty of Science, University Nottingham Malaysia Campus, (ii) Department of Parasitology, Department of Medical Microbiology, and Department of

Chemistry, University of Malaya and (iii) Faculty of Pharmacy, UiTM Puncak Alam for their kind support, excellent co-operation, good motivation and guidance. At the same time, sincere thanks to Assoc. Prof. Dr. Vijay Kumar Prajapathi, Dr. Mohd Ridzuan Mohd Razak, Dr. Mohammed Khaled Break, and Dr. Hassan Mahmood Jindal for providing me the cell lines with software accessibility for data analysis.

Last but not least, I would like to thank to my main supporters: my parents, husband, siblings, course mates and friends for their constant encouragement, spiritual support and for having faith and trust in me to complete the Ph.D. study. To my son, you are my upmost strength.

Abstract

Increasing mortality and morbidity rates caused by *Plasmodium, Leishmania,* and drug-resistant bacteria worldwide have become a major problem. Current drugs do seem less effective, toxic, expensive, and require long periods of administration. WHO recommends the development of new antiparasitic and antibacterial agents. Therefore, research on Malaysian medicinal plants has been performed by our researchers in search of new natural products in the past five decades, offering them a great opportunity to discover new chemical structures in drug discovery.

In 2015, a survey was done to evaluate and document the use of medicinal plants used by local people in day-to-day practice in two preserved villages (Kuala Kangsar and Manong) in the State of Perak, Peninsular Malaysia, which afforded the collection of 6 medicinal plants from three different families in the class Magnoliopsida (Dicotyledons). The collected plants were identified as *Chilocarpus costatus* Miq. (Apocynaceae), *Leuconotis eugeniifolia* (Wall. ex. G. Don) A.DC (Apocynaceae), *Tabernaemontana peduncularis* Wall (Apocynaceae), *Uvaria grandifolia* Roxb. ex Hornem. (Annonaceae), *Artabotrys suaveolens* (Blume) Blume (Annonaceae), and *Diospyros wallichii* King & Gamble (Ebenaceae). To the best of our knowledge, detail pharmacological studies regarding antiparasitic, antibacterial, antioxidant, and cytotoxic activities reported on these six Malaysian medicinal plants are very scanty. Thus, the general objective of this study was to identify natural products

from rare Malaysian medicinal plants with antiplasmodial, antileishmanial, antibacterial, antioxidant or cytotoxic activities.

Parasites such as *Plasmodium falciparum* and *Leishmania donovani* are responsible for infectious disease with over 200 million reported cases and thousands annual deaths. Therefore, *in vitro* antiparasitic activities and cytotoxic activities were performed on both chloroform extracts and compounds by the microtetrazolium test (MTT), Histidine-Rich Protein II (HRPII) assay, and malaria SYBR Green I-based fluorescence (MSF) assay with quercetin, vincristine, chloroquine, dihydroartemisinin, and miltefosine as positive controls. Based on the results obtained, 11 out of 12 extracts tested showed good antiplasmodial activities against *P. falciparum* K1 chloroquine-resistant strains with IC_{50} below 10 µg/mL.

The highest antiplasmodial activities against *P. falciparum* K1 chloroquine-resistant strains were recorded on both stems and leaves chloroform extracts of *C. costatus* with IC_{50} values of 1.15 and 0.85 µg/mL, respectively. The antileishmanial analysis on plant chloroform extracts revealed that 3 of the 12 extracts showed good antileishmanial activities against promastigote *in vitro,* which were stems *A. suaveolens,* stems *C. costatus*, and leaves *D. wallichii* with IC_{50} values of 17.33, 17.32, and 7.6 µg/mL, accordingly. The remaining exhibited moderate activities against *L. donovani* BHU-1251 promastigote strains. We also examined the potential toxicity of the extracts against mammalian cells prior to antileishmanial and antiplasmodial testing to assess the toxicity of these potential antiparasitic agents. Protozoan parasites such as *L.*

donovani and *P. falciparum* can cause lung diseases (Vijayan, 2008). Thus, a cytotoxicity study of extracts against human lung epithelial cells (MRC-5) was performed. Four of the 12 extracts tested were toxic to human cells with CC_{50} below 20 µg/mL, which were stems *T. peduncularis* (11.5±3.8 µg/mL), stems *L. eugeniifolia* (15.5±3.4 µg/mL), stems *D. wallichii* (15.0±2.0 µg/mL), and stems *U. grandiflora* (10.0±6.1µg/mL).

Pathogenic bacteria have become more resistant to conventional antibiotics, leading to an increase of nosocomial bacterial infection worldwide. The most frequent nosocomial bacterial infections are bloodstream infection (BSI), urinary tract infection (UTI), surgical site infection (SSI), pneumonia, gastroenteritis, meningitis, and respiratory infection. Common pathogens found to be responsible for these infections include *Escherichia coli, Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa, Staphylococcus epidermidis,* Methicillin-resistant *Staphylococcus aureus* (MRSA), and *Klebsiella pneumoniae* (KP). Hence, antibacterial assessment on these seven pathogenic bacteria was done using the disc diffusion method, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) as well as synergy and time killing assay with cefotaxime and vancomycin as positive controls.

C. costatus extract had moderate antibacterial activity against *S. epidermidis* with MIC values of 187.5 ± 0.20 µg/mL and was inactive against the other six bacteria tested with MIC values above 1500 µg/mL. The remaining 11 chloroform extracts were inactive against all the bacterial strains tested. Since

only stem *C. costatus* extract displayed antibacterial activities in MIC, therefore, MBC/MIC ratio, time-killing, and synergistic effects were studied only on this extract. *C. costatus* stem extract was found to be bactericidal. Time killing assay was done for 4 hours. In the time killing assay, *C. costatus* stem chloroform killed *S. epidermidis* bacteria faster than cefotaxime and vancomycin. It also was found that *C. costatus* stem chloroform reacts synergistically with cefotaxime (FICI 0.5), however, no synergistic effect was observed with vancomycin (FICI 1.1) when combined against *S. epidermidis.*

Free radicals are involved in the pathogenesis of many human diseases. The investigation of the antioxidant properties of plant extracts was achieved through the assessment of total phenolic content and iron reducing antioxidant power (FRAP) assay with gallic acid and $FeSO_4$ as positive controls. All chloroform extracts had low total phenolic and FRAP content.

Two plant extracts (*C. costatus* stem chloroform and *U. grandiflora* leaves chloroform) were selected in the present study for further isolation and bioactivity, as they were very active and had good antiplasmodial, antileishmanial, and antibacterial activities. Therefore, isolation of the compounds was done by High Performance Liquid Chromatography (HPLC) and resulted in the isolation of three compounds: pinoresinol, zeylenol, and ferrudiol. To the best of our knowledge, this is the first report on the occurrence of pinoresinol and ferrudiol in *C. costatus* Miq. and *U. grandiflora* Roxb. ex Hornem, respectively.

These three compounds were then tested for their bioactivities. Pinoresinol displayed no antileishmanial and antiplasmodial activities, and was not toxic to human lung, MRC-5 cells. However, pinoresinol acted synergistically with cefotaxime against *S. epidermidis.* Meanwhile, both zeylenol and ferrudiol were inactive against *L. donovani.*

In conclusion, the findings of our study indicated that *C. costatus* stem chloroform and its major constituents, pinoresinol may be used as potential candidates for the development of antibiotic potentiators.

List of paper publications

a) Related to the study

Azman, N.S.B.N., Mahboob, T., Tan, T.C., Samudi, C., Nissapatorn, V. and Wiart, C., 2017. Plant-based therapy-How does it work on parasites? *Walailak Journal of Science and Technology (WJST)*, *15*(8), pp.551-559. **(Scopus indexed paper)**.

Azman, N.S.N., Hossan, M.S., Nissapatorn, V., Uthaipibull, C., Prommana, P., Jin, K.T., Rahmatullah, M., Mahboob, T., Raju, C.S., Jindal, H.M. and Hazra, B., 2018. Anti-infective activities of 11 plants species used in traditional medicine in Malaysia. *Experimental Parasitology*, *194*, pp.67-78. **(ISI indexed paper)**

b) Non-related to the study

Mahboob, T., Azlan, A.M., Shipton, F.N., Boonroumkaew, P., **Azman, N.S.N.**, Sekaran, S.D., Ithoi, I., Tan, T.C., Samudi, C., Wiart, C. and Nissapatorn, V., 2017. Acanthamoebicidal activity of periglaucine A and betulinic acid from *Pericampylus glaucus* (Lam.) Merr. *in vitro*. *Experimental Parasitology*, *183*, pp.160-166. **(ISI indexed paper)**

Jaturas, N., Mahboob, T., **Azman, N.S.B.N.**, Tian-Chye, T. and Nissapatorn, V., 2017. Malaria in children: diagnostic tools in resource-limited settings. *Journal of Pediatric Infectious Diseases*, *12*(04), pp.249-255. **(ISI indexed paper)**

Kwan, T.K., Shipton, F., **Azman, N.S.**., Hossan, S., Jin, K.T. and Wiart, C., 2016. Cytotoxic Aporphines from *Artabotrys crassifolius*. *Natural Product Communications*, *11*(3), pp.389-392. **(ISI indexed paper)**

List of poster presentations

a) Related to the study

PGR Student Conference Support Poster Competition.

University of Nottingham Malaysia Campus, Semenyih, Malaysia.

15th September 2017. (Faculty Level)

Title: *In vitro* anti-leishmanial activities of Malaysian medicinal plants.

Nadiah Syafiqah Nor Azman

5th National and International Conferences of the Medical Technology 2017

"Infectious Diseases and Innovation for Clinical Diagnosis".

Twin Lotus, Nakhon Si Thammarat, Thailand.

28th -29th March 2017. (The best outstanding poster award)

Title: Natural Products against *Acanthamoeba, Leishmania* and *Plasmodium* - a Global Review.

Nadiah Syafiqah Nor Azman, Tooba Mahboob, Christophe Wiart and Veeranoot Nissapatorn

b) Non-related to the study

3rd Microbiology and Infectious Diseases Asia Congress. Singapore.

11th -12th October 2016.

Title: Evaluation of Amoebicidal Potential of Malaysian Medicinal Plants *In vitro.*

Tooba Mahboob, Abdul-Majid Azlan, **Nadiah Syafiqah Nor Azman**, Guo-Jie Brandon Mong, Tian-Chye Tan, Chandramathi Samudi Raju, Shamala Devi Sekaran, Veeranoot Nissapatorn and Christophe Wiart.

List of oral presentations

a) Related to the study

5th National and International Conferences of the Medical Technology 2017 "Infectious Diseases and Innovation for Clinical Diagnosis".

Twin Lotus, Nakhon Si Thammarat, Thailand. 28-29th March 2017. (International Level)

Title: *Leishmania donovani* – A Search for Novel Natural Therapy

Nadiah Syafiqah binti Nor Azman, Tooba Mahboob, Vijay Kumar Prajapati, Rajan Kumar Pandey, Banasri Hazra, Veeranoot Nissapatorn and Christophe Wiart

3 Minute Thesis Competition.

University of Nottingham Malaysia Campus, Malaysia. 6th September 2016.

(International Level)

Title: Leishmaniasis.

Nadiah Syafiqah Nor Azman

Faculty research talk.

University of Nottingham Malaysia Campus, Malaysia. 13th July 2017. (Faculty Level)

Title: *In vitro* pharmacological activities of the Malaysian rainforest medicinal plants.

Nadiah Syafiqah Nor Azman

Faculty research talk.

University of Nottingham Malaysia Campus, Malaysia. 21st September 2017. (Faculty Level)

Title: Anti-leishmanial compounds from selected Malaysian medicinal plants.

Nadiah Syafiqah Nor Azman

b) Non-related to the study

5th National and International Conferences of the Medical Technology 2017 "Infectious Diseases and Innovation for Clinical Diagnosis".

Twin Lotus, Nakhon Si Thammarat, Thailand. 28-29 March 2017. (International Level)

Title: *Acanthamoeba* – from Malaysian Plants to Natural Amoebicidal Agents

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List of figures

List of figures **Page**

List of figures **Page**

List of tables

List of tables **Page**

List of tables **Page**

List of appendices

List of appendices **Page**

List of appendices **Page**

List of abbreviations

A

ADME	Absorption, Distribution, Metabolism and Excretion
AFC	Automatic Fraction Collector
$AlCl_3$	Aluminium Chloride
ANOVA	Analysis of Variance
ASEAN	Association of Southeast Asian Nations
ATCC	American Type Culture Collection

B

BHI	Brain Heart Infusion Broth
BOD	Bio-Oxygen Demand
BSI	Bloodstream infection

C

CAT	Catalase
CC_{50}	Cytotoxic concentration at 50% activity
CDC	Centre for Disease Control and Prevention
CFU	Colony forming unit
CFU/mL	Colony forming unit per milliliter

D

d	Doublet
DAD	Diode Array Detector

DMSO	Dimethyl sulfoxide

E

EC_{50}	Effective concentration at 50 % activity
EDTA	Ethylene diamine tetra-acetic acid
EIMS	Electron Impact Mass Spectrometry
ELISA	Enzyme-linked immunosorbent assay

F

FBS	Fetal bovine serum
FC	Folin-Ciocalteu
$FeCl_3$	Ferric chloride
$FeCl_3.6H_2O$	Iron (III) chloride hexahydrate
$FeSO_4.7H_2O$	Iron (II) sulphate heptahydrate
FIC	Fractional Inhibitory Concentration
FICI	Fractional Inhibitory Concentration Index
FRAP	Ferric Reducing Antioxidant Power
FRIM	Forest Research Institute Malaysia

G

GAE	Gallic acid equivalent
GDB	Global Burden of Disease
GPx	Glutathione peroxidase
GRx	Glutathione reductase
GSH	Oxidizing reduced glutathione

H

^{1}H NMR	Proton nuclear magnetic resonance
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
HCI	High-content imaging
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
HRPII	Histidine-Rich Protein II
HTS	High-throughput screening

I

IC_{50}	Inhibitory concentration at 50 % activity
ICL	Immunology Consultants Laboratory, Inc.
IgM	Immunoglobulin M

M

m	Multiplet
MBC	Minimum Bactericidal Concentration
MEM	Minimum Essential Medium
MEP	Mevalonic acid and non-mevalonate pathway
MHA	Müller-Hinton agar
MHB	Müller-Hinton broth
MIC	Minimum Inhibitory Concentration
MR4	Malaria Research and Reference Reagent Resource Center, USA

MRSA	Methicillin Resistant *Staphylococcus Aureus*
MS	Mass spectroscopy
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

N

NMR	Nuclear magnetic resonance
NNN	Novy-McNeal-Nicolle medium

O

O_2^-	Superoxide anion radical
OD	Optical density

P

PBPs	Penincillin-Binding Proteins
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline-Tween 20
PCR	Polymerase Chain Reaction
PGPR	Plant-Growth Promoting Rhizobacteria

R

RBCs	Red blood cells
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI 1640	Roswell Park Memorial Institute 1640 medium

S

s	Singlet
SD	Standard deviation
SDX/PYR	Sulfadoxine and pyrimethamine
SI	Selectivity Index
SOD	Superoxide dismutase
SSI	Surgical site infection

T

TLC	Thin Layer Chromatography
TMB	3,3', 5,5'-tetramethylbenzidine
TPC	Total Phenolic Content
TPTZ	2,4,6-tris(2-pyridyl)-1,3,5-triazine

U

UNMC	University of Nottingham Malaysia Campus
UTI	Urinary tract infection

Chapter 1: Introduction

1.1. Background of the study

Pharmacological activities are used to evidence the beneficial or adverse effect of a drug on living organism. Pharmacological activities of natural products depend on their interactions with proteins, enzymes, receptors, nucleic acid, or bio-membranes (Wink, 2015). Evaluation of the pharmacological activity of a biopharmaceutical drug can be done through *in vitro* and/or *in vivo* assays.

In vitro assays are needed to demonstrate the affinity of the biopharmaceutical to the target, while *in vivo* function to establish the potential biological activity in appropriate animal models (Colerangle, 2013). Much information can be obtained regarding the mechanism of the product and its potential for clinically relevant activities through these pharmacological studies (Colerangle, 2013).

Since the 1970s, many investigations on medicinal plants have been done and natural products demonstrated interesting pharmacological activities (Brantner and Grein, 1994; Sasidharan *et al.,* 2011). The interest of plants is enormous in pharmacological, physiological, and biochemical studies (Williamson *et al.,* 1996). A pharmacopeia survey showed that 20-25% of all medicines available in pharmaceutical industries today are derived from natural sources such as herbs, flowers and underutilized plants (Elvin-Lewis, 2001).

In this research study, we aim to identify natural products from rare Malaysian medicinal plants with antiplasmodial, antileishmanial, antibacterial, antioxidant or cytotoxic activities.

1.2. Problem statement

Increasing mortality and morbidity rate caused by *Plasmodium*, *Leishmania*, and pathogenic bacteria globally has become a major problem. The current pharmacological agents seem less effective, toxic, and expensive (Sasidharan *et al.*, 2011). Natural product may offer safer, more efficient, and cheaper drugs.

1.3. Objectives

1.3.1. General objective

There is an urgent need to find alternative therapeutic agents, particularly from natural resources to combat infectious diseases. As we know, malaria and leishmaniasis have been listed as some of the major life-threatening diseases in Indonesia, India, Bhutan, and Malaysia (WHO Southeast Asia, 2015). Meanwhile, 75% of nosocomial bacterial infections present in developing countries occur mainly in Southeast Asia (WHO, 2011). The general objective is to find antiparasitic and antibacterial natural products from these six Malaysian medicinal plants: *Chilocarpus costatus* Miq., *Tabernaemontana*

peduncularis Wall., *Artabotrys suaveolens* (Blume) Blume, *Leuconotis eugeniifolia* (Wall. ex. G. Don) A.DC., *Diospyros wallichii* King & Gamble, and *Uvaria grandiflora* Roxb. ex Hornem. We also aim to find antibiotic potentiators from these plants. At the same time, we also aim to assess the cytotoxic and antioxidant properties of these plants. To the best of our knowledge, detail pharmacological studies especially antiparasic, antibacterial, antioxidant, and cytotoxic activities reported on these six Malaysian medicinal plants: *Chilocarpus costatus* Miq., *Tabernaemontana peduncularis* Wall., *Artabotrys suaveolens* (Blume) Blume, *Leuconotis eugeniifolia* (Wall. ex. G. Don) A.DC., *Diospyros wallichii* King & Gamble, and *Uvaria grandiflora* Roxb. ex Hornem. are very scanty.

1.3.2. Specific objectives

The ultimate goal of the research study is to contribute to drug discovery. Therefore, the specific objectives of this study are:

1) To extract leaves and stems with chloroform (to selectively extract mid-polar molecules) and to determine the extraction yields. Mid-polar molecules have higher ‘‘drugability’’ (oral absorption).

2) To determine the antiparasitic activities of the chloroform extracts of stems and leaves by *in vitro* antileishmanial, antiplasmodial, and cytotoxicity assays.

3) To investigate the antibacterial activities of the chloroform extracts of leaves and stems *in vitro*.

4) To evaluate the antioxidant properties of the chloroform extracts of leaves and stems.

5) To identify and test compounds from the most potent plant extracts by *in vitro* pharmacological tests.

1.4. Expected outcomes

The expected outcome is the identification of a molecule with antiparasitic, or antibacterial, or antibiotic potentials activities.

1.5. Significance of the study

The finding is not only in depth study but also horizontal scale experiment focusing on the isolation of active constituents from Malaysian medicinal plants. This may benefit the society, considering that natural products play an important role in finding novel antiplasmodial, antileishmanial, and antibacterial agents. In brief, isolated molecules could potentially be used for drug development.

Chapter 2: Literature review

2.1. Plants as natural product approach in drug discovery

Plants have been used widely as therapeutic medicines by ancient civilization as their main source of drugs (Rates, 2001). Since 1984, much research has been done on medicinal plants to provide greater structural diversity than standard combinatorial chemistry. Plants are well known for synthesizing active secondary metabolites against a wide range of targets (Harvey, 2000; Tan *et al.,* 2001).

About a quarter of drugs prescribed worldwide come from natural products (Rates, 2001). Examples of drugs from natural plant products include artemisinin from *Artemisia annua* and quinine from Chincona tree. They have great potentials as antiparasitic leads (Iwu *et al.,* 1999; Kayser *et al.,* 2003). However, a reduction of funding and interest on natural products by pharmaceutical companies worldwide over the past few years has been observed (Butler, 2004).

Nevertheless, there is a rising interest in alternative medicine and therapeutic use of natural products in recent years, mainly in Asian countries (Sasidharan *et al.,* 2011). Malaria, leishmaniasis, and nosocomial bacterial infections are significant encumbrance to public health and global economies (Cragg *et al.,* 2012).

Natural products research study allows the identification of chemicals with structural diversity to treat infectious diseases (Hosseinzadeh *et al.,* 2015).

Clinical trials need to be done to prove the efficiency of the drugs in treating the disease to ensure patient safety. There are currently more than 100 natural products still in clinical trials and no less than 100 similar projects in the preclinical testing stage (Sasidharan *et al.,* 2011;Harvey, 2008).

2.2. Plants in Malaysia

In Malaysia, research on medicinal plants has been performed by our researchers (regardless of whether they are from government-funded universities, research institutes, or health industries) in search of novel natural products and bioactive compounds for the past five decades (Ahmad *et al.,* 2016). There is also increased public interest in the use of herbal medicine. Peninsular Malaysia is rich with medicinal plant (Andaya and Andaya, 2016). A variety of untapped medicinal plants used as traditional medicine can be found in Malaysia, for example, *C. costatus, T. peduncularis, A. suaveolens, L. eugeniifolia, D. wallichii* and *U. grandiflora*, and many others.

However, their *in vitro* pharmacological activities have not been fully explored, despite the use of several hundred plants for the treatment of infectious diseases, wound healing, viral infections, and bacterial infections (Burkill, 1966). For example, periglaucine A from *Pericampylus glaucus* grown in Malaysia possesses good anticancer, antimicrobial, antioxidant, antiamoebicidal, and wound healing activities that may be useful against protozoan diseases (Sasidharan *et al.,* 2011; Kifayatullah and Sarker, 2016; Shipton *et al.,* 2017; Mahboob *et al.,* 2017). However, the effectiveness of these

plants against malaria, leishmaniasis, and nosocomial bacterial infections needs further studies (Jones *et al.*, 2008).

At the same time, the problem of side effects associated with modern medicines further justifies the need to investigate medicinal plants for their pharmacological activities. For instance, Amphotericin B (Guerin *et al.*, 2002) and pentavalent antimonial such as sodium stibogluconate (Omollo *et al.*, 2011) evoke headache and nausea. Therefore, it is urgent to find alternative drugs to conventional therapy. The isolation of active compounds from plants can help such drugs.

2.3. Selection of Malaysian medicinal plants studied

Plants selection can be done either based on botanical, ethnopharmacological, or chemotaxonamical criteria (Chu, 2014; Wiart, 2007; Lattif *et al.*, 1984). There are six Malaysian medicinal plants selected in this study, classified into three families: Apocynaceae, Annonaceae and Ebenaceae.

2.3.1. Family Apocynaceae

2.3.1.1. *Chilocarpus costatus* Miq.

Members of *Chilocarpus* genus are classified in the family Apocynaceae and order Gentianales (Simões *et al.*, 2007). The synonyms are *Chilocarpus aurantiacus* Ridl., *C. costatus var. borneensis* Markgr., *Chilocarpus cuneifolius*

Kerr, *Chilocarpus diepenhorstii* Miq., *Chilocarpus maingayi* Dyer ex Hook. f., and *Chilocarpus* Blume. The vernacular name is ‘‘getah gerip puteh’’. It is found abundantly in India, Malaysia, Philippines, Borneo, Thailand, Burma, Myanmar, and New Guinea (Markgraf, 1971; Simões *et al.,* 2007; Middleton, 2007).

Members of *Chilocarpus* genus have aryllated seeds and a smooth ruminate, thick endosperm (Simões *et al.,* 2007). Their leaves veins are prominent beneath (Middleton, 2007). Their corolla tube is about 6 to 21.5 mm in length (Middleton, 2007). *Chilocarpus costatus* is a climber which is 20 m long. The branchlets are glabrous. The petiole is 5 to 20 mm long. The blade is obovate. The inflorescence is axillary. The flowers are densely congested. The corolla tube is tubular, oval, and orange. The fruits are globular follicules. *Chilocarpus* fruits are dehisced into two carpels. Its mesocarp is scarcely fleshy, seeds in a scanty pulp, and the endocarp are hard and dry (Figure 2.1.) (Markgraf, 1971).

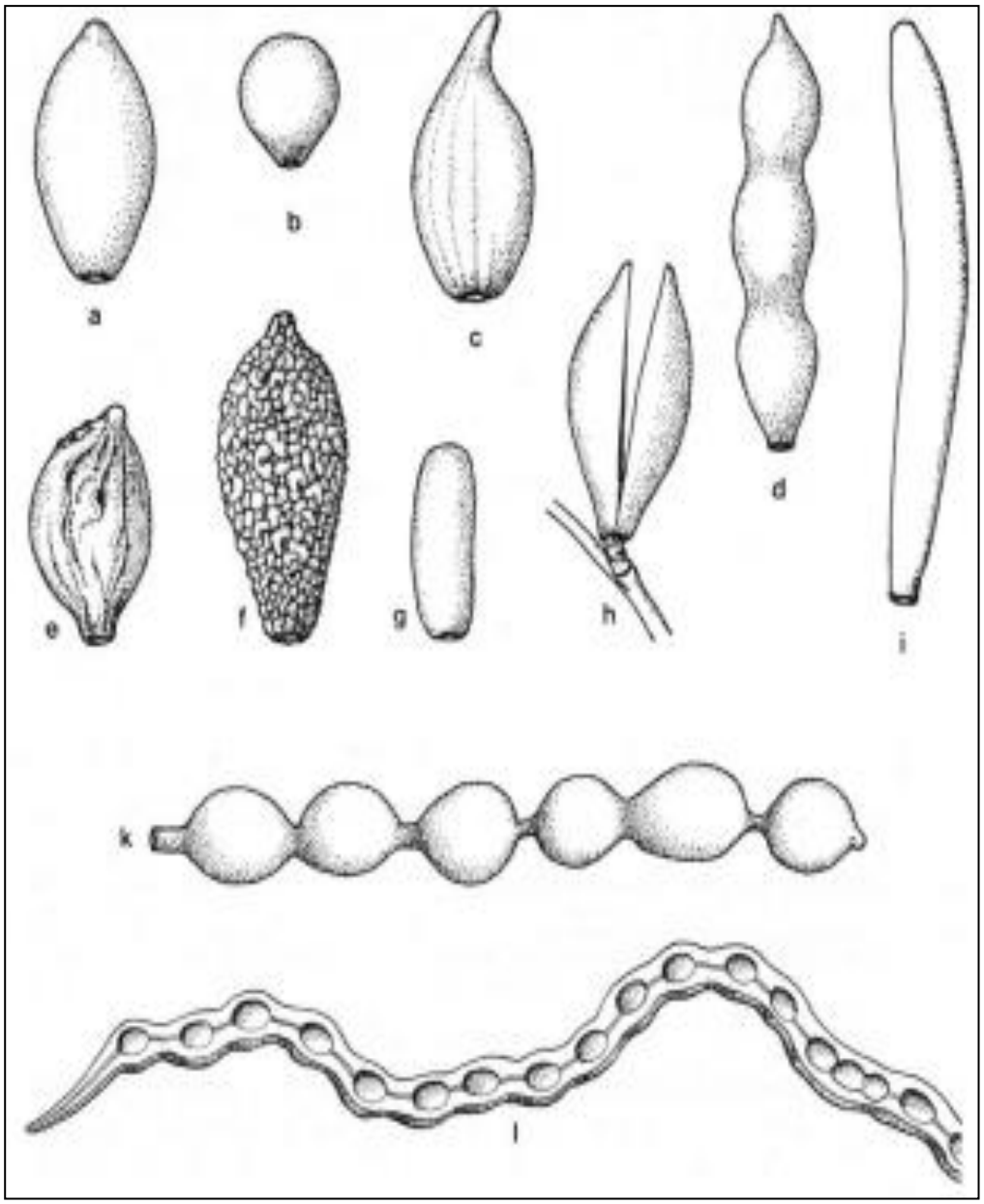


Figure 2.1. Fruits of *Chilocarpus* species. (a) Miq. (b) *embelioides* K. and G. (c) *rostatus* Markgr. (d) *beccarianus* Pierre (e) *vernicosus* Bl. (f) *tuberculatus* Markgr. (g) *decipiends* Hook. f. (h) *suaveolens* Bl. (i) *kuchingensis* Markgr. (k) *cospicuus* (Steen.) Markgr. (l) *torulusus* (Boerl.) Markgr. (Source: Markgraf, 1971)

Members of the genus *Chilocarpus* have been used traditionally. For instance, *C. malabaricus* Bedd. is used by local people in India to treat skin disease by rubbing the leaves on the skin (Prasad *et al.*, 2013). Local people in Perak used stems of *C. costatus* Miq. for wound healing purposes (unpublished data).

So far, there are no reports available on the possible pharmacological activities of *C. costatus* Miq. This is the first detailed study on the pharmacological activity of this plant. *C.malabaricus* Bedd. is antimicrobial to *E. coli* and *S. aureus* (Prasad *et al.,* 2013). US patent (Asiedu *et al.,* 2014) claimed that *Chilocarpus* species would be useful to AIDS.

No chemical constituents have been reported for *C. costatus* Miq. This is the first detailed study done on *Chilocarpus costatus* constituents.

C. costatus was collected in a local rainforest in Manong, Perak, Malaysia in December 2014 and sent to Mr. Kamarudin Saleh from Forest Biodiversity Division, Forest Research Institute Malaysia (FRIM) Herbarium Kepong, Kuala Lumpur, Malaysia in August 2015 for plant species identification (PID 320815-18; UNMC-81).

2.3.1.2. *Tabernaemontana peduncularis* Wall.

T. peduncularis Wall. belongs to the *Tabernaemontana* genus, Apocynaceae family, and Gentianales order (USDA, 2013). The synonyms are

Ervatamia graciliflora (Wall.) Lace, *Ervatamia langbianensis Lý*, *Ervatamia peduncularis* (Wall.) King & Gamble, *Ervatamia repeunsis* Pierre ex Spire, *Tabernaemontana collignonae* Van Heurck & Müll.Arg., *Tabernaemontana graciliflora* Wall., and *Tabernaemontana repoevensis* Pierre ex Pit. The vernacular name is ‘‘deranjang’’. It is found Myanmar, Thailand, Cambodia, Vietnam, and Malaysia (USDA, 2013).

T. peduncularis Wall. is a shrub which can grow from 1 to 15 m tall. Members of the genus *Tabernaemontana* have opposite leaves with milky sap. The flowers are fragrant and are 1 to 5 cm in diameter. The leaves are deep green, 15 cm long, and 5.1 cm wide. The roots have a bitter taste (USDA, 2013).

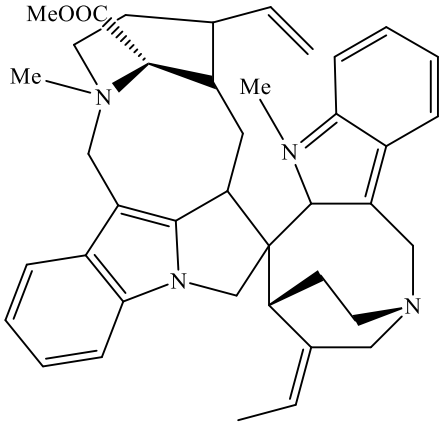
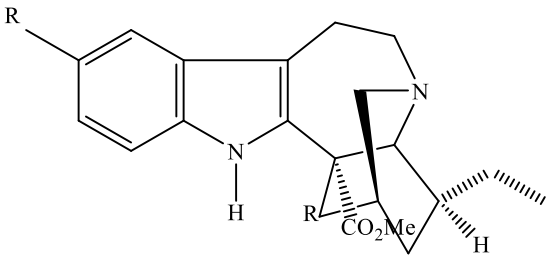
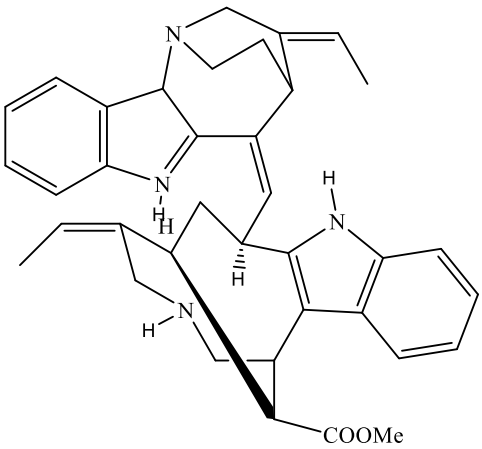
Tabernaemontana genus has been used traditionally. Some of the uses have been verified, others invalidated, while several remain undefined (Silveira *et al.,* 2017). For instance, the flower of *T. divaricata* (L.) R. Br. ex is used traditionally by the East Indian community to treat eye and measles problems (Das, 2016). The root of *T. recurva* Roxb. is rubbed over the body by native Indian in treating fever (Rahman *et al.,* 2007).

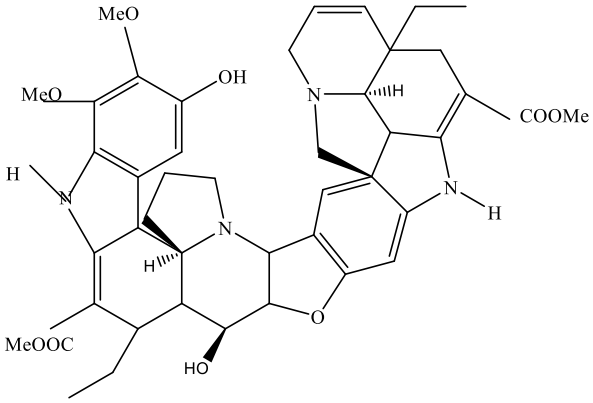
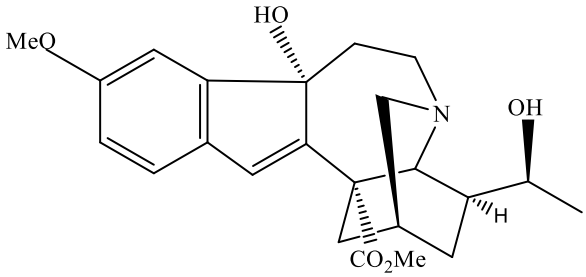
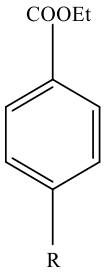
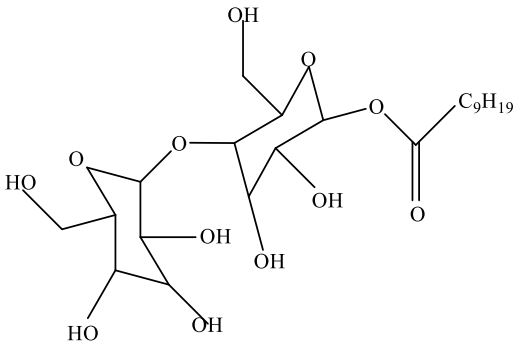
Some research has been done on the pharmacological activities of the genus *Tabernaemontana.* Hydroethanol extract from the leaves of *T. catharinensis* displayed high radical scavenging activities (Boligon *et al.,* 2013). Besides, antitumor activities against NCI-H460 (human lung cancer) cells and SF-268 (human glioblastoma) cells have been reported from the methanol extracts from stems of *T. angulata* (Marinho *et al.,* 2016). Methanolic extracts

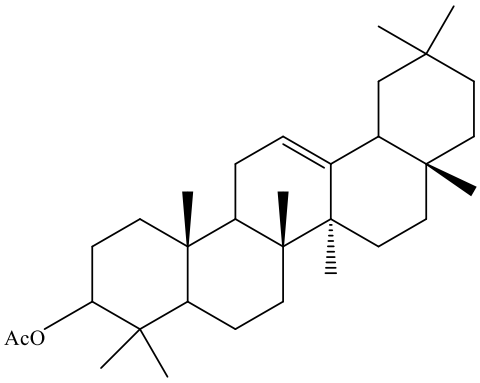
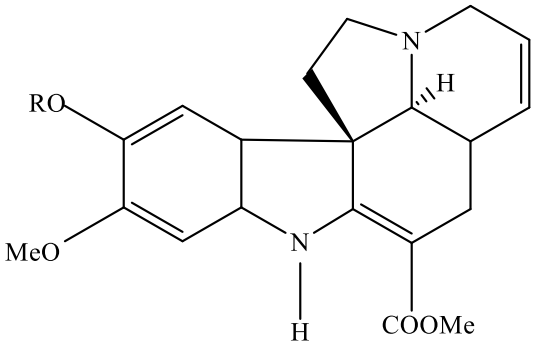
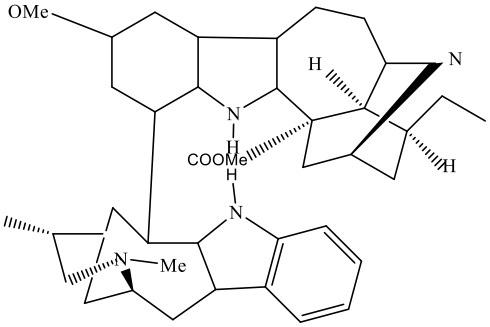
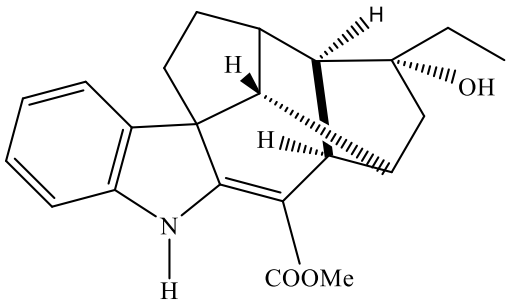
of *T. divaricata* aerial displayed analgesic and antipyretic activities (Kanthlal *et al.,* 2011). *T. divaricata* showed antioxidant, anti-infectious, and anti-tumour activities, as well as analgesic and anti-cholinergic activities (Pratchayasakul *et al.,* 2008). Hydroethanol stem bark extract of *T. crassa,* ethanol leaves extract of *T. divaricata,* aqueous extracts of *T. pachysiphon,* and ethanol extracts of *T. solanifolia* caused cytotoxic effects (Silveira *et al.,* 2017).

Members of the genus *Tabernaemontana* produce indole alkaloids such as monogagaine, voacangine, vobparicine, conophylline, voacristine hydroxyl indolenine, ethyl-4-n-octyl benzoate, digalactosyl deconate, b-Amyrin acetate, jerantinine A, ervadivaricatine A, and pandine, most of which have good pharmacological activities, as shown in Table 2.1. (Silveira *et al.,* 2017). Although *Tabernaemontana* species potentially presents several biologically active compounds (Table 2.1.), some species are not known for their chemical or biological properties. To date, there is no research on the pharmacological activity of this plant.

Table 2.1. Chemical constituents of the genus *Tabernaemontana* and their activities (Source: Silveira *et al.*, 2017)

Chemical constituents	**Isolated from**	**Therapeutic uses**
Monogagaine 	*T. chippi* and *T. dichotoma*	Antibacterial properties
Voacangine 	*T. citrifolia*	Antibacterial, anti-angiogenic properties
Vobparicine 	*T. chippi*	Antibacterial properties

Conophylline 	*T. microphylla*	Anti-hepatic, anti-diabetic properties
Voacristine hydroxy indolenine 	*T. microphylla*	Antioxidant properties
Ethyl-4-n-octyl benzoate 	*T. divaricata*	Antioxidant properties
Digalactosyl deconate 	*T. divaricata*	Antioxidant properties

b-amyrin acetate 	*T. amygdalifolia*	Enzyme inhibitors
Jerantinine A 	*T. corymbosa*	Anti-cancer
Ervadivaricatine A 	*T. divaricata*	Anti-tumour
Pandine 	*T. bufalina*	Anti-inflammatory

T. peduncularis was collected in a local rainforest in Manong, Perak, Malaysia in December 2014 and sent to Mr. Kamarudin Saleh from Forest Biodiversity Division, Forest Research Institute Malaysia (FRIM) Herbarium Kepong, Kuala Lumpur, Malaysia in August 2015 for plant species identification (PID-330815-18; UNMC-82).

2.3.1.3. *Leuconotis eugeniifolia* (Wall. ex. G. Don) A. DC.

L. eugeniifolia is classified under *Leuconotis* genus, family Apocynaceae, and order Gentianales (Endress and Bruyns, 2000). The synonyms are *Leuconotis cuspidata* Blume, *Melodinus eugeniifolius* Wall. ex G. Don. and *Leuconotis griffithii.* The vernacular names are ‘‘akar getah garah’’, ‘‘akar gerip putih’’, ‘‘akar getah sundik’’, and ‘‘gegrip sundik’’. It is found in Peninsular Malaysia, Singapore, and Borneo.

L. eugeniifolia have sparsely lenticellated to densely pubescent branches, narrow ovate to linear sepals. A single flower and petals which are acute to acuminate apex. The stamens are 6.4 to 6.7 mm long . The fruit is ovoid pear shaped and strongly wrinkled (Lu *et al.*, 2014).

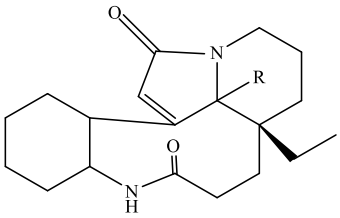
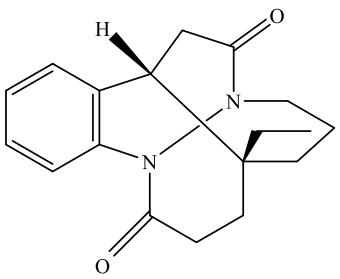
L. eugeniifolia has been used traditionally as a source of rubber before the widespread use of *Hevea brasiliensis,* and externally used to treat yaws.

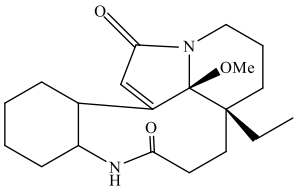
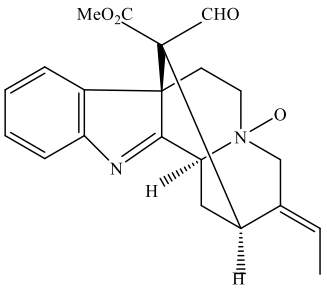
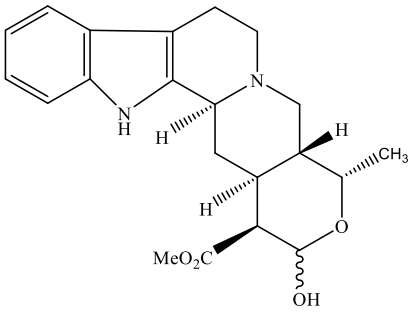
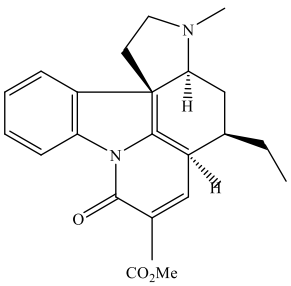
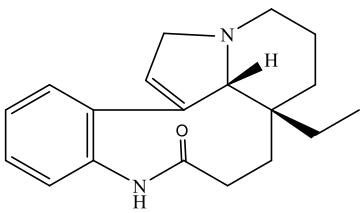
Chloroform extract of *L. eugeniifolia* leaves against *P. falciparum* 3D7 showed weak antimalarial effect with IC_{50} above 500 μg/ml (Khaw *et al.*, 2015).

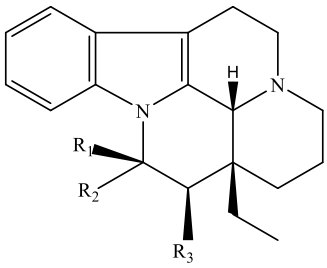
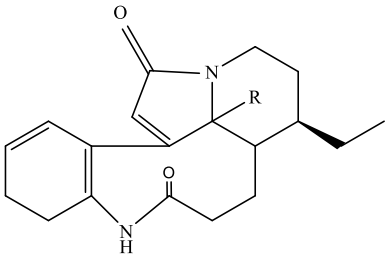
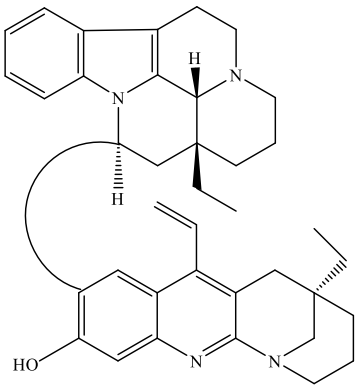
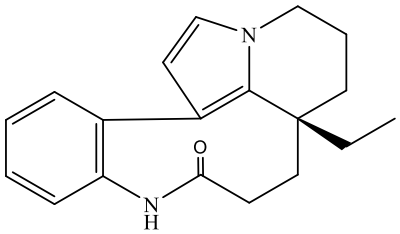
However, other publications reported that members of the genus *Leuconotis* have antimalarial (Wang *et al.*, 2013) and anticancer (Lu *et al.*, 2014) activities. Members of the genus *Leuconotis* also have activity against meningitis and rheumatic disease (Goldberg and Stoltz, 2011).

Few alkaloids have been isolated from stem, leaves, and bark of *L. eugeniifolia* such as leuconicine B, dihyrorhazinilam, eburnamine, leuconalam, epi-leuconalam, leucophyllidine and rhazinilam as shown in Table 2.2. (David *et al.*, 1997; Gan, 2013).

Table 2.2. Chemical constituents of *L. eugeniifolia* in the different part of plants (Source: David *et al.*, 1997; Gan, 2013)

Chemical constituents	**Plant part**	**References**
Leuconalam  R= β-OH	Leaves, bark and stems	David *et al.*, 1997; Gan, 2013
Leuconoxine 	Leaves and stems	Gan, 2013

21-O-Methylleuconalam 	Leaves and stems	Gan, 2013
Rhazinaline N-oxide 	Leaves	Gan, 2013
Yohimbine and *B*-yohimbine 	Leaves and stems	Gan, 2013
Leuconicine B 	Bark	Gan, 2013
5,21-Dihydronizilam 	Bark	David *et al.*, 1997; Gan, 2013

Eburnamine  R^1=OH, R^2,R^3=H	Bark	Gan, 2013
Epi-leuconalam  R= α-OH	Bark	Gan, 2013
Leucophyllidine 	Bark	Gan, 2013
Rhazinilam 	Bark	David *et al.*, 1997; Gan, 2013

L. eugeniifolia was collected in a local rainforest in Manong, Perak, Malaysia in December 2014 and sent to Mr. Kamarudin Saleh from Forest Biodiversity Division, Forest Research Institute Malaysia (FRIM) Herbarium Kepong, Kuala Lumpur, Malaysia in August 2015 for plant species identification (PID 350815-18; UNMC-84).

2.3.2. Family Annonaceae

2.3.2.1. *Artabotrys suaveolens* (Blume) Blume

A. suaveolens Blume (Blume) is classified under genus *Artabotrys,* family Annonaceae, and order Magnoliales. The synonyms are *Artabotrys blumei* Hook. f. & Thomson, *Artabotrys corniculatus* (Blanco) Merr. *Artabotrys monogynus* Merr., *Artabotrys parviflorus* Miq., *Artabotrys rolfei* S. Vidal, *A. suaveolens* var. *parviflorus* (Miq.), *Artabotrys trigynus* Merr., *Unona corniculata* Blanco, and *Unona suaveolens* Blume. The vernacular names are ‘‘akar mempisang’’, ‘‘xiang ye zhao’’, ‘‘nga pye yin’’, ‘‘manaranchitan akar cenana’’, ‘‘bahai balangan’’, ‘‘susong kalabau’’, ‘‘akakai’’, and ‘‘kai’’. It is found in India, Myanmar, Thailand, Malaysia, Indonesia, and Philippines.

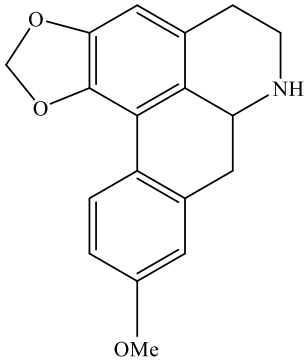
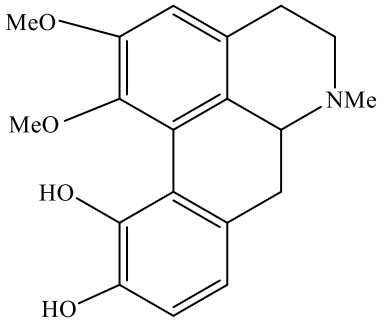
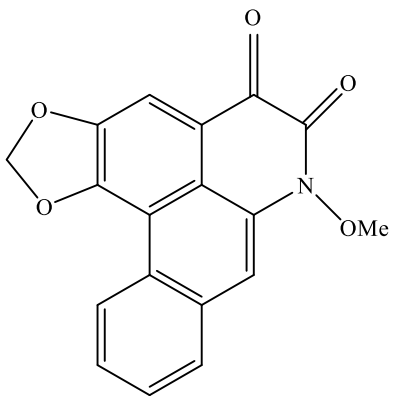
A. suaveolens (Blume) Blume is a woody climber with hook-shaped inflorescences, belonging to the Annonaceae family, which can grow up to 25 m long. The dark green leaves are simple, alternating, and 5-8.5 x 3-3.5 cm long. The flowers of members of the genus *Artabotrys* are 1 to 1.5 cm in diameter and develop 6 lobes. The fruits are bright green in color and contain a single seed.

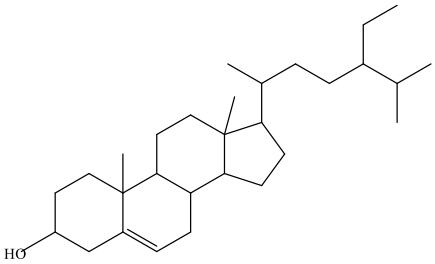
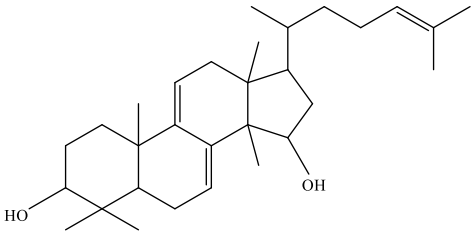
The bark and roots are used traditionally as emmenagogue (herbs which stimulate menstruation and blood flow in the pelvic area and uterus) (Aguilar, 2001). The leaves are used for treating cholera (Ong *et al.,* 2011). The twigs are used to make ropes (Wiart, 2007).

Antifungal and anti-aflatoxigenic activities were reported from the leaves of *A. odoratissimus* (Srivastava *et al.,* 2009). Good antibacterial activities were found in *A. hexapetalus* and *A. uncinatus* leaves methanolic extract against *Staphylococcus aureus, Bacillus megaterium, Pseudomonas aeruginosa,* and *Escherichia coli* (Gothandam *et al.,* 2010; Sowjanya *et al.,* 2013). High antimalarial activity against *P. falciparum* K1 strains has been observed with *A. uncinatus* (Szpilman *et al.,* 2005). Anticancer activities have been displayed in the stembark of *Artabotrys zeylanicus* against leukaemia cell lines (Wijeratne *et al.,* 1995; Ding *et al.,* 2006).

A. suaveolens shelters suaveoline (phenolic alkaloid), artabotrine (non-phenolic alkaloid), catecholic aporphine (alkaloid), isocorydine (benzylisoquinoline alkaloids), and artabotrinine (Table 2.3.) (Maranon, 1929; Barger and Sargent, 1939; Leboeuf *et al.,* 1982; Teo *et al.,* 1990).

Table 2.3. Chemical constituents of *A. suaveolens* in different part of plants (Source: Maranon, 1929; Barger and Sargent, 1939; Leboeuf *et al.*, 1982; Teo *et al.*, 1990)

Chemical constituents	**Plant part**	**References**
Artabotrinine 	Bark	Barger and Sargent, 1939; Leboeuf *et al.*, 1982
Suaveoline 	Bark	Barger and Sargent, 1939; Leboeuf *et al.*, 1982; Teo *et al.*, 1990
Artabotrine 	Roots and stem bark	Maranon, 1929; Barger and Sargent, 1939; Leboeuf *et al.*, 1982

B-sitosterol 	Not specified	Teo *et al.,* 1990
Polycarpol 	Not specified	Teo *et al.,* 1990

A. suaveolens was collected in a local rainforest in Manong, Perak, Malaysia in December 2014 and sent to Mr. Kamarudin Saleh from Forest Biodiversity Division, Forest Research Institute Malaysia (FRIM) Herbarium Kepong, Kuala Lumpur, Malaysia in August 2015 for plant species identification (PID 340815-18; UNMC-83).

2.3.2.2. *Uvaria grandiflora* Roxb. ex Hornem

U. grandiflora is classified under the *Uvaria* genus, Annonaceae family, and Magnoliales order. The synonyms are *Uvaria rubra*, *Uvaria rhodantha, Uvaria platypetala,* and *Uvaria purpurea.* The vernacular names are ‘‘pisang’’, ‘‘red hot poker’’, ‘‘pisang akar’’, ‘‘pisang tanduk’’, and ‘‘akar larak’’.It is found in Myammar, Vietnam, Laos, Sri Lanka, Thailand, Cambodia, Sumatra, Peninsular Malaysia, Singapore, Philippines, Borneo, and Java.

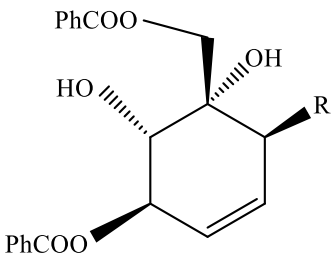
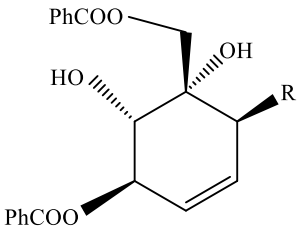
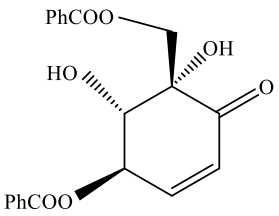
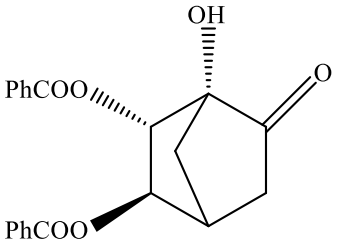
U. grandiflora is a climber shrub which is up to 10 m long. Its foliage is alternate with thin membranous blades which are oblong to oblong-obovate in shape, shiny dark green above, and pale green below. The flowers are 7 to 10 cm in diameter, with broadly ovate sepals. The fruits are cylindrical ripe carpels.

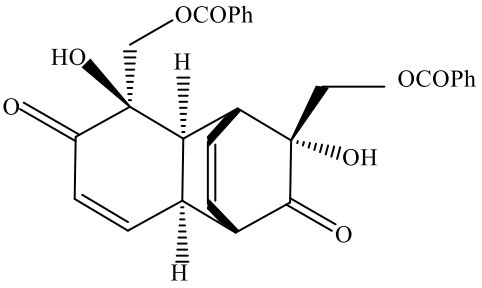
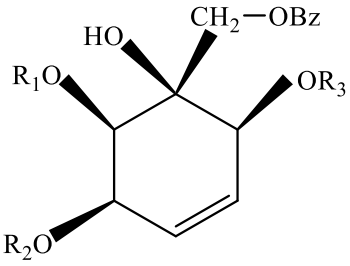
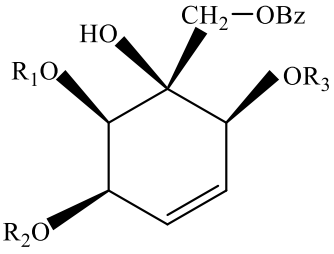
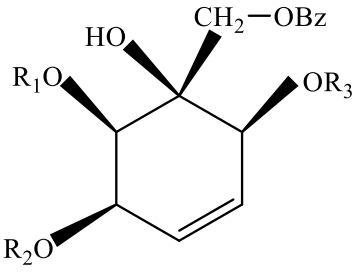
U. grandiflora was used traditionally as a source of rattan, edible aromatic fruits, treat abdominal pains, stomachache, skin diseases, and putrefaction (Aziz *et al.,* 2016).

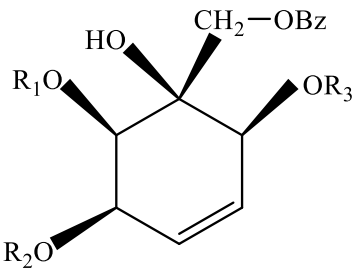
Stems and leaves of *U. grandiflora* displayed high radical scavenging activities (Aziz *et al.,* 2016). Stembarks of *U. grandiflora* have been reported active against HCT-116 colon carcinoma cells (Golshan *et al.,* 2012).

Few chemical constituents have been isolated from *U. grandiflora* such as zeylenol, chlorohydrin pipoxide, zeylenone, grandiflorone, grandifloracin (Yong-Hong *et al.,* 1997), uvariganol A, uvariganol B, uvariganol C, and uvariganol D (Xi-Ping *et al.*, 1995) (Table 2.4.).

Table 2.4. Chemical constituents of *U. grandiflora* in the different part of plants (Source: Maranon, 1929; Barger and Sargent, 1939; Leboeuf *et al.*, 1982; Teo *et al.*, 1990)

Chemical constituents	**Plant part**	**Reference**
Zeylenol  R= OH	Stem and leaves	Yong-Hong *et al.*, 1997
Chlorohydrin pipoxide  R= Cl	Stem and leaves	Yong-Hong *et al.*, 1997
Zeylenone 	Stem and leaves	Yong-Hong *et al.*, 1997
Grandiflorone 	Stem and leaves	Yong-Hong *et al.*, 1997

Grandifloracin 	Stem and leaves	Yong-Hong *et al.*, 1997
Uvariganol A  R_1=H, R_2=H, R_3=Bz	Roots	Xi-Ping *et al.*, 1995
Uvariganol B  R_1=Ac, R_2=Bz, R_3=H	Roots	Xi-Ping *et al.*, 1995
Uvariganol C  R_1=H, R_2=Bz, R_3=EtOH	Roots	Xi-Ping *et al.*, 1995

Uvariganol D  R1=Ac, R2=Bz, R3=EtOH	Roots	Xi-Ping *et al.*, 1995

U. grandiflora was collected by Assoc. Prof. Dr. Christophe Wiart from the School of Pharmacy, Faculty of Science, University of Nottingham Malaysia Campus (UNMC) in February 2015. Plants were identified and deposited in UNMC (UNMC-64).

2.3.3. Family Ebenaceae

2.3.3.1. *Diospyros wallichii* King & Gamble

D. wallichii King & Gamble is classified under the *Diospyros* genus, Ebenaceae family, and Ericales order. The synonyms are *Diospyros bakhuisii* Boerl & Koord-Schum and *Diospyros pulchrinervia* Kostem. The vernacular names are ‘‘kayu baleh’’, ‘‘kayu malam’’, ‘‘kayu tutup kebali’’, ‘‘mauhi’’, ‘‘lapad perurut’’, and ‘‘tubai buah’’. It is found in Southeast Asia, mainly in India, Burma, Thailand, Peninsular Malaysia, Sumatra, and Borneo (Arifullah *et al.,* 2014).

D. wallichii is a tree 20 to 32 m tall and 56 cm in diameter. Young twigs

are hairy. The leaves are rusty-hairy, oblong-elliptic, and acuminate apex. The inflorescences are slender 1 to 2 cm long with 9 flowers. The female calyx of flowers comprises 4 valvate lobes. The fruits are globose.

Lundayeh people in Sabah use the leaves and shoots of *D. wallichii* for treating yellow skin traditionally, by washing and rubbing the leaves externally.

Extracts of fruits and stembarks of *D. wallichii* have been reported active against HCT-116 colon carcinoma cells (Golshan *et al.,* 2012). Besides, they have also been reported to have high cytotoxicity, antimicrobial, and antioxidant activities (Arifullah *et al.,* 2014). *D. wallichii* has also good antibiotic and antiseptic properties (Nematollahi *et al.,* 2011). Few phenolic compounds have been isolated from *D. wallichii* such as gallic acid, methylgallate, ellargic acid, kaempferol, and quercetin (Arifullah *et al.,* 2014).

D. wallichii was collected by Assoc. Prof. Dr. Christophe Wiart from the School of Pharmacy, Faculty of Science, University of Nottingham Malaysia Campus (UNMC) in February 2015. Plants were identified and deposited in UNMC (UNMC-63).

Chapter 3: Plant collection and extraction

3.1. Introduction

3.1.1. Selection and identification of plants

Selection of suitable plants is crucial and important for isolation of biologically active compounds as candidates for pharmaceutical agent**s** (Tan, 2015). Each plant chosen is unique and contains different types of secondary metabolites.

Selected plants are placed in herbaria used by professional botanists for plant identification. This identification step is extremely important in conducting a research. Some plants may look similar but belong to different species with different chemical profiles (Shipton, 2017). Examination of plant phenotypes is done carefully by comparison with voucher specimen to confirm identity (Nesbitt *et al.*, 2010).

3.1.2. Plant extraction

The basic phase of general utilization of biologically active compounds from plant sources started with proper preparation of the extracts or pure compounds by grinding the plant into a fine powder**.** The plant is ground into a fine powder to rupture the cells, increase the surface area of the plant material

and allow chemicals to dissolve faster (Rogers and Bendich, 1989; Azwanida, 2015; Shipton, 2017).

Besides, a proper extraction method is critical, since it has a major and crucial impact on yields (Azmir *et al.,* 2013). There are few common extraction methods including maceration, infusion, digestion, decoction, percolation, supercritical fluid, and phytonic process (Chu, 2014).

At the same time, the choice of solvent determines the type of phytochemicals extracted. Polarity results from the distribution of electrons across molecules such as lone pair electrons (electronegative electrons) and π electrons (aromatic rings, carbon-carbon double bond, hydroxyl and carbonyl groups) (Mustafa Din, 2014).

Natural products of plants are non-polar, mid-polar, or polar. When seeking bioactive constituents, the extraction of a plant with solvents of different polarities allows to extract selectively compounds with different polarities (Tan, 2015). The yield of extract depends on few factors: part of the plant used, choice of the solvents, and selection of extraction procedures (such as temperature, pressure, and time of extraction) (Hernández *et al.,* 2009).

3.2. Materials and methods

3.2.1. Chemicals and reagents

Analytical grade n-hexane, chloroform, methanol, and acetone were purchased from RCI Labscan Co., Ltd (Bangkok, Thailand).

3.2.2. Equipment and software

Heavy duty electrical grinder (Waring, USA) and aluminium collection blender (Philips, China) were used to pulverize dried plant materials into powder form. Filtrates of the extracts were concentrated to dryness under reduced pressure at 40°C with the aid of rotary evaporator (Buchi Labortechnik AG, Switzerland) and desiccator equipped with the manual build-in vacuum pump, Thomas Scientific Inc., (New Jersey, USA). The obtained dry extracts were weighed using analytical balance, Sartorius AG (Göttingen, Germany).

3.2.3. Plant collection and identification

Four types of fresh Malaysian medicinal plants were collected from local rainforest in Kuala Kangsar and Manong, Perak, Malaysia (coordinate 4.61°N 100.87°E and 4.77 °N 100.87°E) (Figure 3.1) and sent to Mr. Kamarudin Saleh, Forest Biodiversity Division, Forest Research Institute Malaysia (FRIM), FRIM Herbarium Kepong, Kuala Lumpur, Malaysia for identification. The other two plant species which came from the same location were collected and

authenticated from Assoc. Prof. Dr. Christophe Wiart from School of Pharmacy, Faculty of Science, University of Nottingham Malaysia Campus (UNMC).

Basically, the local rainforest in Kuala Kangsar and Manong was chosen for sampling because it is a preserved primary rainforest with a huge plant biodiversity. Some of the flowering plant species have been used by local people as food and medicinal sources. Basically, the collection of plants was based on chemotaxonomical and ethnopharmacological criteria: if plants (i) have been used to treat infectious disease (Mahboob *et al.*, 2017), (ii) possess fruits or flowers (Wiart, 2007), (iii) sufficient amount for extraction and (iv) have not or poorly been studied for pharmacological activity.

Voucher herbarium specimens with vernacular names, collecting localities, and dates of collections were deposited and labelled at the Department of Pharmacy, Faculty of Science, University of Nottingham Malaysia Campus. The extracted plant samples were stored in the Department of Parasitology, Faculty of Medicine, University Malaya at -20°C until further use.

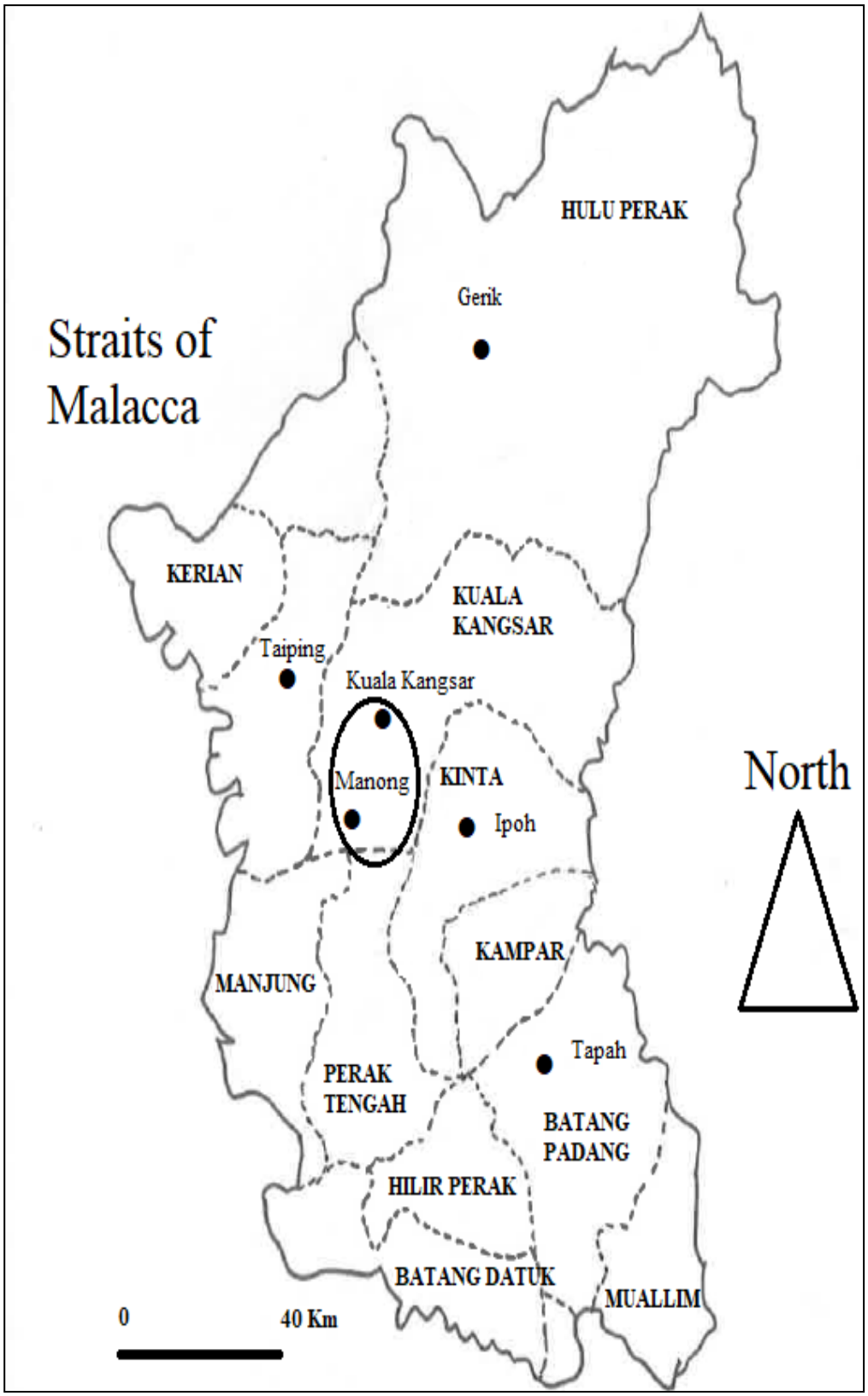


Figure 3.1. Location collection of plants

3.2.4. Plant sequential extraction

Leaves and stems were separated and air-dried at room temperature for 2 weeks. The dried materials were then finely pulverized using an electrical blender and the powders obtained were weighted with top loading balance. Two hundred grams of dried plant powders of stems and leaves were soaked at room temperature with hexane, chloroform, and methanol, respectively (Shipton, 2017).

Each extraction was performed in triplicate by macerating the plant powder-to-solvent ratio of 1:5 (w/v) for 3 days at room temperature (Shipton 2017). The respective liquid extracts were subsequently filtered through Whatman® qualitative filter paper No. 1 using aspirator pump (EW-35031-00, 18 L/min, 9.5l Bath, 115 VAC). The filtrates were concentrated to dryness under reduced pressure at 40°C using rotary evaporator.

3.2.5. Extraction yield of the plant extracts

The dry extracts obtained were weighed with an analytical balance and stored in tightly closed glass scintillation vials (Kimble, USA) at -20°C until further use. The yields of plant extracts (%) were calculated using the following formula (Tan, 2015):

$$\text{Plant extract yield (\%)} = \frac{\text{Mass of dry extract (in grams)}}{\text{Mass of dried plant powder (in grams)}} \times 100$$

3.3. Results and discussion

3.3.1. Collection of medicinal plants

Survey for evaluation and documentation of the use of medicinal plants used by local people in day-to-day practice in two preserved villages (Kuala Kangsar and Manong) in the State of Perak, Peninsular Malaysia (Figure 3.1) afforded the collection of 6 medicinal plants from 3 different families in the Class Magnoliopsida (Dicotyledons): Annonaceae, Apocynaceae, and Ebenaceae (Table 3.1) (Takhtajan, 2009). *C. costatus* Miq., *L. eugeniifolia* (Wall. ex. G. Don) A. DC. and *T. peduncularis* Wall belong to the Apocynaceae Juss. (1789). *U. grandifolia* Roxb. ex Hornem. and *A. suaveolens* (Blume) Blume belong to the Annonaceae Juss. (1789). *D. wallichii* King & Gamble belongs to the Ebenaceae Gürke (1890).

Table 3.1. Traditional therapeutic properties of 6 medicinal plants from Malaysia

Family, genus species and authority	Voucher No.	FRIM No.	Collection date	Locality	Common name	Part used	Medicinal use
Apocynaceae:							
C. costatus Miq.	035	PID 320815-18	23/6/2015	Kuala Kangsar	*Getah gerip puteh*	Stem	Wounds
L. eugeniifolia (Wall. ex. G. Don) A. DC.	036	PID 350815-18	18/6/2015	Manong	*Akar getah garah*	Leaf	Yaws
T. peduncularis Wall.	063	PID-330815-18	19/6/2015	Manong	*Deranjang*	Stem	Bacterial infection
Annonaceae:							
U. grandifolia Roxb. ex Hornem.	048	N/A	15/6/2016	Manong	*Pisang*	Leaf	Wounds
A. suaveolens (Blume) Blume	064	PID 340815-18	20/6/2015	Manong	*Akar mempisang*	Leaf	Bacterial infection
Ebenaceae:							
D. wallichii King & Gamble	049	N/A	15/6/2016	Manong	*Kayu baleh*	Leaf	Viral infection

3.3.2. Extraction yield of the crude extracts

Secondary metabolites from leaves and stems were extracted by successive sequential maceration in solvent starting from hexane, chloroform, and methanol in sequence, respectively. The percentage yields of the extracts were expressed as per dry weight of plant material, calculated from the formula of Tan (2015).

Table 3.2 presents plant extract yield values obtained between plant species, from the lowest 0.01% (*C. costatus* stem hexane extract) to the highest 6.44% (*A. suaveolens* stem methanol extract). Based on the results, each different plant species had different yields. Among the type of solvent used, the highest yield was obtained mainly from methanolic extracts, while moderate yield on most chloroform extracts and the lowest yield on most hexane extracts. The more polar the solvent, the higher the yield obtained (Tan, 2015; Shipton, 2017).

In short, extraction yield act as a tool in determining the solvent efficiency (Aspe and Fernandex, 2011; Tsai *et al.,* 2012). We choose chloroform to extract plant parts because: (i) it does not extract tannins (which give false positive pharmacological activities) and (ii) we are looking for mid-polar constituents which according to Lipinski have better ‘‘drugability’’(Harborne, 1998; Surade and Blundell, 2012). Therefore, only chloroform extracts were used for all experiments because of their selectively mid-polar constituents. Mid-polar molecules have better ‘‘drugability’’ (absorption, distribution, metabolism, and

excretion (ADME) as per Lipinski´s rule of 5 (Surade and Blundell, 2012). Molecules which are mid-polar have higher chance of being active orally.

Table 3.2. The extraction yield of the crude extracts

Plants	Part of plants	Weight of plant powder (in g)	Plant dry extracts (in g)			Dry yield of the crude extracts (%)		
			Hexane	Chloroform	Methanol	Hexane	Chloroform	Methanol
C. costatus	Leaves	200	1.99	1.76	2.83	0.99	0.88	1.41
	Stems		0.02	5.63	0.37	0.01	2.81	0.18
T. peduncularis	Leaves	200	1.46	2.72	4.91	0.73	1.36	2.45
	Stems		1.36	0.03	3.54	0.68	0.01	1.77
A. suaveolens	Leaves	200	1.13	3.78	11.19	0.57	1.89	5.59
	Stems		0.10	2.85	12.88	0.05	1.42	6.44
L. eugeniifolia	Leaves	200	1.43	0.04	1.10	0.72	0.99	0.55
	Stems		3.55	2.89	1.51	1.78	1.44	0.75
D. wallichii	Leaves	200	0.67	2.47	1.93	0.34	1.23	0.96
	Stems		0.22	2.12	3.00	0.11	1.06	1.50
U. grandiflora	Leaves	200	1.64	1.85	1.89	0.82	0.92	0.94
	Stems		0.33	2.56	2.38	0.17	1.28	1.19

3.4. Conclusion

Collection of plants in Perak afforded 6 dicotyledons from three different families of the Magnoliopsida class: Annonaceae, Apocynaceae, and Ebenaceae. Each of the plants was separated into two parts: stems and leaves and extracted. The extraction yields obtained using sequential solvent varied between plants and parts. The highest yield was obtained with methanolic stem of *A. suaveolens* extract (6.44%) and the lowest yield was obtained with hexane extract of *C. costatus* extract (0.01%). The more polar the solvent, the higher the yield obtained (Tan, 2015; Shipton, 2017). Chloroform extracts were used for all experiments because we target mid-polar molecules.

Chapter 4: Antiparasitic activities of plant chloroform extracts

4.1. Introduction

4.1.1. Malaria and leishmaniasis diseases

Malaria and leishmaniasis are considered as some of the most lethal infectious disease in the world according to Global Burden of Disease (GDB) study in 2015 (Deribew *et al.*, 2017). In 2016, about 216 million cases of malaria cases were reported in 91 countries worldwide with 445 thousand deads including children (WHO, 2016). The same year, about 700,000 to 1 million new cases of leishmaniasis were reported worldwide, with 20,000 to 30,000 deaths (WHO, 2016).

Malaria and leishmaniasis have also been listed as major life-threatening diseases in Southeast Asian (WHO Southeast Asia, 2015). In 2015, approximately, 1.4 million cases of malaria occurred in Southeast Asia (SEA) with the majority in Bangladesh, Myanmar, Bhutan, Nepal, Korea, Sri Lanka, India, Thailand, Indonesia, and Malaysia (WHO Southeast Asia, 2015). Roughly, 200 million cases of leishmaniasis have been reported in Southeast Asia, mostly in Indonesia, Bangladesh, India, Nepal, and Bhutan (WHO Southeast Asia, 2015).

Malaria is caused by *Plasmodium*, transmitted to people through the bite of infected female *Anopheles* mosquitoes (WHO, 2016). Meanwhile,

leishmaniasis or known as kala-azar is caused by *Leishmania*, transmitted to people through the bites of infected female phlebotomine sand fly (WHO, 2016).

4.1.2. Type of causative agent for the parasite

4.1.2.1. Malaria

There are five species of *Plasmodium* parasite that cause malaria in humans: *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium knowlesi,* and *Plasmodium malariae* (Coura *et al.,* 2006). Falodun *et al.* (2014) reported that *Plasmodium falciparum* (*P. falciparum*) is responsible for more than 200 million of clinical malarial cases around the globe, with majority of death from tropical and subtropical areas in ASEAN countries. Therefore, *P. falciparum* species will be focused on this research study.

4.1.2.2. Leishmaniasis

There are two *Leishmania* species that cause leishmaniasis in humans: *Leishmania donovani* and *Leishmania infantum* (Chappuis, 2007; Zulfiqar *et al.,* 2017). *Leishmania donovani* (*L. donovani*) species are found in East Africa, and Southeast India while *Leishmania infantum* species (sometimes recognized as *L. chagasi*) are found in Europe, North Africa, and America (Chappuis, 2007). Chappuis (2007) reported that *L. donovani* is the most pathogenic species. Therefore, *L. donovani* species will be used in this research study.

4.1.3. Symptoms of the parasitic infection

4.1.3.1. Symptoms for malaria

The seriousness of malaria depends on the type of *Plasmodium* parasite species, parasite strains, and level of human immunity (Coura *et al.*, 2006). The basic symptoms of malaria include headache, fever, shivering, joint pain, prostration (kneeling), vomiting, haemolytic anaemia, clinical jaundice, haemoglobin in the urine, and retinal damage (WHO, 2000). Multiple convulsions (seizures), deep breathing, and respiratory distress with paroxysm (sudden cold with shivering) are also evoked (WHO, 2000).

4.1.3.2. Symptoms for leishmaniasis

Visceral leishmaniasis is the most severe among all three types of leishmaniasis (visceral, cutaneous and mucocutaneous leishmaniasis) (Chappuis, 2007; WHO, 2016). Clinical symptoms for leishmaniasis include fever, weight loss, loss of appetite, fatigue, anaemia, weakness, substantial liver, spleen swelling, hepatosplenomegaly, pancytopenia, lymphadenopathy, cytopenia, and hypergammaglobulinemia (Buckner and Schwartz, 2017; Zulfiqqar *et al.*, 2017). Patients will suffer intercurrent infections such as pulmonary tuberculosis, dysentery, and gastrointestinal haemorrhage (Buckner and Schwartz, 2016).

4.1.4. Life cycle of parasite

4.1.4.1. Life cycle of *P. falciparum*

The life cycle of *P. falciparum* begins with the introduction of an asymptomatic liver stage, followed by a symptomatic blood stage (Dalai *et al.*, 2015). As illustrated in Figure 4.1, the life cycle of *P. falciparum* starts in human by the introduction of the parasite through the bite of infected female *Anopheles* mosquito (Enomoto *et al.*, 2012). The sporozoite from the site of injection reaches the liver and infects hepatocytes and multiplies to produce thousands of merozoites. The invasion of erythrocytic cycle of *P. falciparum* begins when merozoites enter the bloodstream and infect red blood cells (RBCs), producing different stages in infected blood, such as ring-infected, gametocytes, trophozoites (ring-shaped, vacuolated, and uninucleated), and schizonts (segregation of trophozoites) (Table 4.1). The cycle of parasites within the RBCs repeats after 48 hours.

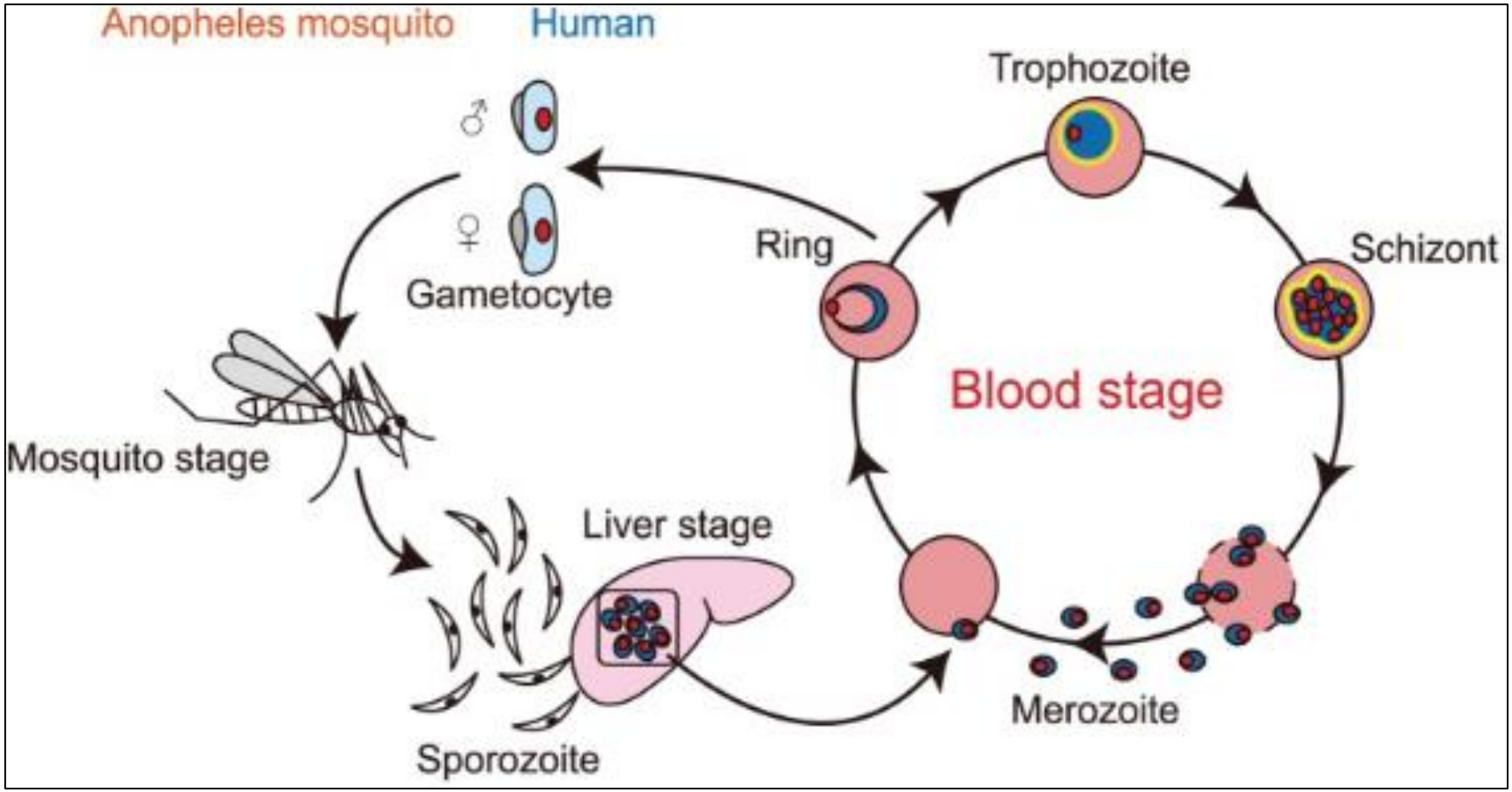
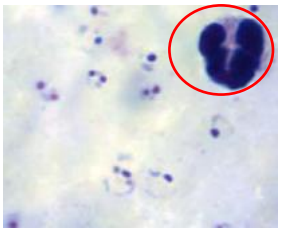
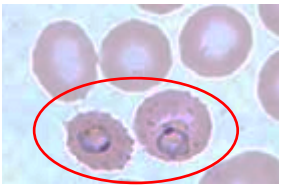
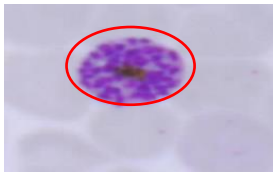
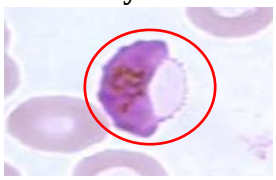


Figure 4.1. The life cycle of *P. falciparum* (Source: Enomoto *et al.*, 2012)

Table 4.1. Morphological observation of the blood-stages of *P. falciparum* (Source: CDC, 2016)

Morphology	Characterization
Ring 	*P. falciparum* rings have a delicate cytoplasm and one or two small dots of chromatin. Red blood cells that are infected are not enlarged. Occasional forms (rings appearing on the periphery of the red blood cells) may be present.
Trophozoites 	*P. falciparum* mature trophozoites have denser cytoplasm compared to younger rings. As trophozoites grow, they retain their ring-like/ amoeboid shape with some trace amounts of yellow pigment that can be seen within the cytoplasm.
Schizonts 	*P. falciparum* schizonts are seldom seen in peripheral blood. Mature schizonts have 8 to 24 small merozoites, dark pigment, clumped in one mass.
Gametocytes 	*P. falciparum* gametocytes are crescent or sausage (shape) with chromatin in a single mass (macrogamete) or diffuse (microgamete).

4.1.4.2. Life cycle of *L. donovani*

The life cycle of *L. donovani* needs two hosts: human and sand flies. Two different forms exist in the life cycle of *L. donovani*: promastigote (found in the midgut of the female phlebotomine sand flies) and amastigote (found in the macrophages of the human host) (Table 4.2). As illustrated in Figure 4.2, the life cycle of *L. donovani* starts by the bite of infected female phlebotomine sand flies. Infected sand flies inject the infective stage of promastigotes from its proboscis into the development of their eggs during the blood meal.

The phagocytosis by macrophages and other mononuclear phagocytic cells transforms promastigotes into amastigotes (round and non-motile). Amastigotes then multiply and invade other cells, such as dendritic cells, neutrophils, and fibroblasts, continuing parasite replication. The anthroponotic transmission (from an infected human to non-infected humans) of leishmaniasis occurs when another sand fly sucks blood from the infected human host and bites another human (Zulfiqar *et al.*, 2017).

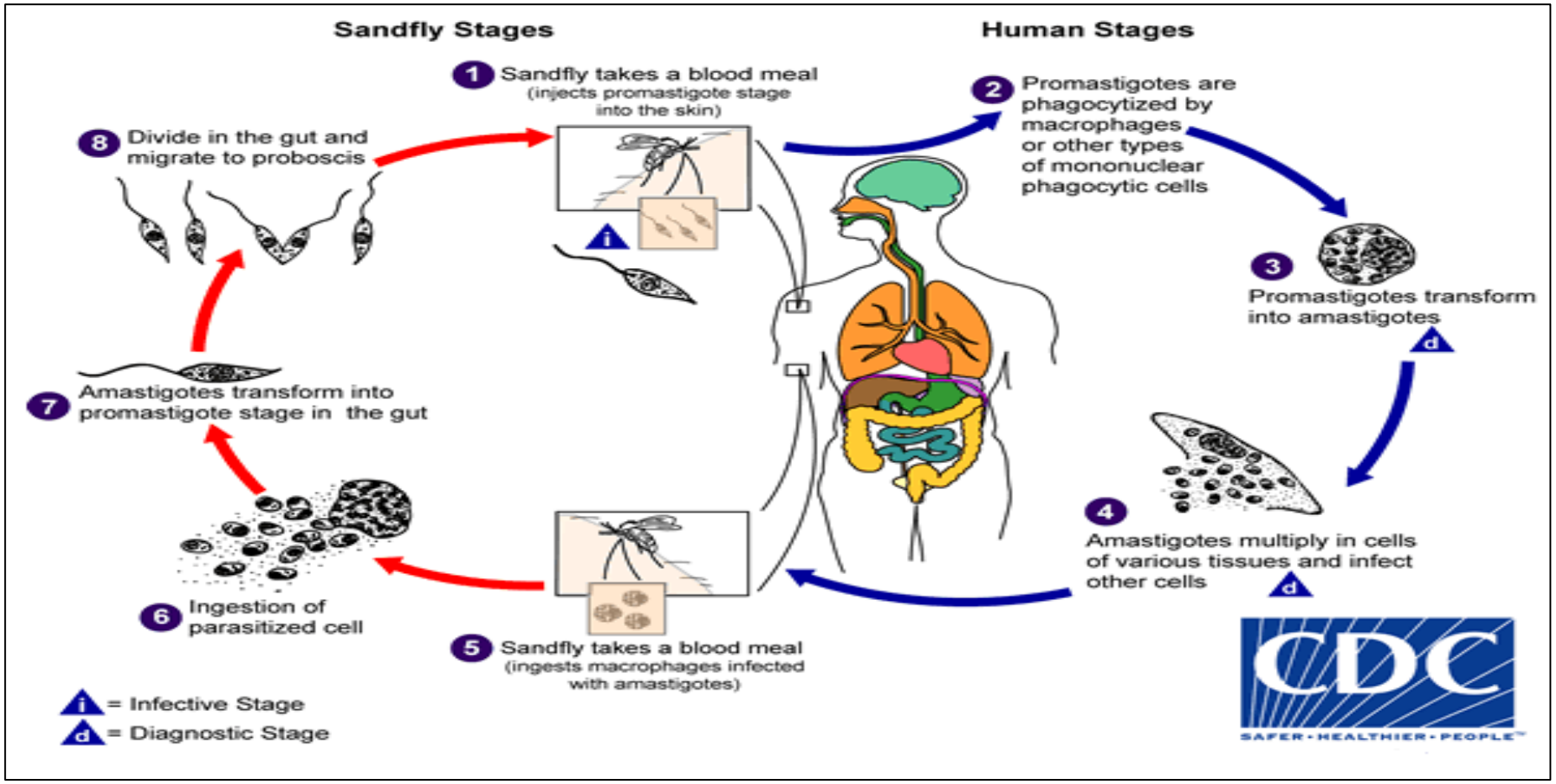
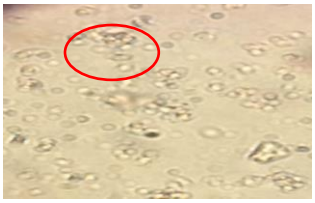
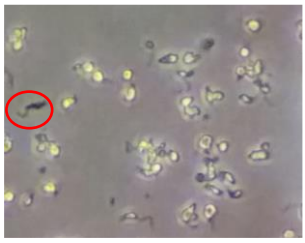


Figure 4.2. The life cycle of *L. donovani* (Source: CDC, 2016)

Table 4.2. Morphological observation of the *L. donovani* stage (Source: CDC, 2016)

Morphology	Characterization
Amastigote 	Amastigotes of *L. donovani* are divided by binary fission at 37°C, in a flagellar stage, which occurs in the vertebrae host. They are round or oval (2 to 4µm along the longitudinal axis) and their nucleus is relatively larger and situated centrally.
Promastigote 	Promastigotes of *L. donovani* are divided by binary fission at 25 to 27°C, in a flagellar stage, which occurs in the female sand fly. They are spindle in shape (15 to 20µm in length and 1 to 2µm in width) and their nucleus is smaller and situated in the middle of the cell.

4.1.5. Modern treatment

4.1.5.1. Modern treatment for malaria

Methods for controlling the transmission of malaria diseases involve insecticide-treated mosquito nets, indoor residual spraying, and chemotherapy (Wright, 2005). Chemotherapy remains the main control of malaria with chloroquine, quinine, halofantrine, mefloquine, and artemisinin. However, these drugs start to be ineffective for *P. falciparum* (Ukwe *et al.,* 2010). Combination of drugs however can be effective. For instance, chloroquine with

pyrimethamine (Dondero *et al.*, 1976) and sulfadoxine with pyrimethamine (SDX/PYR) (Black *et al.,* 1981; Ponnampalam, 1982) may increase therapeutic efficacy, decrease the level of cytotoxicity, and delay the emergence of antiplasmodial drug resistance (Ukwe *et al.*, 2010).

However, resistance to *P. falciparum* has been reported (Ukwe, 2010). The life cycle of *P. falciparum* makes it difficult to develop effective vaccines (Dalai *et al.*, 2015; Snow *et al.,* 2005; Walochnik and Duchêne, 2016). In addition, Ukwe *et al.* (2010) reported that modern antiplasmodial drugs have their own limitations, such as low dosage level and high cost to implement (Ukwe *et al.*, 2010).

4.1.5.2. Modern treatment for leishmaniasis

There are several treatments and formulations available in order to control leishmaniasis. However, the main therapy in controlling leishmaniasis includes pentavalent antimonial. Since the 1970s, pentavalent antimonial drugs like sodium stibogluconate and meglumine antimoniate have been used as leishmaniasis first-line treatment until banned in certain countries (such as India and Nepal) due to high resistance issues (Prajapati and Pandey, 2017). Amphotericin B in the 1990s is considered as the best alternative chemotherapeutic methods for leishmaniasis first-line treatment (Prajapati and Pandey, 2017).

However, amphotericin B is highly toxic, unstable at high temperatures, and costly (Rajagopal, 2014). Miltefosine, paromomycin, and pentamidine were introduced in the 2000s as first oral drugs for the treatment of visceral leishmaniasis. Nowadays these drugs are facing resistance (Prajapati and Pandey, 2017; Zulfiqar *et al.*, 2017). The application of combination therapy (for example sodium stibogluconate with paromomycin, liposomal amphotericin B with miltefosine, and the combination of three drugs of miltefosine, liposomal amphotericin B with paromomycin) instead of monotherapy alone in treating the leishmaniasis disease prevented parasite resistance (Zulfiqar *et al.*, 2017).

4.1.6. Natural antiparasitic agents/ traditional medicines

The widespread use of traditional and herbal medicines from plants seems to be an alternative for parasitic diseases, especially in endemic areas (Rahman *et al.*, 1999). For example, antiplasmodial and antileishmanial agents derived from natural products are the best option for treating malaria and leishmaniasis as they can be easily accessible within that marginalized area. In fact, urgent need to treat diseases forced people in endemic areas to use traditional herbal medicines, mainly from plants (Falodun *et al.*, 2014). Considering this great potential of plants and the need to find novel alternative drugs for the disease, we attempted herewith to isolate antiparasitic agents.

Plants are important potential source of antimalarial medicines. Quinine and artemisinin are main examples. These drugs are derived from the traditional plants, namely *Cinchona* tree and *Artemisia annua* (Nguyen-Pouplin *et al.*,

2007). Other plants are *Microglossa pyrifolia*, *Clerodendrum rotundifolium*, *Corymbia citriodora*, *Calotropis procera*, *Annona squamosal*, *Holarrhena pubescens*, *Tabernaemontana elegans*, *Vangueria infausta*, *Stephania rotunda*, *Brucea javanica*, *Zanthoxylum chalybeum*, *Cyperus articulates*, *Cissampelos pareira*, *Erythrina caffra*, *Ochna schweinfurthiana*, *Fuerstia Africana*, *Satureja parvifolia*, *Cinchona succirubra*, and *Nauclea latifolia* (Azman *et al.*, 2017).

Thousands of plants and natural products have been screened for antiplasmodial activity and demonstrated antiplasmodial activities *in vitro* (Nugroho *et al.*, 2012). These include aporphine alkaloids from *Tinospora crispa* (L.) Hook. f. and Thomson (family Menispermaceae) (Ahmad *et al.*, 2016), indole alkaloids from *Leuconotis griffithii* (Retz.) Gardner ex Thwaites (family Apocynaceae), flavonoids from *Kaempferia parviflora* Wall. ex Baker (family Zingeriberaceae) (Yenjai *et al.*, 2004), and pentacyclic triterpenes from *Diospyros peregrina* (Gaertn.) Gurke (Kamaraj *et al.*, 2012).

A study in endemic areas from poor countries revealed that most of patients use plants as alternative remedies for leishmaniasis (Mahboob, 2018). Plants such as *Ocimum gratissimum*, *Anisomeles malabarica*, *Ricinus communis*, *Polyalthia suaveolens*, potato tuber, *Opuntia ficus indica* are actively against promastigotes *L. donovani* (Azman *et al.*, 2017).

4.1.7. Techniques used to study antiparasitic activities

The Histidine-Rich Protein II (HRPII) assay, SYBR green, and nanotechnologies are used to investigate antiplasmodial activities. Each method varies in term of duration, solvents, and procedures used. However, the end results are similar. In 1976, Trager and Jensen established an *in vitro* culture technique for *P. falciparum.* Nowadays, the technique is used widely for culturing *P. falciparum.* In this chapter, ring-infected stage was selected for HRPII assay as per Noedl *et al.* (2002), Razak *et al.* (2014) and Bozdech *et al.* (2003).

Methods used for screening *Leishmania* parasites include direct counting of promastigotes and intracellular amastigotes, absorbance and fluorescence, flow cytometry, radionucleotide uptake assay, reporter gene assays, use of transgenic *Leishmania* for *in vivo* real-time imaging, high-throughput screening (HTS), high-content imaging (HCI), as well as *ex vivo* screening assay. Stabilization of *Leishmania* parasitic cells was done in the Novy-MacNeal-Nicolle (NNN) medium (Ladopoulos *et al.,* 2015).

Subsequently, *Leishmania* parasites were transferred to the RPMI 1640 medium supplemented with fetal bovine serum (Ladopoulos *et al.,* 2015). Cells were maintained at 24 to 26°C prior to promastigotes. Promastigotes were chosen as per Allahverdiyev *et al.* (2011).

4.2. Materials and methods

4.2.1. Chemicals and reagents

Analytical grade dimethyl sulfoxide (DMSO), Giemsa stain, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), chloroquine, artemisinin, quinine, and mefloquine (>98% purity) were bought from Sigma-Aldrich Corporation (Missouri, USA). Vincristine (>98% purity) was obtained from ChemFaces Ltd. (Hubei, China). Miltefosine was a kind gift from Professor Shyam Sundar, Banaras Hindu University, India.

SYBR Green I was purchased from Takara BioMedicals (Tokyo, Japan). Immunoglobulin M (IgM) capture antibody was purchased from ICL Inc. (Newberg, USA). Roswell Park Memorial Institute (RPMI) 1640 Medium, Minimum Essential Medium (MEM), Novy-MacNeal-Nicolle (NNN) media, Glutamine-Penicillin-Streptomycin, Horseradish Peroxidase, Phosphate Buffer Saline-Tween 20 (PBST), 3,3',5,5'-tetramethylbenzidine (TMB) chromogen, Sorbitol, Trypan blue solution and glycerol (>99% purity) from various brand such as GIBCO, Invitrogen and Life Sciences were purchased from Thermo Fisher Scientific (Massachusetts, USA). Fetal Bovine Serum (FBS) was purchased from Biosera Ltd. (Kansas, USA).

4.2.2. Cell line and parasites

Two parasites: *P. falciparum* K1 chloroquine-resistant; MRA-159, MR4 (Virginia, USA) and *L. donovani* BHU-1251 (Rajasthan, India) strains together with normal human lung epithelial cell line MRC-5 ATCC®CCL-171™ were used. They were kept in liquid nitrogen in specific media until further anti-parasitic use.

4.2.3. Equipment and software

All absorbance-based biological assays using 96-well plates were measured using multifunctional microplate readers, Victor^2 1440, Perkin Elmer (Turku, Finland) and Spectramax M5, Molecular devices (California, USA) at different wavelength prior to the respective experiments.

The data were then analysed using different software: HN Nonlin v1.1 (specific for antiplasmodial activities), IC_{50}/IC_{90} software (specific for antileishmanial activities), GraphPad Prism v.7, and Microsoft Excel (specific for cytotoxicity assay).

4.2.4. Maintenance of cell and parasite cultures

Two parasitic cell lines (*P. falciparum* K1 chloroquine-resistant strain and *L. donovani* BHU-1251 strain) and 1 human lung epithelial cell line (MRC-5 ATCC®CCL-171™ strain) were maintained as follows:

***P. falciparum* K1 chloroquine-resistant strain** was thawed in RPMI 1640 media containing 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium bicarbonate ($NaHCO_3$), gentamycin, and fresh prepared O^+ human blood following Razak *et al.* (2014) method. Next, the cells were grown in continuous culture as described by Trager and Jensen (1976 and 1977) for few times in complete RPMI 1640 medium. The cell culture was synchronized to one stage according to Lambros and Vanderberg (1979) after it achieved 8% parasitemia using 5% Sorbitol solution.

***L. donovani* BHU-1251 strain** was thawed according to Prajapathi *et al.* (2013) method, stabilized first in NNN medium in accordance to Ladopoulus *et al.* (2015) method, and cultured to promastigote stage at 26°C in RPMI 1640 medium supplemented with Fetal Bovine Serum and L-Glutamine-Penicillin-Streptomycin-Solution later after reached stationary phase of 2.0 x 10^5 cells/mL as per described by Prajapathi *et al.* (2013) method.

Normal human lung epithelial cell line MRC-5 ATCC®CCL-171™ was thawed, grown and maintained in RPMI 1640 medium supplemented with

Fetal Bovine Serum and L-Glutamine-Penicillin-Streptomycin-Solution following Break *et al.* (2018) method.

4.2.5. Anti-parasitic activities

4.2.5.1. Antiplasmodial assay

Toxicity of extracts against chloroquine-resistant *P. falciparum* K1 strain was evaluated using the Histidine-Rich Protein II ELISA technique following Noedl *et al.* (2002). Chloroquine and dihydroartemisinin were used as positive control drugs, while media and parasite act as negative controls.

In summary, about 200 µL of each control and dilution of crude extracts at 1% parasitemia and 2% haematocrit were seeded into 96-well plates, respectively. The plates were then incubated in 5% CO_2 incubator at 37°C for 72 hours. After 3 days, the plates were stored in -80°C for overnight and thawed the next day at room temperature to lyse the ring-infected red blood cells (RBCs). This plate was labelled as Plate A.

On the other hand, 96 well plates (Plate B) were used for HRPII *P. falciparum*. In this case, 100 µL of 1 µg/mL immunoglobin M (IgM) capture antibodies in phosphate buffer saline (PBS) solution were added to the 96 well plates and incubated at 4°C for overnight. The cells were discarded the next day and rinsed with 0.05% PBS-Tween 20 (PBST) 3 times. Two hundred µL of the 2% bovine serum albumin in phosphate buffer saline solution was added to each

well, correspondingly and incubated for 2 hours at room temperature, to block unbound sites of the plates. Next, the 96 well plates were rinsed again 3 more times with 200 µL of PBST.

In HRPII assay, 100 µL of the previous freeze-thawed crude extracts with parasitic cells and standard drugs control (from Plate A) were added into each of the well plates of Plate B and incubated in a humidity chamber for 1 hour at room temperature. Then, Plate B was rinsed again using 0.05% PBST for 3 times and 100 µL of horseradish peroxidase-conjugated detector antibody (0.2 µg/mL in Phosphate Buffer Saline solution) was added. The wash step using PBST was done 3 times before adding 100 µL of 3,3',5,5'-tetramethylbenzidine (TMB) chromogen to each well. The plate was incubated for 10 min at room temperature under dark conditions and 50 µL of 1M sulphuric acid was subsequently inserted. The absorbance of the plates was measured at 485 nm using a UV-Vis spectrophotometer. The percentage of growth inhibition was calculated as follows:

$$\% \text{ inhibition} = \frac{\text{O.D. of untreated control - O.D. of treated set}}{\text{O.D. of untreated control}} \times 100$$

All experiments were performed in triplicate. Results were expressed as the concentration reducing the number of live parasites by 50% (IC_{50}) in µg/mL.

4.2.5.2. Antileishmanial assay

The method of Prajapati *et al.* (2013) was used to determine the *in vitro* toxicity of extracts or compounds against *L. donovani* BHU-1251 strain. Parasite cells were maintained in complete RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% L-Glutamine-Penicillin-Streptomycin-Solution at 26°C temperature in BOD incubator. Stationary stage of *L. donovani* promastigotes was used for the antileishmanial assay.

About 100 µL of parasite cultures (1×10^5 promastigotes) were added to each well plate. Media and parasites were used as negative control, while miltefosine was used as positive control. The same ratio of crude extracts (concentration range from 0.3125 to 200 µg/mL) was added and allowed to interact with the promastigote cells for 72 hours at 26°C in the BOD incubator. MTT assay was performed by adding and incubating 10 µL of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide reagent dissolved in Phosphate Buffer Saline solution (5 mg/mL) into each of the 96 well plates including the controls for 4 hours. The mixture was then removed and added with 100 µL DMSO. The absorbance was measured at 485 nm to check the viability of promastigotes. All experiments were performed in triplicate and results were expressed as the concentration reducing the number of living promastigotes by 50% (IC_{50}) in µg/mL.

4.2.5.3. Cytotoxicity assay

Prior to antileishmanial and antiplasmodial testing, it was necessary to evaluate the potential toxicity of chloroform extracts against mammalian cells in order to calculate the selectivity index (SI) value. The MTT assay was performed according to Mosmann (1983) with slight modifications to study the effect of toxicity. Protozoan parasites can cause tropical parasitic lung diseases such as pulmonary leishmaniasis (caused by *L. donovani*) and pulmonary malaria (caused by *P. falciparum*), which affect the main airways and parenchyma (Vijayan, 2008). Therefore, the toxicity of fractions and compounds to the mammalian cell line was tested using the MRC-5 human lung epithelial cell line.

First, MRC-5 cells were grown and cultured in both complete RPMI 1640 medium and minimal essential medium supplemented with 10% Fetal Bovine Serum (FBS) and 1% L-Glutamine-Penicillin-Streptomycin-Solution using a humidified incubator with 5% CO_2, individually. Next, Trypsin-EDTA was used to detach MRC-5 cells and count concentrations. The highest concentration of MRC-5 cells grown between the two media was selected for the entire experiment.

About 100 µL of MRC-5 (5×10^3 cells) were added to each well and allowed to attach for 24 hours before removing medium and adding plant fractions at various concentrations. At the same time, untreated cells with medium alone were used as negative controls while cells treated with quercetin

and vincristine were used as positive control drugs. Next, the same ratio of crude extracts and fractions (concentration range from 0.3125 to 100 µg/mL) was added and allowed to interact with MRC-5 cells for 48-72 hours at 37°C in the 5% CO_2 incubator.

The MTT assay was performed by adding 10 µL of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide reagent dissolved in phosphate buffer saline solution (5 mg/mL) into each of the 96 well plates at 37°C in a 5% CO_2 incubator. After incubating for 4 hours, the medium was removed from each well and 50 µL of DMSO was then added to each well and thoroughly mixed with a pipette before incubating for 10 minutes. The absorbance was read at 535 nm using a microplate reader (Mosmann, 1983; Shipton *et al.,* 2017; Mahboob *et al.,* 2017). The selectivity index (SI), was calculated as follow:

$$\text{Selectivity index (SI)} = \frac{CC_{50} \text{ human lung epithelial cell line}}{IC_{50} \text{ of parasite cell line}}$$

4.2.6. Statistical analysis

Data are presented as mean values ± standard deviation (SD), obtained from at least three determinations and analysed through Microsoft Excel program. The growth curve of *Plasmodium* parasite was analysed using the HN Nonlin v1.1 software and the growth curve of *Leishmania* parasite was performed using IC_{50}/IC_{90} software. The cytotoxicity growth curve was

completed using GraphPad Prism v.7 (California, USA) with additional of ANOVA analysis, where mean separation between positive and negative controls was carried out using Tukey's pairwise comparison at $P<0.05$ via SPSS software v.21 (Chicago, USA).

4.3. Results and discussion

4.3.1. Antiplasmodial activity of plant chloroform extracts

The antiplasmodial effects of chloroform plant extracts against chloroquine-resistant *P. falciparum* K1 strain were examined by HRPII assay (Razak *et al.*, 2014). Chloroquine was used as positive standard exhibiting IC_{50} value > 100 nM confirming that *P. falciparum* was resistant to chloroquine (Basco *et al.*, 2007). From the results obtained (Table 4.3), 11 extracts out of 12 tested showed IC_{50} below 10 µg/mL. According to Rasoanaivo *et al.* (2011), this is an indication of good antiplasmodial activity (Table 4.3). Cos *et al.* (2006) also suggested that IC_{50} below 100 µg/mL indicates cytotoxicity to the parasite cells.

Chloroform extracts exhibited *in vitro* antiplasmodial activity against chloroquine-resistant *P. falciparum* K1 strains in the increasing order (from inactive to the most active) as follows: stems *L. eugeniifolia* > leaves *T. peduncularis* > stems *A. suaveolens* > stems *U. grandiflora* > stems *D. wallichii* > leaves *A. suaveolens* > leaves *U. grandiflora* > leaves *L. eugeniifolia* > leaves *D. wallichii* > stems *T. peduncularis* > stems *C. costatus* > leaves *C. costatus.*

The readings of IC_{50} for the chloroform extracts in the increasing order were > 15.70, 5.41, 4.66, 4.54, 4.47, 4.36, 3.78, 3.03, 1.73, 1.32, 1.15, and 0.85 µg/mL, respectively.

Leaves of *L. eugenifolia, D. wallichii,* and leaves of *C. costatus,* as well as stems of *T. peduncularis,* and *C. costatus* exhibited good antiplasmodial activities. Members of *Leuconotis* genus such as *L. griffiithi* (Nugroho *et al.,* 2012), members of *Diospyros* genus such as *D. melanoxylon* genus (Saxena *et al.,* 2011; Olusola and Olumuyiwa, 2018), and members of *Tabernaemontana* genus such as *T. elegans* and *T. arborea* (Ramanitrahasimbola *et al.,* 2001; Ramalhete *et al.,* 2008; Bapela *et al.,* 2014; Marinho *et al.,* 2016) showed good antiplasmodial activities against *Plasmodium.* To the best of our knowledge, good antiplasmodial activities from these 6 medicinal plants are being reported for the first time. Selectivity index (SI) above 5 indicates selective toxicity for protozoa activity (Zirihi *et al.,* 2005).

4.3.2. Antileishmanial activity of plant chloroform extracts

The antileishmanial effects of chloroform extracts against *L. donovani* BHU-1251 strains were measured through MTT assay (Prajapati *et al.,* 2013). Miltefosine used as a positive standard, showed IC_{50} of 4.65 µM (Prajapathi *et al.,* 2013).

According to Bero *et al.* (2011), extracts are considered as having good antileishmanial activity with IC_{50} values $\leq$ 20 µg/mL. Three out of 12 chloroform

extracts had good antileishmanial activities against promastigote *in vitro.* These were stems of *A. suaveolens,* and *C. costatus,* and leaves *D. wallichii* (Table 4.4). The remaining had moderate activities. In short, the ascending order of chloroform extracts that exhibited *in vitro* antileishmanial activity against *L. donovani* BHU-1251 strains (least active to most active) was as follows: leaves *C. costatus* > stems *L. eugeniifolia* > stems *U. grandiflora* > stems *T. peduncularis* > stems *D. wallichii* > leaves *A. suaveolens* > leaves *U. grandiflora* > leaves *T. peduncularis* > leaves *L. eugeniifolia* > stems *A. suaveolens* > stems *C. costatus* > leaves *D. wallichii.* The readings of IC_{50} for the chloroform extracts in the ascending order were 94.25, 90.6, 74.74, 66.36 ,59.37, 49.94 ,40.51, 27.30 ,21.76, 17.33, 17.32, 7.6 µg/mL, respectively.

The extract of leaves of *T. peduncularis,* leaves of *L. eugeniifolia*, stems of *A. suaveolens,* and *C. costatus,* and leaves of *D. wallichii* showed good antileishmanial activities. Members of *Tabernaemonta* genus such as *T. corymbosa* (Roshan-Jahn *et al.,* 2018) and members of *Artabotrys* genus such as *A. venustus* and *A. hexapetalus* are active against *Leishmania. Chilocarpus* and *Leuconotis* species are reported for the first time for good antileishmanial activities. To the best of our knowledge, good antileishmanial activities from these 6 medicinal plants are being reported for the first time. According to Zirihi *et al.* (2005), **s**electivity index (SI) above 5 indicates selective toxicity for protozoan activity.

4.3.3. Cytotoxicity of plant chloroform extracts

Prior to antileishmanial and antiplasmodial testing, it was necessary to evaluate the potential toxicity of chloroform extracts against mammalian cells in order to calculate the selectivity index (SI) value. Thus, chloroform extracts were screened *in vitro* to examine their toxicity towards normal human lung epithelial cells. Human lung cells (MRC-5) were selected in this chapter as *L. donovani* and *P. falciparum* can cause lung diseases such as pulmonary leishmaniasis and pulmonary malaria (Vijayan, 2008; Russo *et al.*, 2003).

The American National Cancer Institute defines crude extracts as toxic for human cells when CC_{50} values are below 20 µg/mL (Hashim *et al.*, 2011). From the results obtained in Table 4.3, 4 out of the 12 chloroform extracts tested were toxic to human cells. These are stems of *T. peduncularis,* stems of *L. eugeniifolia*, stems of *D. wallichii,* and stems of *U. grandiflora.* In fact, stems for *L. eugeniifolia, D. wallichii,* and *U. grandiflora* are not interesting, as they have SI values below 5.

According to Zirihi *et al.* (2005), **s**electivity index (SI) above 5 indicates selective toxicity for protozoa. The results obtained showed that 9 chloroform extracts tested had selectivity index (SI) above 5, except for stems of *L. eugeniifolia, D. wallichii,* and stems *of U. grandiflora* which had SI below 5. Therefore, 8 extracts can be considered as antiplasmodial hits. These are stems and leaves of *C. costatus*, leaves of *T. peduncularis,* and *A. suaveolens*, stems of *A. suaveolens,* leaves of *L. eugeniifolia*, *D. wallichii,* and leaves *U. grandiflora*.

All the 12 chloroform extracts had selectivity index below 5 for antileishmanial activity.

Table 4.3. Cytotoxicity, antileishmanial, and antiplasmodial activities of plant chloroform extracts (µg/mL)

Extract	Cytotoxicity MRC-5 [b] (CC_{50})	Antileishmanial activity *L. donovani* [b] (IC_{50})	Antiplasmodial activity *P. falciparum* [b] (IC_{50})
Leaves *C. costatus*	67.5±14.1	94.25±2.29 (SI: 0.72)	0.85±3.7 (SI: 79.32)
Stems *C. costatus*	27.5±6.1	17.32±0 (SI: 1.59)	1.32±1.5 (SI: 20.72)
Leaves *T. peduncularis*	36.0±5.1	27.30±0 (SI:1.32)	5.41±4.0 (SI: 6.65)
Stems *T. peduncularis*	11.5±3.8	66.36±0 (SI: 0.17)	1.15±1.0 (SI: 9.99)
Leaves *A. suaveolens*	34.0±4.5	49.94±0.02 (SI: 0.68)	4.36±0.6 (SI: 7.79)
Stems *A. suaveolens*	53.5±5.5	17.33±0 (SI: 3.09)	4.66±0.8 (SI: 11.46)
Leaves *L. eugeniifolia*	39.0±3.7	21.76±3.47 (SI: 1.79)	3.03±1.3 (SI: 12.83)
Stems *L. eugeniifolia*	15.5±3.4	90.6±0.07 (SI: 0.17)	>15.70±5.9 (SI: 0.99)
Leaves *D. wallichii*	34.2±1.7	7.6±0 (SI: 4.51)	1.73±0.4 (SI: 19.73)
Stems *D. wallichii*	15.0±2.0	59.37±0 (SI: 0.25)	4.47±0.2 (SI: 3.35)
Leaves *U. grandiflora*	25.6±2.3	40.51±0 (SI: 0.63)	3.78±1.0 (SI: 7.27)
Stems *U. grandiflora*	10.0±6.1	74.74±0 (SI: 0.13)	4.54±1.5 (SI: 2.20)
Vincristine[a]	2.4 ± 1.5	N.T.	N.T.
Miltefosine[a]	N.T.	1.8 ± 2.5	N.T.
Chloroquine[a]	N.T.	N.T.	> 31.9
Dihydroartemisinin[a]	N.T.	N.T.	0.001± 0.6

Each value represents mean ± standard deviation of 3 independent experiments. [a]Positive control drug. [b]MRC-5: Normal human lung cell; *L. donovani*; *Leishmania donovani* BHU-1251 strain; *P. falciparum: Plasmodium falciparum* K1 chloroquine-resistant strain. SI: Selectivity Index

4.4. Conclusion

Antiparasitic screening of twelve extracts stems and leaves from six Malaysian rainforest plants showed promising growth inhibition on *Plasmodium falciparum* and *Leishmania donovani*. Further isolation work could be done on *C. costatus* and *U. grandiflora* Roxb. ex. Hornem.

Chapter 5: Antibacterial activities of plant chloroform extracts

5.1. Introduction

5.1.1. Nosocomial bacterial infection disease

In 2015, nosocomial bacterial infections were announced as top deadliest disease in hospitals and healthcare facilities worldwide (Khan *et al.*, 2015). According to WHO (2011), about 75% of nosocomial bacterial infections occur in developing countries, mainly in Southeast Asia including Malaysia. Nosocomial bacterial infections also occur in Sub-Saharan Africa, Latin America, and Africa (Khan *et al.*, 2017). Nosocomial bacteria are often Gram-negative and/ or Gram-positive bacteria that infect the urinary tract, surgical wounds, or bloodstream of hospitalized patients (WHO, 2016). Thus, WHO recommends the development of new antibacterial agents to control nosocomial bacterial infection as well as the bacterial resistance (WHO, 2014).

For example, the resistance of Gram-positive *Staphylococcus epidermidis* to cefotaxime has been problematic in hospital settings for decades (Raad *et al.*, 1998; Tacconelli *et al.*, 2007; Azman *et al.*, 2018). However, approvals of new antibacterial compounds by the FDA have been decreasing (Blaskovich *et al.*, 2017). New antibacterial agents need to be found and therefore, a focus of antibacterial study will be covered in this chapter.

5.1.2. The importance of new chemotherapeutic agents

Despite common accessibility to modern and traditional antibacterial treatments, bacterial infection remains a significant threat to public health worldwide (Abranches *et al.,* 2013; Bow, 2013). Increasing mortality and morbidity rates occur in Southeast Asia due to the ineffectiveness of the existing antibacterials and the evolution of antibiotic resistance (Tan, 2015). Penicillin, fluoroquinolone, chloramphenicol, and vancomycin are less effective. The evolution of bacterial resistance relative to current modern antibiotics is a result of bacterial mutation or bacterial gene transfer (Khajuria *et al.,* 2014; Bradley *et al.,* 2015).

The widespread and misuse of antibiotics in medicine has caused the mutation of several pathogenic bacterial strains and the acquisition of additional survival strategies (Tzialla *et al.*, 2012). For this reason, the search for alternative antibacterial agents with diverse chemical structures as well as new mechanisms of action are needed to prevent the new and re-emerging bacterial infections. The development of new chemotherapeutic agents should be based on a high therapeutic index with selective toxicity.

This can be achieved by disrupting the cell membrane and inhibiting certain enzymes essential for bacterial growth and survival without causing similar effects to their eukaryotic host (Rajagopal, 2014). Next, a chemotherapeutic agent should reach the site of the infection. This can be

affected by few factors, including stability of the drugs, lipophilic or hydrophilic nature, and susceptibility of pathogen to the particular chemotherapeutic agent.

5.1.3. Pathogenic and non-pathogenic bacteria

Pathogenic and non-pathogenic bacteria can be found everywhere including soil, plants, water, and animals as well as in humans. In short, pathogenic bacteria are harmful bacteria that can cause infectious diseases in humans, whereas non-pathogenic bacteria are beneficial to some hosts and essentials to others (Rajagopal, 2014; Shipton, 2017).

Non-pathogenic bacteria play an important role in our life. For instance, non-pathogenic bacteria in the soil such as Plant-Growth Promoting Rhizobacteria (PGPR) help in enhancing the availability and uptake of plant nutrients and suppress them from harmful pathogens (Babalola, 2010; Bhattacharyya and Jha, 2012). Non-pathogenic bacteria in the human gut, such as lactobacilli and bifidobacteria, also help in improving the digestion of lactose in lactose-intolerant patients, by reducing diarrhea and preventing intestinal infections (Murphy *et al.,* 2009; Saulnier *et al.,* 2009). Non-pathogenic bacteria in fermented food help in suppressing the growth of harmful bacteria and transform chemical compounds from raw materials in food or animal sources into beneficial entities during the fermentation too (Chen *et al.,* 2010; Thapa and Tamang, 2015).

Nevertheless, some bacteria can be harmful and pathogenic. They release toxins such as endotoxins, exotoxins, and siderophores that damage human cells (Shipton, 2017). The most common bacterial pathogens responsible for infectious disease include Methicillin-resistant *Staphylococcus aureus*, and *Klebsiella pneumoniae* (Khan *et al.*, 2015).

5.1.4. Type of bacteria: Gram-negative and Gram-positive bacteria

Bacteria consist of single cells which are few micrometres in length. The three common basic bacterial shapes are cocci (round-shaped bacteria), bacilli (cylindrical and capsule-shaped bacteria), and spirilla (spiral-shaped bacteria). Bacteria can be classified into two classes, either as Gram-positive or Gram-negative bacteria (Fayaz *et al.*, 2010; Shipton, 2017). Gram-positive and Gram-negative are distinguished through Gram staining, as Gram-positive appear dark purple color (Fayaz *et al.*, 2010; Shipton, 2017).

Since Gram-positive bacteria have a thick cell wall containing many layers of peptidoglycan and teichoic acids, the crystal violet remains in it. Examples of Gram-positive bacteria include Methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus aureus* (*S. aureus*), *Staphylococcus epidermidis* (*S. epidermidis*), and *Enterococcus faecalis* (*E. faecalis*).

In contrast, Gram-negative bacteria have thin cell wall consisting of lipopolysaccharides and lipoproteins and no peptidoglycan present, so they will not be able to retain the Gram stain. Examples of Gram-negative bacteria include

Klebsiella pneumoniae (*K. pneumoniae*), *Escherichia coli* (*E. coli*), and *Pseudomonas aeruginosa* (*P. aeruginosa*) (Barghouthi, 2011; Shipton, 2017).

5.1.5. Symptoms of bacterial infection

Symptoms of bacterial infection vary depending on the type of bacteria. Bloodstream infection (BSI), urinary tract infection (UTI), surgical site infection (SSI), pneumonia, gastroenteritis, meningitis, and respiratory infection are often associated with nosocomial bacterial (Weinstein, 2005; Khan *et al.,* 2015; Khan *et al.,* 2017). Table 5.1 shows bacterial symptoms often associated with nosocomial bacterial infection.

Table 5.1. General characteristics and bacterial strains symptoms (Source: Weinstein, 2005; Khan *et al.*, 2015; Khan *et al.*, 2017)

Bacterial strains	**General characteristics**	**Pathologies**
K. pneumoniae	Gram-negative, rod-shaped, non-flagellated, non-forming spores, facultative anaerobic bacteria.	Urinary tract infection (UTI)
P. aeruginosa	Gram-negative, rod-shaped, single flagellum, non-forming spores, facultative anaerobic bacteria.	Surgical and wound infection, urinary tract infection (UTI), pneumonia, cystic fibrosis and bacteremia
E. coli	Gram-negative, rod-shaped, flexible flagella, non-forming, facultative anaerobic bacteria.	Urinary tract infection (UTI), septicemia, pneumonia, neonatal meningitis, peritonitis and gastroenteritis
E. faecalis	Gram-positive, cocci in shape, non-flagellated, non-forming spores, facultative anaerobic bacteria.	Endocarditis, septicemia, urinary tract infection (UTI) and meningitis
S. epidermidis	Gram-positive, cocci in shape, non-flagellated, non-forming spores, facultative anaerobic bacteria.	Catheter infections, urinary tract infections (UTI), septicemia, endocarditis, fever, headache, fatigue, anorexia and dyspnea.
S. aureus	Gram-positive, cocci in shape, non-flagellated, non-forming spores, facultative anaerobic bacteria.	Food poisoning (due to ingestion of enterotoxins) and scalded skin syndrome (due to toxic shock syndrome)
MRSA	Gram-positive, cocci in shape, non-flagellated, non-forming spores, facultative anaerobic bacteria, resistant to methicillin.	Endocarditis, pneumonia, bacteremia, bone and joint infections and infected implants

5.1.6. Modern treatment

Current antibacterial treatment includes the prescription of variety of antibiotics, depending on the type of bacterial infection. Methicillin-resistant *S. aureus* and *E. coli* infections occurs in both hospital and community settings worldwide. Penicillin G was used as an effective therapeutic agent in the treatment of *S. aureus* as well as *P. aeruginosa* until a penicillin resistance problem arises (Hiramatsu *et al.,* 1997; Lowy, 2003).

The development of methicillin resistance, in particular also became a problem until the discovery of the glycopeptide vancomycin (Slaughter *et al.,* 1996; Appelbaum, 2006). However, the widespread and misuse of vancomycin against *Staphyloccoccus,* including *S. epidermidis, S. aureus,* and MRSA, has led to the emergence of vancomycin-resistant strains within coagulase-negative *Staphyloccocci*, both from inside and outside of the infected hospitalized patients (Appelbaum, 2006). Vancomycin resistance occurs due to the accumulation of peptidoglycans amounts, leading to the mutation and thickening of cell wall (Hiramatsu *et al.,* 1997).

Cefotaxime (β-lactam antibiotic) is a third-generation class of cephalosporin. It is commonly used in the treatment of Gram-positive and Gram-negative bacteria, including those resistant to classic β-lactam antibiotics such as penicillin, ampicillin, amoxicillin, and ceftriaxone (Campillo *et al.,* 2001; Patterson, 1995; The American Society of Health-System Pharmacists, 2018). β-lactam antibiotics act by targeting and binding to the cell wall synthesizing

enzymes (penicillin-binding proteins, PBPs) to interfere with the biosynthesis of bacterial peptidoglycan, resulting in inhibition of cell growth or lysis (Charpentier and Tuomanen, 2000).

5.1.7. Natural antibacterial agents / traditional medicines

Natural products play an essential role in the discovery of antibacterial drugs. Their contribution includes the innovation of drugs completely derived from natural products and models for a discovery of new drugs (Rajagopal, 2014). At the same time, plants also have been proven to be a source of antibiotics against several bacterial strains, including antibiotic resistant strains when used in combination with other antibiotics (Diarra *et al.*, 2013; Mustafa-Din, 2014). Medicinal plants synthesize a fascinating array of low molecular weight molecules with structures completely unrelated to antibiotics.

Traditional antibiotic structures have been almost depleted to the point that antibacterial research is literally needing new chemotypes that could be found using new and different research approaches (Azman *et al.*, 2018). Plants are known to have antibacterial potentials. For example *Acacia nilotica*, *Syzygium aromaticum, Cinnamum zeylanicum*, *Terminalia arjuna*, *Eucalyptus globulus, Terminalia chebula, Myrtus communis, Dianthus coryophyllus, Glycyrrhiza glabra, Ramunculus asitaticus Rhus coriaria, Rheum ribes, Chrozophora verbasafalia, Ephedra intermedia, Cinnamomum zeylanicum,* and *Citrullus colcocynthis* showed good activities against nosocomial bacterial strains, such *E. coli* and *K. pneumoniae* (Shahidi Bonjar, 2004; Khan *et al.*, 2015).

5.1.8. Techniques used to study antibacterial activities

Antibacterial activity can be studied through a variety of *in vitro* and *in vivo* methods. The common methods include disc diffusion assay, microdilution broth assay, time killing assay, and synergistic assay. Disc diffusion is a qualitative technique, since it only gives a rough idea of the presence or absence of substance with antibacterial activity. Whereas, microdilution broth is a quantitative since it can precisely determine minimum inhibitory concentration (Vlietinck, 1991; Valgas *et al.,* 2007; Rajagopal, 2014). In the present study, disc diffusion assay was used on a large number of samples allowing them to be quickly screened for antibacterial activities, while MIC and MBC were done to verify and provide more details on the activity of the samples such as concentration details in which they kill or inhibit bacteria (Shipton, 2017). Time killing assay was also performed to allow the study of the rate of an antibiotic at specific concentration in killing bacteria over the time (Ferro *et al.,* 2014). The interactions between the antibiotic and activities of the samples relative to the corresponding bacteria were studied via synergistic assay (Chi and Holo, 2018).

5.2. Materials and methods

5.2.1. Chemicals and reagents

Analytical grade dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich Corporation (Missouri, USA). Standard cefotaxime and vancomycin

(>98% purity) were bought from Sigma-Aldrich Corporation (Missouri, USA). Müeller-Hinton Agar (MHA), Müeller-Hinton Broth (MHB), Brain Heart Infusion Broth (BHI) medium were obtained from Oxoid Ltd (Hampshire, United Kingdom). Glycerol was purchased from R & M Chemicals (Essex, UK).

5.2.2. Bacterial strains

Seven bacterial strains including 4 Gram-positive bacteria and 3 Gram-negative bacteria were used for the entire experiment in this chapter.

- **Gram-positive bacteria**: Methicillin-resistant *Staphylococcus aureus* (MRSA) (clinical isolate), *Staphylococcus aureus* ATCC®25923™, *Staphylococcus epidermidis* ATCC®12228™, and *Enterococcus faecalis* ATCC®29212™.

- **Gram-negative bacteria**: *Klebsiella pneumoniae* (clinical isolate), *Escherichia coli* ATCC®25922™, and *Pseudomonas aeruginosa* ATCC®15442™.

5.2.3. Equipment and software

All absorbance-based biological assays using 96-well plates were measured using multifunctional microplate readers SpectraMax®M3 Multi-Mode Microplate Reader, Molecular Devices (California, USA) at different wavelength prior to the respective experiments.

5.2.4. Maintenance of bacteria

All frozen-stocked bacterial strains were passaged and cultured *in vitro* twice prior to use (CLSI Guidelines, 2011; Khan *et al.,* 2015). This can be achieved by growing the stock of bacterial strains one day before in a sterile MHA plate at 37°C for 24 hours with presence of 5% CO_2. Next, a group of bacterial colonies were streaked out from the MHA plate using sterile inoculating loop and inserted into sterile 1000 µL of the cation-adjusted MHB in sterile micro centrifuge tubes. The final concentration of the bacterial strains cultures in the broth was then adjusted into 1 x 10^8 CFU/mL by setting the optical density OD_{625} to 0.08-0.1, followed by two serial ten dilutions to get 1×10^6 CFU/mL of fresh stock concentration (CLSI Guidelines, 2011; Khan *et al.,* 2015).

For a long-term storage, all the bacteria were cryopreserved in a sterile cryovial tube containing BHI broth and 20% glycerol and kept frozen at -80°C until further use. It should be noted that all bacterial strains need to be freshly prepared prior to each bacterial test such as disc diffusion assay, MIC, MBC, synergy as well as time killing assays.

5.2.5. Disc diffusion assay

The Kirby-Bauer disc diffusion technique (CLSI Guidelines, 2011) was done to evaluate the antibacterial activities of the chloroform extracts against ATCC and clinical strains.

Concisely, the density of the corresponding bacteria was standardized at 1×10^8 coliform units (CFU/mL) using Khan *et al.* (2015) and CLSI Guidelines (2011). The adjusted bacterial concentration was then swabbed onto MHA agar surface. Next, 20 µL of plant extracts were loaded onto 6 mm of sterile paper disc and left to dry under a stream of sterile air, before impregnating it in the inoculated agar. The same amount (20 µL) of vancomycin (30 mg/mL), 1% DMSO, and cefotaxime (30 mg/mL) were also loaded onto a sterile paper disc and impregnated on the inoculated MHA agar, which in this case serves as negative and positive controls. The agar was incubated for 24 hours at 37°C in the 5% CO_2 incubator. The appearance of clear zones of bacterial growth inhibition zones around each disk after a 24-hour incubation period indicated that the plant extract has a good antibacterial property. The assay was repeated in triplicate. Determination of the efficacy of each plant extracts was done by measuring the diameters of the zone of inhibition of tested plant extracts (Vambe *et al.,* 2018).

5.2.6. Evaluation of MIC and MBC

Evaluation of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) can be accomplished through the micro dilution method (Razafintsalama *et al.,* 2013; CLSI Guidelines, 2014; Khan *et al.,* 2015; Razafintsalama *et al.,* 2017).

Minimum inhibitory concentration (MIC) was obtained by visualizing the lowest concentration of the sample that completely inhibited bacterial growth in round bottom well-plate (Khan *et al.*, 2015). In this case, a clear liquid broth in the well-plate after 24 hours incubation indicated inhibition of bacterial growth, whereas a turbid liquid agar indicated bacterial growth.

Firstly, the density of the corresponding bacteria was standardized at 1×10^6 coliform units (CFU/mL) using Khan *et al.* (2015) and CLSI Guidelines (2011). Next, 100 µL of the adjusted bacterial suspension was added to each 96-round bottom well plate. Then, a serial dilution of the plant extract samples was made by adding 100 µL of the sample to the first 96-well plates, mixing the contents to the well. Then, 100 µL of the mixture was added to the second 96-round bottom well plate and the process was repeated until 8 different concentrations were made, ranging from 1500 µg/mL to 11.78 µg/mL. Positive controls of vancomycin and cefotaxime were also made using this step. Wells containing only the MHB were used as the negative controls. The plates were then incubated for 24 hours at 37°C in the incubator in the presence of 5% CO_2. The lowest concentration of sample where no bacterial growth was visible was recorded as the MIC. The experiments were done in triplicate.

Thereafter, 10µl from each well that had no bacterial growth from MIC tests after the 24-hour incubation was deposited on the MHA plate using sterile pipette and L-shaped bacterial spreader. The agar plates were then incubated in the 5% CO_2 incubator for 24 hours at 37°C. The number of the viable bacterial colonies were counted and recorded. In this case, determination of Minimum

Bactericidal Concentration (MBC) was obtained from the lowest concentration at which no colony growth was observed on the agar plates after 24-hour incubation at 37°C (Razafintsalama *et al.,* 2013; CLSI Guidelines, 2014; Khan *et al.,* 2017; Razafintsalama *et al.,* 2017).

5.2.7. Synergy test

Staphylococcus epidermidis ATCC®12228™ cell and *C. costatus* Miq. stem chloroform were used for the synergy tests. Assessment of synergistic effect between mixture of plant *C. costatus* Miq. stem chloroform with standard drugs (vancomycin and cefotaxime) was achieved through the checkerboard titration method described in Khan *et al.* (2017) and Vambe *et al.* (2018) method with slightly modifications.

It is the norm to start with the crude extracts as it helps to narrow down the active constituents, leading to isolation of potent compounds with synergistic activities. In order to prepare a range of concentrations that allow the detection of synergism, indifference/additive, and antagonism, the experiment was started by adding 50 µL of eight serials standard drug B (vancomycin or cefotaxime) to 4 × MIC, followed by 50 µL of a fixed 0.25 × MIC Drug A (*C. costatus* Miq. stem chloroform plant extracts) into each of the 96-round bottom well plate to generate a mixture of eight drugs at different ratios. Later, 100 µL of bacterial suspension (1×10^6 CFU/mL) was inserted into each well, mixed well and incubated for 24 hours at 37°C in 5% CO_2 incubator. The experiment was done in triplicate. The results obtained were observed and recorded.

Two drugs have synergistic effect when FIC ≤ 0.5, or both drugs have indifference effects when 0.5 < FIC ≤ 4.0 or the two drugs have antagonistic effect when FIC > 4.0 (Khan *et al.*, 2015).

Thus, the fractional inhibitory concentration (FIC) index of each mixture were computed and analysed using the formula as follows:

$$\text{FIC index} = \frac{\text{MIC of Drug A in mixture}}{\text{MIC of Drug A alone}} + \frac{\text{MIC of Drug B in mixture}}{\text{MIC of Drug B alone}}$$

5.2.8. Time killing assay

The time required by plant extracts or compound to kill the bacterial growth was performed as previously described (Khan *et al.,* 2015). Based on MIC results, *S. epidermidis* was grown overnight on Müeller-Hinton Agar at 37°C, with 5% CO_2, and the concentration was adjusted into 1×10^6 CFU/mL using Khan *et al.* (2015) and CLSI Guidelines (2011) methods. Next, 100 µL of antibiotics and plant extracts (vancomycin, cefotaxime, and *C. costatus* stem chloroform) at MIC were combined with adjusted bacterial *S. epidermidis* (1×10^6 CFU/mL) in Müeller-Hinton Broth and incubated in the 5% CO_2 incubator at 37°C. An aliquot of 10 µL of the mixture was removed at different time intervals (1 hour, 2 hours, 3 hours, and 4 hours) and mixed on the sterile Müeller-Hinton agar plates using sterile L-shaped bacterial spreader. The agar plates were labelled and incubated for 24 hours in the 5% CO_2 incubator at 37°C.

Vancomycin and cefotaxime were used as positive controls. The assay was done in triplicate. Viable colonies obtained after 24 hours incubation were counted and recorded.

5.2.9. Statistical analysis

Data reported as mean ± standard deviation (SD), obtained from at least three determinations were analysed through Microsoft Excel program. The bacterial growth curve was plotted with standard deviation and 95% confidence interval. The growth curve of bacteria was analysed using one-way ANOVA, and mean separation between treatments was carried out using Tukey's pairwise comparison at $P<0.05$ via SPSS software v.21 (Chicago, USA).

5.3. Results and discussion

5.3.1. Disc diffusion and MIC micro dilution assays

Antibacterial activities of chloroform extracts against 4 Gram-positive bacteria: Methicillin-resistant *S. aureus* (MRSA), *E. faecalis*, *S. aureus*, *S. epidermidis*, and 3 Gram-negative bacteria: *E. coli*, *P. aeruginosa*, and *K. pneumoniae* were screened through disc diffusion and broth micro dilution techniques. Cefotaxime and vancomycin were used as positive controls.

The results of disc diffusion assay are displayed in Table 5.2. The results of MIC micro dilution assay are shown in Table 5.3. There is no correlation

between disc diffusion and and MIC (Mayrhofer *et al.*, 2008; Hoelzer *et al.*, 2011). In line, we did not observe any correlation between the zone of inhibition and MIC (Hammer *et al.*, 1999; Chomnawang *et al.*, 2005; Klančnik *et al.*, 2010; Shipton, 2017).

In disc diffusion assay, chemicals that have antibacterial activities with inhibition zones of 7 mm and above can be counted as potential positive antibacterial agents (Nascimento *et al.*, 2000; Rajagopal, 2014). There was no recorded zone of inhibition for all extracts against *E. faecalis*. However, the remaining six bacterial pathogens showed several positive antibacterial results (Table 5.2).

For MRSA strains, stems of *L. eugeniifolia* and leaves of *U. grandiflora* had inhibition zones of 8.0 and 7.7 mm, respectively. For *S. aureus* strains, stems of *T. peduncularis, A. suaveolens,* and *L. eugeniifolia* had inhibition zones of of 9.0, 9.3, and 6.7 mm, respectively. For *S. epidermidis* strains, stems *U. grandiflora* had zone of inhibition of 7.3 mm. For *E. coli* strains, stems *C. costatus,* stems *A. suaveolens,* and leaves *A. suaveolens* had zones of inhibition of 7.3, 7.0, and 7.0 mm, accordingly. For *P. aeruginosa* strains, both leaves and stems of *A. suaveolens,* both leaves and stems of *L. eugeniifolia,* leaves of *D. wallichii,* and stems *of U. grandiflora* presented inhibition zones of 7.0, 7.0, 7.0, 7.0, 8.0, and 7.7 mm, respectively. For *K. pneumoniae* strains, stems *T. peduncularis,* and leaves *D. wallichii* showed antibacterial activities with an inhibition zone of 6.7 mm.

Fabry *et al.* (1998) defined extracts with MIC < 8000 µg/mL as active, while Kuete (2010) and Cos *et al.* (2006) stated that extracts with MIC < 100 µg/mL are active. Rajagopal (2014) defined extracts with MIC > 625 µg/mL as weakly active. Stem extract of *C. costatus* had a moderate activity against *S. epidermidis*, with MIC value of 187.5 ± 0.3 µg/mL, but was inactive against the other six bacteria tested with MIC values above 1,500 µg/mL (Table 5.3). The other 11 extracts were inactive with MIC values above 1,500 µg/mL (Table 5.3).

Table 5.2. Disc diffusion assay of plant chloroform against seven bacterial strains

Plant extract	Zone of inhibition (mm)						
	Gram-positive bacteria				Gram-negative bacteria		
	MRSA	*E. faecalis*	*S. aureus*	*S. epidermidis*	*E. coli*	*P. aeruginosa*	*K. pneumoniae*
Leaves *C. costatus*	-	-	-	-	-	-	-
Stems *C. costatus*	-	-	-	-	7.3 ± 0.6	-	-
Leaves *T. peduncularis*	-	-	-	-	-	-	6.7 ± 0.6
Stems T. *peduncularis*	-	-	9.0 ± 0.0	-	-	-	-
Leaves *A. suaveolens*	-	-	-	-	7.0 ± 0.0	7.0 ± 0.0	-
Stems *A. suaveolens*	-	-	9.3 ± 0.6	-	7.0 ± 0.0	7.0 ± 0.0	-
Leaves *L. eugeniifolia*	-	-	-	-	-	7.0 ± 0.0	-
Stems *L. eugeniifolia*	8.0 ± 0.0	-	6.7 ± 0.6	-	-	7.0 ± 0.0	-
Leaves *D. wallichii*	-	-	-	-	-	8.0 ± 0.0	6.7 ± 0.6
Stems *D. wallichii*	-	-	-	-	-	-	-
Leaves *U. grandiflora*	7.7 ± 0.6	-	-	-	-	-	-
Stems *U. grandiflora*	-	-	-	7.3 ± 0.6	-	7.7± 0.6	-
Cefotaxime [a]	15.0 ± 0.0	29.0± 0.0	15.0 ± 0.0	26.0± 0.0	29.0± 0.0	26.0± 0.0	29.0± 0.0
Vancomycin[a]	7.3 ± 0.6	9.3± 0.6	15.0 ± 0.0	17.0± 0.0	9.3± 0.6	9.3 ± 0.6	13.0± 0.0

Each value represents mean ± standard deviation of 3 independent experiments. [a]Antibiotic standard. [b]*Staphylococcus aureus* (ATCC®25923™); MRSA: Methicillin-resistant *Staphylococcus aureus* (clinical isolate); *Staphylococcus epidermidis* (ATCC®12228™); *Enterococcus faecalis* (ATCC®29212™); *Escherichia coli* (ATCC®25922™); *Pseudomonas aeruginosa* (ATCC®15442™); *Klebsiella pneumoniae* (clinical isolate).

Table 5.3. Minimum inhibitory concentration of plant chloroform against seven bacterial strains

Plant extract	Minimum inhibitory concentration (µg/mL)						
	Gram-positive bacteria				Gram-negative bacteria		
	MRSA	***E. faecalis***	***S. aureus***	***S. epidermidis***	***E. coli***	***P. aeruginosa***	***K. pneumoniae***
Leaves *C. costatus*	>1500	>1500	>1500	>1500	>1500	>1500	>1500
Stems *C. costatus*	>1500	>1500	>1500	**187.5± 0.3**	>1500	>1500	>1500
Leaves *T. peduncularis*	>1500	>1500	>1500	>1500	>1500	>1500	>1500
Stems T. *peduncularis*	>1500	>1500	>1500	>1500	>1500	>1500	>1500
Leaves *A. suaveolens*	>1500	>1500	>1500	>1500	>1500	>1500	>1500
Stems *A. suaveolens*	>1500	>1500	>1500	>1500	>1500	>1500	>1500
Leaves *L. eugeniifolia*	>1500	>1500	>1500	>1500	>1500	>1500	>1500
Stems *L. eugeniifolia*	>1500	>1500	>1500	>1500	>1500	>1500	>1500
Leaves *D. wallichii*	>1500	>1500	>1500	>1500	>1500	>1500	>1500
Stems *D. wallichii*	>1500	>1500	>1500	>1500	>1500	>1500	>1500
Leaves *U. grandiflora*	>1500	>1500	>1500	>1500	>1500	>1500	>1500
Stems *U. grandiflora*	>1500	>1500	>1500	>1500	>1500	>1500	>1500
Cefotaxime [a]	1500± 0.03	11.7 ± 0.1	<11.7	<11.7	93.7±0.03	11.7±0.3	N.T.
Vancomycin[a]	11.7±0.3	11.7±0.3	11.7±0.1	11.7±0.3	11.7±0.1	375±0.3	11.7±0.3

Each value represents mean ± standard deviation of 3 independent experiments. [a]Antibiotic standard. [b]*Staphylococcus aureus* (ATCC®25923™); MRSA: Methicillin-resistant *Staphylococcus aureus* (clinical isolate); *Staphylococcus epidermidis* (ATCC®12228™); *Enterococcus faecalis* (ATCC®29212™); *Escherichia coli* (ATCC®25922™); *Pseudomonas aeruginosa* (ATCC®15442™); *Klebsiella pneumoniae* (clinical isolate).

5.3.2. MBC assay and MBC/MIC ratio

Upon identifying possible antibacterial action, the effect of antibacterial activity either bacteriostatic or bactericidal can be determined through the ratio of MBC/MIC (Shipton, 2017; Khan *et al.*, 2017). A bactericidal effect is obtained if the ratio MBC/MIC is less than or equal to 4 (Rajagopal, 2014; Khan *et al.*, 2017; Shipton, 2017). A bacteriostatic effect is obtained if the ratio MBC/MIC is more than 4 (Rajagopal, 2014; Khan *et al.*, 2017; Shipton, 2017). Only *C. costatus* stem chloroform showed good MIC results in the present study. Therefore, MBC study was done on this extract. The results revealed that *C. costatus* stem chloroform is bactericidal (Table 5.4). Bactericidal agent acts by killing bacteria. Meanwhile, bacteriostatic agent acts by inhibiting or slowing down bacterial growth (Rajagopal, 2014; Shipton, 2017).

Table 5.4. Minimum bactericidal concentration (MBC) of plant chloroform extracts against bacterial *S. epidermidis*

Plant extracts	MBC (µg/mL)	MBC/MIC ratio	Conclusion
Leaves *C. costatus*	N. T	N/A	N/A
Stems *C. costatus*	**1000 ± 0.3**	3.3	Bactericidal
Leaves *T. peduncularis*	N. T	N/A	N/A
Stems T. *peduncularis*	N. T	N/A	N/A
Leaves *A. suaveolens*	N. T	N/A	N/A
Stems *A. suaveolens*	N. T	N/A	N/A
Leaves *L. eugeniifolia*	N. T	N/A	N/A
Stems *L. eugeniifolia*	N. T	N/A	N/A
Leaves *D. wallichii*	N. T	N/A	N/A
Stems *D. wallichii*	N. T	N/A	N/A
Leaves *U. grandiflora*	N. T	N/A	N/A
Stems *U. grandiflora*	N. T	N/A	N/A
Vancomycin[a]	N. T	N/A	N/A
Cefotaxime[a]	N. T	N/A	N/A

Each value represents mean ± standard deviation of 3 independent experiments.
[a]Antibiotic standard. N.T.: not tested. N/A: not available**.**

5.3.3. Synergy test

Further, the synergistic effects of stem chloroform of *C. costatus* extract was done because this extract showed good MIC activities. Wright (2000) proposed to develop agents that "resist" resistance as a strategy to antibiotic resistance. Based on the synergy results in Table 5.5 below, it was found that *C. costatus* stem chloroform reacts synergistically with cefotaxime, however no

synergistic effects were observed with vancomycin when combined against *S. epidermidis.*

Table 5.5. Fractional inhibitory concentration index (FICI) of stem chloroform *C. costatus* with standard antibiotics against *S. epidermidis* (ATCC®12228™)

Extract	Antibiotics	FICI[a]	Effect
Stem *C. costatus*	Cefotaxime	**0.5**	Synergistic
	Vancomycin	1.1	Indifferent

*FICI: fractional inhibition concentration index. Values are means of triplicates.

[a]FICI ≤ 0.5 represent synergistic effect; > 0.5 and ≤4.0 represent indifference; > 4.0 represent antagonistic effect.

5.3.4. Time killing assay

The time killing assay was performed on stem chloroform extracts of *C. costatus* and standard antibiotics. The remaing extracts were not studied further. This is because this extract showed antibacterial activities. The results obtained for the time-killing study (Figure 5.1) and MBC values (Table 5.4) are consistent with the bactericidal concentration as displayed by the growth curve of this time-kill study. Bacterial survival decreased in the presence of antibiotics with time. The extract of *C. costatus* stem chloroform killed *S. epidermidis* faster than the standard antibiotics (vancomycin and cefotaxime). This may be due to the fact that *S. epidermidis* developed resistance to cefotaxime and vancomycin (Charpentier and Tuomanen, 2000; Appelbaum, 2006).

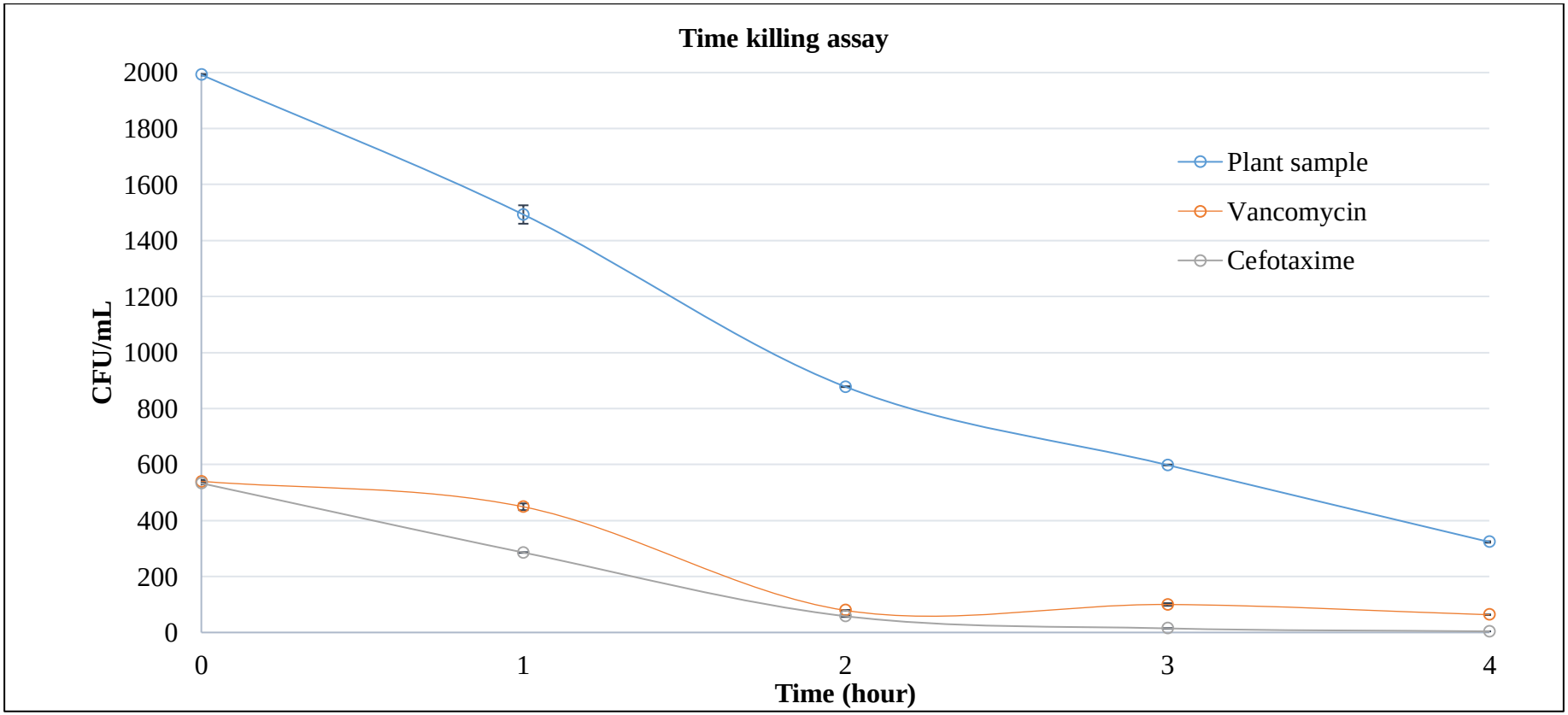


Figure 5.1. Time killing assay of cefotaxime, vancomycin and plant sample

5.4. Conclusion

Chloroform extract of *C. costatus* Miq. stem possesses antibacterial and interesting synergistic effects with cefotaxime.

Chapter 6: Antioxidant properties of plant chloroform extracts

6.1. Introduction

6.1.1. Chronic and degenerative diseases

Although oxygen and free radicals are important in life processes, the production of an excess of oxygen and free radicals is harmful. The situation becomes worst when the overloading of free radicals and oxygen cannot be neutralized, hence ‘‘oxidative stress’’ (Pham-Huy *et al.,* 2008). Oxidative stress is an imbalance resulting from the formation and neutralization of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the cellular redox process (Pham-Huy *et al.,* 2008).

Oxidative stress can cause chronic and degenerative diseases such as cancer, autoimmune disorders, Alzheimer’s disease, Parkinson, ageing, cataract, rheumatoid arthritis, cardiovascular, and neurodegenerative diseases (de Oliveira *et al.,* 2009; Jindal and Mohamad, 2012; Shipton, 2017). In South-East Asia, cardiovascular diseases cause an estimated 3.6 million deaths annually (WHO Southeast Asia, 2011). Nevertheless, the human body has several mechanisms to overcome oxidative stress. For example, the production of antioxidants such as glutathione and ubiquinol (co-enzyme Q), both by naturally and externally supplied (through food and supplements) helps the body to fight oxidative stress by preventing and repairing the damage triggered by ROS and RNS as well as enhancing the immune defence.

6.1.2. Mechanism of action

Antioxidants are molecules that slow down or inhibit oxidation. Thus, antioxidants provide protections to the cells from oxidative stress. There are few factors that affect oxidation: the presence of oxygen and transition metal ions, moisture, heat, and light. To prevent oxidation, antioxidants work by transferring electrons to free radicals. Once the transfer electron is completed and valence electrons are paired, free radicals lose their reactivity and no other radicals can be generated from the reaction (Riley, 1994; Rajagopal, 2014).

6.1.3. Type of antioxidant

Antioxidants are classified into two groups: endogenous (naturally produced in the body) and exogenous (externally supplied through foods and supplements) antioxidants (Pham-Huy *et al.*, 2008). Endogenous antioxidants are either enzymatic or non-enzymatic. The main enzymatic antioxidants that directly involve ROS and RNS neutralization and protect the body from free radical damage are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GRx) (Pham-Huy *et al.*, 2008). SOD catalyses the dismutation of superoxide anion radical (O_2^-) into hydrogen peroxide (H_2O_2) through the reduction process. H_2O_2 is transformed into water (H_2O) and oxygen (O_2) by CAT and GPx. GPx enzyme not only removes H_2O_2, but also reduces lipid and non-lipid hydroperoxide while oxidizing reduced glutathione (GSH). Non-enzymatic antioxidants that directly involve ROS and

RNS neutralization are metabolic antioxidants. These are produced by metabolism in the body.

Examples of metabolic antioxidants include lipoic acid, glutathione L-arginine, co-enzyme Q10, melatonin, uric acid, bilirubin, metal-chelating proteins, and transferrin (Droge, 2002; Willcox *et al.*, 2004). Nutrient antioxidants are exogenous antioxidants. Nutrient antioxidants are consumed through food and supplements. Examples of nutrient antioxidants include vitamin E, vitamin C, carotenoids, trace metals (selenium, manganese and zinc), flavonoids, omega-3, and omega-6 fatty acids (Miguel, 2010).

6.1.4. Free radicals

Free radicals are highly reactive chemical entities that can damage tissues, resulting in chronic and degenerative diseases. Free radicals are endogenous (from normal metabolic reactions) or exogenous (through tobacco smoke, air pollution, radiation exposure, metabolism of solvents, drugs, haze, and pesticides) (Machlin and Bendich, 1987). Free radicals react with polyunsaturated fatty acids in cellular membranes, nucleotides in DNA, and sulfhydryl bonds in proteins (Machlin and Bendich, 1987). Some antioxidants act as defence agents against free radical damage, such as tocopherol (vitamin E), ascorbic acid (vitamin C), beta-carotene, glutathione, uric acid, and bilirubin (Machlin and Bendich, 1987).

6.1.5. Oxygen

Excess in oxygen can cause oxidative stress. Oxidative stress is the results of oxidation. Rajagopal (2014) described oxidation as the loss of electron, while the reduction is the gain of an electron. Oxygen is well known for its high electronegativity and hard to be oxidized. Nevertheless, oxygen can still be oxidized as the energy level of oxygen has a significant effect on the oxidation reaction. Singlet state oxygen ($^1O_2$) has spin-coupled electrons and is a non-radical of high energy species (Decker, 2002; Min and Boff, 2002; Brewer, 2011). It may react with other high electrons of non-radical that contains double bonds (C=C, C=O).

Meanwhile, triplet state oxygen ($^3O_2$) has two unpaired parallel spin electrons and is a non-radical of low energy species (Brewer, 2011). Although having low energy, it is very reactive with radical species. $^3O_2$ is relatively unreactive with most food components. Surprisingly, food components (such as carbohydrates, proteins) are relatively reactive with $^1O_2$ in response to changes of temperature, reduced activation energy and exposure to UV light (Brewer, 2011).

Therefore, singlet oxygen ($^1O_2$) and free radicals cause biological damage to macromolecules and membrane constituents (Brewer, 2011). The presence of natural antioxidants can help in controlling these degradative reactions by interacting with the oxidative compounds, neutralizing them and cell from oxidative damage (Rajagopal, 2014).

6.1.6. Antioxidant flavonoids

Flavonoids are antioxidant compounds comprising flavonols, anthocyanins, isoflavonoids, flavanones, and flavones. All these sub-compounds share a diphenylpropane ($C_6C_3C_6$) skeleton (Carocho and Ferreira, 2013). Flavanones and flavones are commonly found in fruits. Most of flavanone-rich plants also lack anthocyanins. Most flavonoids act as reducing agents, hydrogen donators, singlet oxygen quenchers, superoxide radical scavengers, and metal chelators (Carocho and Ferreira, 2013).

Flavonoids activate antioxidant enzyme, reduce α-tocopherol radicals (tocopheroxyls), inhibit oxidases, mitigate nitrosative stress, and increase levels of uric acid (Carocho and Ferreira, 2013). Examples of flavonoids include catechin, catechin-gallate, quercetin, kaempferol, and rutin. Flavonoids are polyphenolic compounds (Rajagopal, 2014). These compounds are effective in preventing diseases related to oxidative stress (Rajagopal, 2014).

6.1.7. Antioxidant phenolic acids

Phenolic acids comprise hydroxycinnamic and hydroxybenzoic acids (Carocho and Ferreira, 2013). They are ubiquitous and are mainly present as esters and glycosides. Phenolic acids are antioxidants acting as chelators and free radical scavengers, especially against hydroxyl and peroxyl radicals, superoxide anions and peroxynitrites (Carocho and Ferreira, 2013).

6.1.8. Synthetic antioxidants

Industries have developed numerous synthetic antioxidants. Few examples of synthetic antioxidants include butylated hydroxyanisole (BHA), butylated hydroxyl toluene (BHT), metabisulphite, and ethoxyquin (Rajagopal, 2014). However, butylated hydroxyanisole and butylated hydroxyl toluene are toxic (EFSA, 2011).

6.1.9. Natural antioxidants

Since the 1980s, there has been an increasing demand and interest from consumers for natural and organic products in the USA and Europe. As a consequence, there is a decline in the use of synthetic products and greater commercialization of natural products. The natural trend, coupled with the growing market for premium food products, has powered the use of natural antioxidants. Examples of natural antioxidants include tocopherol (vitamin E), natural herbal flavourings and ascorbic acid (vitamin C).

Natural antioxidants from plant sources efficiency and mode of action depend on their chemical structures (Haworth, 2003). As many herbal extracts contain antioxidant properties, hence they are commonly used as food antioxidants. These antioxidant properties are generally linked to the presence of phenolic compounds like carnosic acid, carnosol, rosmanol, or rosmanic acid (Haworth, 2003).

Rosemary, oregano, sage, cloves, ginger, nutmeg, and turmeric are common herbal extracts used as food antioxidants (Haworth, 2003; Embuscado, 2015). Some criteria looked for the selection of antioxidant for food products are lack of toxicity, efficient at low concentrations, no undesirable effects, heat stable, and economical.

6.1.10. Techniques used to study antioxidants

The antioxidant properties of different plant extracts, essential oils, and pure compounds can be determined through *in vitro* assays. There are two types of evaluation of antioxidant assays in foods and plants: (i) assays that examine lipid peroxidation and (ii) assays that evaluate free radical scavenging ability (Miguel, 2010). However, only antioxidant assays that measure radical scavenging ability was performed in the present study.

There are two main methods used for determining free radical scavenging properties: (i) hydrogen atom transfer-reaction based methods and (ii) single electron reaction-based methods (Miguel, 2010). Examples of methods based on the hydrogen atom transfer-reaction include: inhibition of low-density lipoprotein (LDL) induced oxidation, oxygen radical absorbance capacity (ORAC), total radical-trapping antioxidant parameter (TRAP), and crocin-bleaching assays. In this present study, only single electron reaction-based methods were focused because hydrogen atom transfer-reaction based methods are high reactive.

Single electron reaction-based methods involve an oxidant (substrate) that abstracts an electron from the antioxidant, which causes color changes in the substrate. The degree of color change is directly proportional to the antioxidant concentrations and its end-point reaction is achieved when the color changes stop (Huang *et al.*, 2005; Miguel, 2010). Antioxidant activity can be then determined from the plotted graph of absorbance changes against the antioxidant concentration through the slope of the graph curve. Examples of single electron reaction-based methods include: the total phenols assay by Folin-Ciocalteu reagent and the ferric ion reducing antioxidant power (FRAP) assay.

Although there are other assays that can be used to measure the radical scavenging ability for oxidants, Miguel (2010) claimed that results obtained from these different methods are not comparable due to the physical structure of the test systems. In fact, there is no single universal method that can evaluate the antioxidant activities accurately (Tan, 2015).

- Total phenols quantification (Folin-Ciocalteu reagent)

The Folin-Ciocalteu (FC) method quantifies the number of phenolic groups or other possible oxidizable groups present in samples (Becker *et al.*, 2004). The chemical nature of FC remains unknown. Some researchers have stated that FC reagent contains heteropolyphosphotungstates-molybdates; and therefore, there will be blue color when there is an electron-transfer reaction between molybdenum and antioxidant in the solution mixture. Since the reaction

only occurs in an alkaline environment (pH ≈10), the addition of sodium carbonate is very important to complete the reaction.

- Ferric ion reducing antioxidant power (FRAP)

FRAP method uses a ferric salt as an oxidant agent. As FRAP methods occur in an acidic environment, it needs low pH (pH 3.6). The redox reaction of the crude extracts is achieved when there is a reduction of the ferric tripyridyltriazine (Fe (III)-TPTZ) complex into ferrous tripyridyltriazine (Fe (II)-TPTZ) at low pH; that changes the color of yellowish solution into different shades of green and blue.

6.2. Materials and methods

6.2.1. Chemicals and reagents

Analytical grade methanol and dimethyl sulfoxide (DMSO) were purchased from RCI Labscan Co., Ltd (Bangkok, Thailand). Standard (>98% purity) gallic acid, iron (II) sulphate heptahydrate ($FeSO_4.7H_2O$) together with 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ), iron (III) chloride hexahydrate ($FeCl_3.6H_2O$), sodium acetate ($C_2H_3NaO_2$), sodium carbonate (Na_2CO_3), aluminium chloride ($AlCl_3$) and Folin-Ciocalteu's (FC) reagent (>97% purity) were obtained from Sigma-Aldrich Corporation (Missouri, USA).

6.2.2. Equipment

All absorbance reading using 96-well plates were measured with UV-Vis spectrophotometer, SpectraMax®M3 Multi-Mode Microplate Reader, Molecular Devices (California, USA) at different wavelength prior for the respective experiments.

6.2.3. Preparation of plant extracts

Stock of plant extracts (10 mg/mL) was prepared by diluting the dry extracts with 1% DMSO solution.

6.2.4. Total phenolic assay

Total phenolic content of the crude extracts was evaluated using a modified method of Folin-Ciocalteu assay from Wee (2015), to determine the redox properties of polyphenols in the plant crude extracts (Ainsworth and Gillespie, 2007). Through this assay, a blue complex solution was formed as a result of the complex redox reaction between phenolics in the crude plant extracts with the phosphomolybdate and phosphotungstate present in the Folin reagent, which can be visualized under visible-light spectrophotometer (Schofield *et al.*, 2001).

In short, about 0.05 mL of crude aliquot extract was added to the 3.0 mL of distilled water and 0.25 mL of undiluted Folin-Ciocalteu's phenol reagent in

a test tube. After 8 minutes, the reaction mixture was added with 0.7 mL of 7% (w/v) sodium carbonate and 1.0 mL of distilled water and incubated in the water bath at 37°C for 2 hours under dark conditions. Finally, 0.2 mL of the incubated reaction mixture was transferred into the flat bottom 96-well plate and the absorbance of the reaction mixture was read at 765 nm using a UV-Vis spectrophotometer.

The total phenolic content of crude extracts was calculated from a standard calibration curve using gallic acid (GAE) ($y=0.9958x + 0.0026$, $R^2=0.9997$) (Figure 6.1), where mg GAE is equivalent to 10 g fresh weight samples (FW) (Ainsworth and Gillespie, 2007). The concentration of polyphenols in the extracts was derived from a standard curve of gallic acid ranging from 0.2 to 1.0 mg/mL. A well without gallic acid was used as negative standard.

6.2.5. Ferric Reducing Antioxidant Power Assay

The ability of the crude extracts to reduce ferric tripyridyltriazine (Fe (III)-TPTZ) complex into ferrous tripyridyltriazine (Fe (II)-TPTZ) at low pH can be done using a modified Ferric Reducing Antioxidant Power (FRAP) assay from Wong *et al.* (2006). In this case, the reduction of antioxidant in the plant extract changes of yellowish solution into different shades of green and blue in the FRAP assay (Miguel, 2010; Wee, 2015).

Succinctly, FRAP reagent was freshly prepared by combining 300 mM of sodium acetate (pH 3.6), 10 mM of iron (III) chloride hexahydrate ($FeCl_3.6H_2O$)

and 10 mM of 2,4,6-Tri(2-pyridinyl)-1,3,5-triazine (TPTZ) dissolved in 40 mM of hydrochloric acid (HCl) in a ratio of 10: 1:1. Next, about 0.2 mL of crude aliquot extracts (concentration ranging from 1.0 mg/mL to 5. 0 mg/mL) were mixed with the 3 mL of the FRAP reagent and incubated in the water bath at 37°C for 30 minutes under dark conditions together with the blank control. 0.2 mL of the incubated reaction mixture and the blank control was transferred into the flat bottom 96-well plate. Finally, the absorbance of the reaction mixture and the blank control was read at 593 nm using a UV-Vis spectrophotometer.

A calibration curve ($y= 2.553x + 0.0039$, $R^2=0.9997$) (Figure 6.2) was obtained from the standard $FeSO_4.7H_2O$ and was expressed as mg $FeSO_4.7H_2O$ equivalent per 10 g fresh weight samples (FW) (Tan, 2015). The concentration of FRAP content in the extracts was derived from a standard curve of iron (II) sulfate heptahydrate which ranging from 0.2 to 1.0 mg/mL. A well without iron (II) sulfate was used as negative standard.

6.3. Results and discussion

Most of plant herbal extracts contain antioxidants (Tan, 2015). However, there is no single universal method that can evaluate the antioxidant activities (Tan, 2015). Therefore, TPC and FRAP assays were carried out in order to study the antioxidant potential of the plant extracts.

6.3.1. Total phenolic content (TPC)

Results displayed on Table 6.1 shows that chloroform extracts exhibited total phenolic content in the ascending order (lowest to highest): stems *C. costatus* > leaves *T. peduncularis* > stems *U. grandiflora* > stems *T. peduncularis* > stems *L. eugeniifolia* > stems *D. wallichii* > leaves *C. costatus* > leaves *D. wallichii* > stems *A. suaveolens* > leaves *U. grandiflora* > leaves *L. eugeniifolia* > leaves *A. suaveolens*. The readings of total phenolic content for the chloroform extracts in the ascending order were 0.030, 0.030, 0.030, 0.031, 0.031, 0.031, 0.032, 0.032, 0.033, 0.033, 0.34, and 0.044 mg GAE/ 10 g FW, respectively.

The Folin-Ciocalteu assay is commonly used for the quantification of total phenolic content. It is based on the reduction of phosphomolybdic and phosphotungstic acid complexes into blue complex solution in reaction with the presence of phenolic compounds under alkaline conditions (Schofield *et al.*, 2001; Ainsworth and Gillespie, 2007; Mehran *et al.*, 2014;). In this case, the total phenolic content of extracts was quantified from the regression equation of gallic acid calibration curve ($y=0.9958x + 0.0026$, $R^2=0.9997$) (Figure 6.1, Table 6.1). There are three classifications of total phenolic content of the plant extracts: low (less than 10 mg GAE/g), medium (10 > mg GAE/g > 50) and high (more than 50 mg GAE/g) (Maria do Socorro *et al.*, 2010; Tan, 2015).

Based on this classification, it can be said that almost all plant chloroform extracts had low total phenolic content. It is not suprising because chloroform

does not extract well phenolics (Wiart, 2007). The highest total phenolic content obtained was in the leaves *A. suaveolens* extract with 0.044 mg GAE/ 10 g FW.

6.3.2. Ferric Reducing Antioxidant Power (FRAP)

Results presented on Table 6.1 shows that chloroform extracts exhibited FRAP in the ascending order (lowest to highest): stems *C. costatus* > stems *L. eugeniifolia* > leaves *A. suaveolens* > stems *T. peduncularis* > stems *D. wallichii* > stems *A. suaveolens* > leaves *D. wallichii* > stems *U. grandiflora* > leaves *L. eugeniifolia* > leaves *T. peduncularis* > leaves *C. costatus* > leaves *U. grandiflora*. The readings of the total flavonoid content for chloroform extracts in the ascending order were 0.0123, 0.0123, 0.0127, 0.0137, 0.0142, 0.0147, 0.0153, 0.0158, 0.0162, 0.0164, 0.0165, and 0.0193 mg $FeSO_4.7H_2O$ / 10 g FW, respectively.

The FRAP assay is applied to investigate the ferric reducing antioxidant power of the plant extracts. It was based on the ability of plant extracts to reduce the ferric tripyridyltriazine (Fe (III)-TPTZ) complex into ferrous tripyridyltriazine (Fe (II)-TPTZ) at low pH (Wong *et al.,* 2006). The reduction of antioxidant by the plant extract influences changes in the yellowish solution in different shades of green and blue in the FRAP assay (Miguel, 2010; Wee, 2015). Blue color indicates that plant extracts possess higher antioxidant, while green indicated that the plant extracts have low antioxidant activity (Rajagopal, 2014).

In this case, the FRAP of the plant extracts was determined from the regression equation of the $FeSO_4.7H_2O$ calibration curve ($y= 2.553x + 0.0039$, $R^2=0.9997$) (Figure 6.2, Table 6.1). Almost all the extracts had a low FRAP content. The highest FRAP obtained was from *U. grandiflora* leaves with 0.0193 mg $FeSO_4.7H_2O$ / 10 g FW.

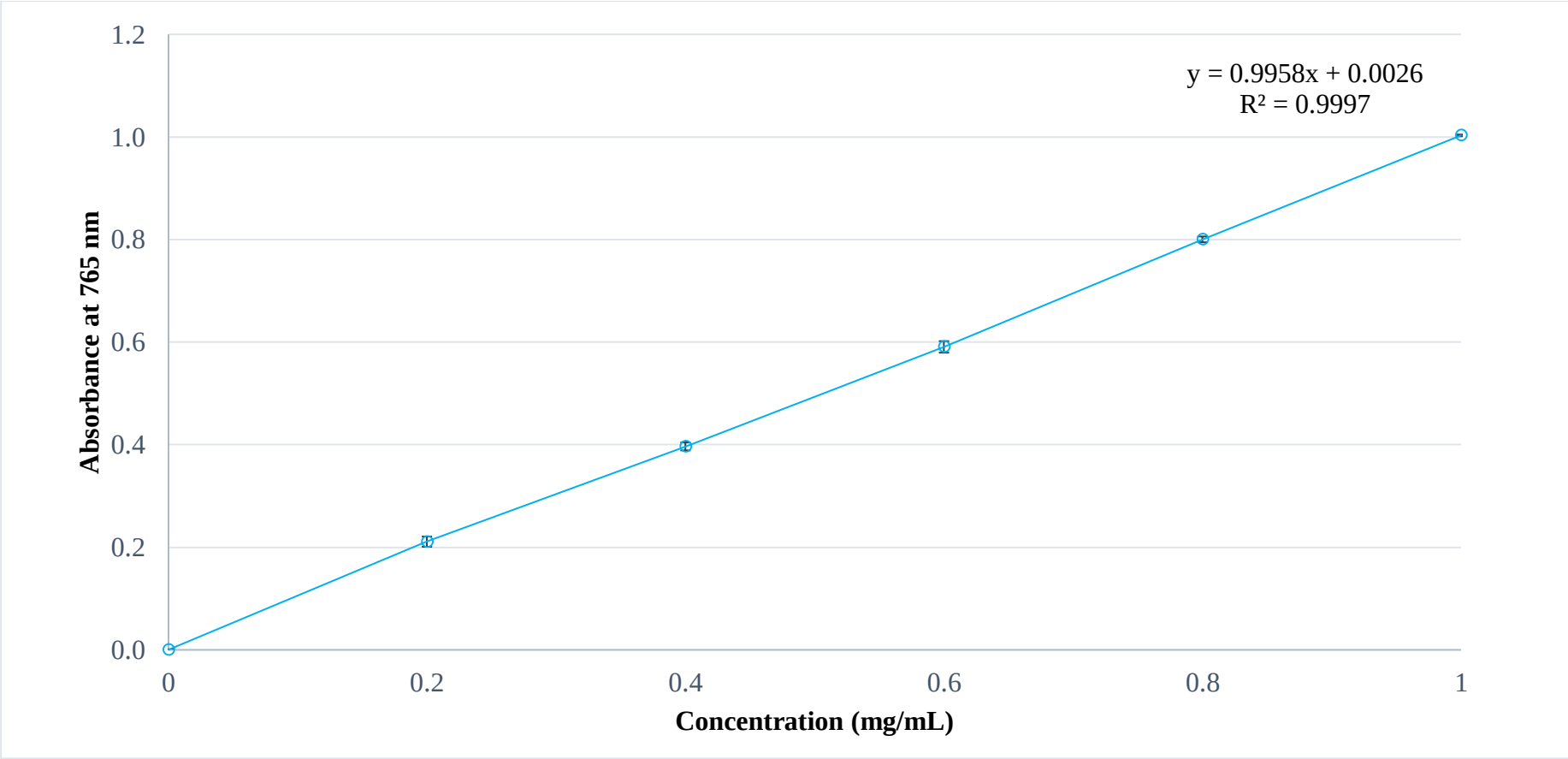


Figure 6.1. Standard curve of gallic acid for the determination of Total Phenolic Content (TPC)

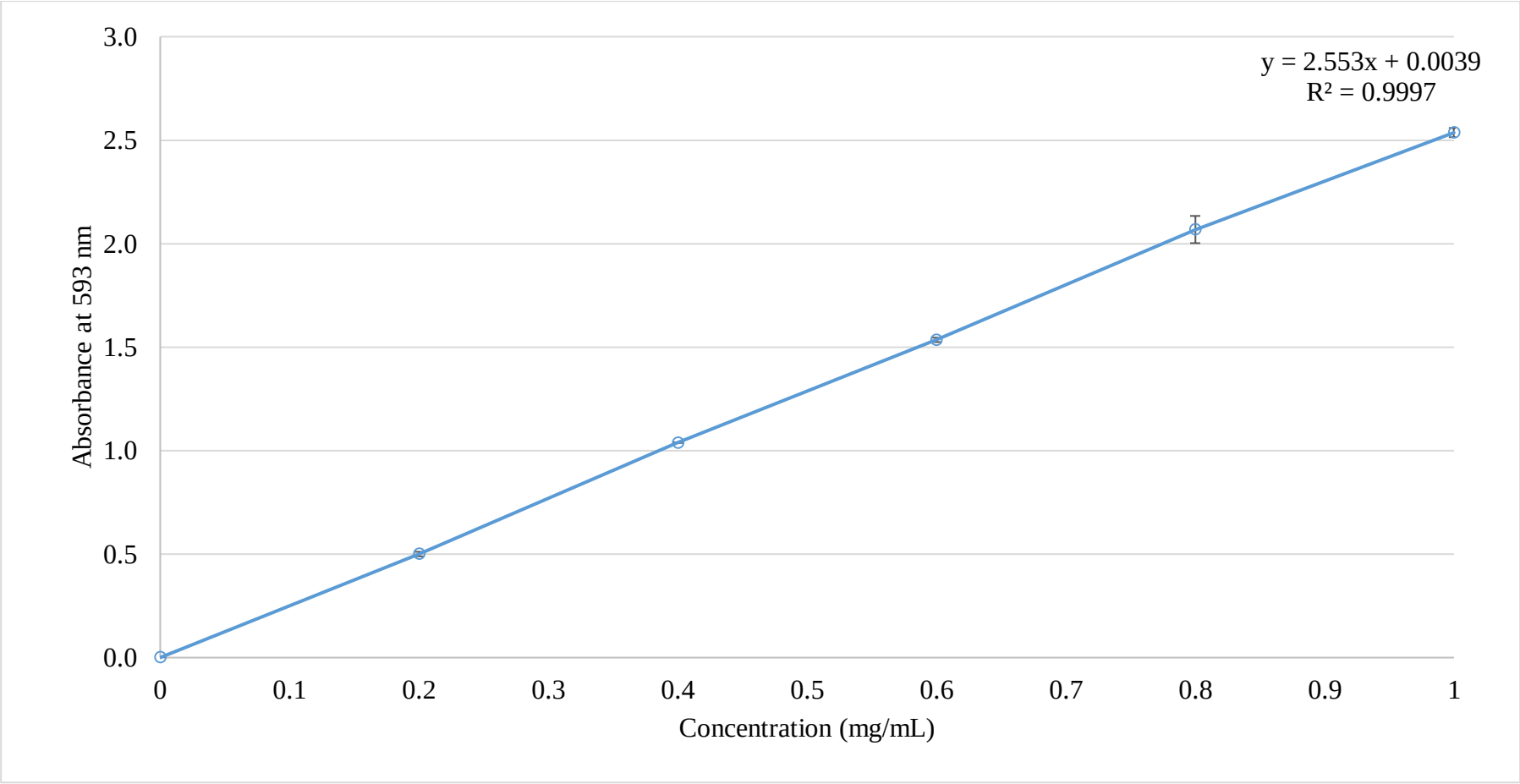


Figure 6.2. Standard curve of $FeSO_4.7H_2O$ for the determination of Ferric Reducing Antioxidant Power (FRAP)

Table 6.1. Total phenolic content and FRAP of plant chloroform extracts from leaves and stems

Plant extracts	Total Phenolic Content (mg GAE/10 g FW)		FRAP (mg $FeSO_4.7H_2O$ /10 g FW)	
	Leaves	Stems	Leaves	Stems
C. costatus	0.032 ± 0.000	0.031 ± 0.000	0.0165 ± 0.008	0.0123 ± 0.001
T. peduncularis	0.031 ± 0.001	0.030 ± 0.000	0.0164 ± 0.007	0.0137 ± 0.003
A. suaveolens	0.044 ± 0.006	0.033 ± 0.001	0.0127 ± 0.001	0.0147 ± 0.005
L. eugeniifolia	0.034 ± 0.002	0.030 ± 0.000	0.0162 ± 0.008	0.0123 ± 0.000
D. wallichii	0.032 ± 0.001	0.030 ± 0.001	0.0153 ± 0.005	0.0142 ± 0.007
U. grandiflora	0.033 ± 0.000	0.031 ± 0.001	0.0193 ± 0.015	0.0158 ± 0.010

* Values for total phenolic content expressed as mg gallic acid equivalent (GAE) per 10 g fresh weight samples (FW).

* Values for total flavonoid content expressed as mg rutin equivalent (RE) per 10 g fresh weight samples (FW).

*Values for FRAP expressed as mg $FeSO_4.7H_2O$ per 10 g fresh weight samples (FW).

* Values expressed as means of two biological replicated experiments and each biological replicate consisted of three technical replicates.

6.4. Conclusion

All chloroform extracts have a low total phenolic and FRAP content. The low positive correlation of the regression curve obtained from the TPC and FRAP assay supports the assumption.

Chapter 7: Isolation and identification of pinoresinol from chloroform extract of stems of *C. costatus* Miq. and zeylenol and ferrudiol from chloroform extract of leaves of *U. grandiflora* Roxb. ex Hornem.

7.1. Introduction

7.1.1. Secondary metabolites from plants

Plants produce a wide variety of chemical compounds that generate a high economic income. Most of plant chemicals (bioactive compounds) used for pharmacological or toxicological purposes are obtained from secondary metabolites (Thorat, 2018; Shipton, 2017). These secondary metabolites are biosynthetically derived from plant primary metabolites such as carbohydrates, amino acids, and lipids through secondary metabolism (Thorat, 2018). In short, secondary metabolites are defined as compounds that do not play a direct role in the daily functioning of plants. Secondary metabolites can be classified into three main categories: terpenes, alkaloids, and phenolic compounds (Thorat, 2018). There are four major pathways to synthesize these secondary metabolites: shikimic acid, malonic acid, mevalonic acid, and non-mevalonate pathway (MEP) (Thorat, 2018).

Terpenes. Terpenes are synthesized through mevalonate and deoxyxylulose pathway using a five-carbon building block called isoprene or short MEP and mevalonic acid pathway. The word ‘terpene’ itself is the generic term to summarize all kinds of isoprene (C_5H_8) polymers and their derivatives

with the general formula $(C_5H_8)_n$, where about 25000 types of terpenes have been identified so far (Thorat, 2018). Terpenes include hemiterpenes (such as prenol), monoterpenes (including iridoids), sesquiterpenes, diterpenes, sesterterpenes, triterpenes (including steroids and saponins), tetraterpenes (including carotenoids and xanthophylls), and polyterpenes (Shipton, 2017). In fact, there are a large number of terpenes forming various kinds of oxygenated derivatives, such as alcohols, aldehydes, ketones, carboxylic acids, esters, and glycosides (Thorat, 2018). Terpenoids are considered volatile and function as communicative chemicals, fragrances and flavouring in industries, and are a source of drugs (Shipton, 2017).

Alkaloids. Alkaloids (basic compounds) are produced from aromatic amino acids (via the shikimic acid pathway) and aliphatic amino acids (via the tricarboxylic acid cycle). Almost 12000 types have been isolated from plants so far (Thorat, 2018). Alkaloids are heterocyclic and come in a wide variety of structures, but they are all organic and nitrogen-containing compounds. The number of substitutions of the hydrogen atoms that are bound to the nitrogen with an alkyl group determine whether the alkaloid is a primary ($R\text{-}NH_2$), secondary (R2-NH), tertiary (R3-N), or quaternary (R4-N) (Shipton, 2017). Most alkaloids are soluble in alcohol and moderately soluble in water. Alkaloids have high pharmacological value, as their pharmacological effects are diverse.

Phenolic compounds. Phenolic compounds are synthesized through the shikimic acid pathway, as well as malonic acid pathway. Approximately 8000 types of phenolic compounds have been identified so far (Thorat, 2018).

Phenolic compounds have an aromatic ring bound to a hydroxyl group and the simplest phenolic compound is phenol, while polyphenols consist of a number of phenolic groups. Phenolics include benzoquinones or other simple phenols and phenolic acids or aldehydes, coumarins, naphthoquinones, xanthonoids, stilbenoids, anthraquinones, flavonoids, lignans, lignins, tannins, and other polyphenols.

7.1.2. Isolation of the bioactive compounds from crude extracts

Several natural products have been developed into very useful drugs. Some have served for the synthesis of derivatives (Baker *et al.*, 2007). Plant-derived molecules generally have highly complex structures that are difficult and not economically practical to synthesize on the industrial scale. Therefore, in such cases, the isolation of bioactive compounds from plants becomes the only possible approach to obtain the compounds (Rajagopal, 2014). Isolation of potentially bioactive compounds from crude extracts can be done by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). The compounds are identified by Nuclear Magnetic Resonance (NMR) and mass analysis (Chu, 2014; Mustafa Din, 2014).

7.1.2.1. High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography (HPLC) is a common analytical method for the isolation of compounds. HPLC has a high degree of adaptability, sensitivity, and reproducibility (Molnár-Perl and Füzfai, 2005).

Therefore, HPLC helps to isolate mostly and indicates the number of compounds present in sample mixture by appropriate standards and references (Molnár-Perl and Füzfai, 2005). HPLC uses a non-polar solid phase, like C-18, and a polar liquid mobile phase, generally a mixture of water and another solvent, such as acetonitrile or methanol (Shipton, 2017). C18 is a hydrophobic resin and has a high affinity to non-polar compounds (Shipton, 2017).

7.1.3. Structure elucidation and identification of the compounds

Identification of isolated compounds is much easier when this compound is known, because one ought to compare preliminary spectroscopic data of the compound with published data (Rajagopal, 2014). Identification of an unknown compound requires isolation of the compound from complex mixtures in sufficient amount for spectroscopic analyses (Boiteau *et al.,* 2018). Therefore, elucidation the structure of isolated compounds has become the last step in most studies of extraction and isolation of natural product.

Conventional methods used for structure elucidation are ultraviolet-visible spectroscopy (UV-vis), infrared spectroscopy (IR), mass spectrometry (MS), and nuclear magnetic resonance (NMR) (Rajagopal, 2014). Chu (2014) stated that nuclear magnetic resonance (NMR) and mass spectroscopy (MS) offer specific information on the molecular mass, carbon skeleton, and proton distribution and polarity of compounds, which help to obtain correct structures. Therefore, Nuclear Magnetic Resonance (NMR) and mass spectrophotometry (MS) were used in this study.

7.2. Materials and methods

7.2.1. Chemicals and reagents

Analytical hydrochloric acid (HCl), diethyl ether, dichloromethane, acetone, diethanolamine, and dimethyl sulfoxide (DMSO) were purchased from RCI Labscan Co., Ltd (Bangkok, Thailand). HPLC grade acetonitrile and methanol were bought from Thermo Fisher Scientific (Massachusetts, USA). Filtered ultrapure water 18Ω, PURELAB Flex, ELGA (High Wycombe, UK) was obtained from the respective HPLC lab.

7.2.2. Isolation and identification of the pure compounds

7.2.2.1. Pinoresinol (1) from *C. costatus* Miq. stem chloroform (UNMC-035)

Chloroform extract of stems of *C. costatus* Miq. (Apocynaceae) (5 g) was passed into high-pressure liquid chromatography (HPLC) machine (Dionex Ultimate 3000, Thermo Fisher (Florida, USA) equipped with a binary pump, diode array detector (DAD), autosampler, and automatic fraction collector (AFC) with further analysis using Chromeleon v.6 software. Acetonitrile/ ultrapure water (90: 10 ratio) was used as the mobile phase at a flow rate of 1 mL/min, detected by DAD at 210 nm UV wavelength, injection of 100 µL/min, at 37°C, using a reverse phase Hypersil™ ODS C18 column (size 250 × 4.6 mm, particle size 5 µm, Thermo Fisher, USA). A gradient system was applied to the

separation: 20-80% acetonitrile in 14 min, 80% acetonitrile in 3 min, 80-20% acetonitrile in 1 min, and 20% in 7 min.

Collecting a major peak at retention time of 9 min allowed the isolation of compound 1 (3 mg). The molecular mass of this compound was determined via an LC-TOF-MS system, MicroTOF QIII (Bruker Daltonic, Germany) in combination with a Thermo Scientific Dionex Ultimate 3000 UHPLC system. Further identification of the compound was achieved through Nuclear Magnetic Resonance (NMR) using the Bruker AVANCE-500 MHz NMR spectrometer with 5 mm BBO probe. It was identified as pinoresinol after comparing its ^{1}H-NMR and EIMS data with those from literature (Guz and Stermitz, 2000; Diep *et al.*, 2007).

7.2.2.2. Zeylenol (2) and ferrudiol (3) from *U. grandiflora* Roxb. ex Hornem. leaves chloroform (UNMC-048)

Chloroform extract of leaves of *U. grandiflora* Roxb. ex Hornem. (Annonaceae) (1.5 g) was injected into HPLC (Agilent 1200, Santa Clara, USA) equipped with a binary pump, diode array detector (DAD), autosampler, and automatic fraction collector (AFC) with further analysis, using LC 1260 DAD Open Lab software. Acetonitrile/ ultrapure water (90:10 ratio) was used as mobile phase at a flow rate of 1 mL/min, detected by DAD at 210 nm UV wavelength, injection of 60 µL/min, at 37°C, using reverse phase C18 column of Biphenyl 100A (size 150 x 4.6 mm, particle size 5 µm, Kinetex® Phenomenax, USA). A gradient system was used for separation: 10% acetonitrile in 2 min, 10-

75% acetonitrile in 12 min, 75% acetonitrile in 10 min, 75-100% acetonitrile in 4 min, 100% acetonitrile in 4 min, 100-10% acetonitrile in 2 min, and 100% acetonitrile in 3 min.

Two peaks were collected at a retention time of 11.01 min and 16.25 min, allowing the isolation of compound 2 (2 mg) and compound 3 (1.5 mg), respectively. The molecular mass of compounds was analysed through QTOF high resolution MS system (6600 Triple-TOF-Electrospray Quadrupole-quadrupole-TOF AB Sciex, Singapore). Further identification of the compounds was achieved by Nuclear Magnetic Resonance (NMR) using Bruker AVANCE HDX III 600 MHz NMR spectrophotometer with 5 mm cryoprobe. Those peaks were identified as zeylenol and ferrudiol based on their ^{1}H-NMR and EIMS data which were in agreement with those of Jolad *et al.* (1981), Kijjoa *et al.* (2002), Wirasathien *et al.* (2006).

7.3. Results and discussion

7.3.1. Selection of the crude extracts for further isolation and identification of compounds

Chloroform extracts of *C. costatus* stem and chloroform extracts of *U. grandiflora* leaves were selected for further separation through chromatography due to their antiplasmodial, antileishmanial, and antibacterial activities. To the best of our knowledge, it is the first phytochemical analysis of *C. costatus*.

7.3.2. High Performance Liquid Chromatography (HPLC) analysis

HPLC analysis of the chloroform extract of stems of *C. costatus* yielded a major compound (**1**) while HPLC analysis of the chloroform extract of *U. grandiflora* leaves yielded major compounds (**2**) and (**3**) (Figure 7.1 and Figure 7.2). These major compounds (**1**), (**2**), and (**3**) were freeze-dried prior to NMR and MS analysis. The presence of additional peaks in the spectra (Figure 7.1 and Figure 7.2) was attributed to the presence of minor constituents. In this case, compound (**1**) was isolated in enough amounts for NMR analysis and further experiments on biological activities e.g. antiplasmodial, antileishmanial, and antibacterial. The amounts obtained for compounds (**2**) and (**3**) were sufficient for both structural elucidation via NMR analysis and a biological assay.

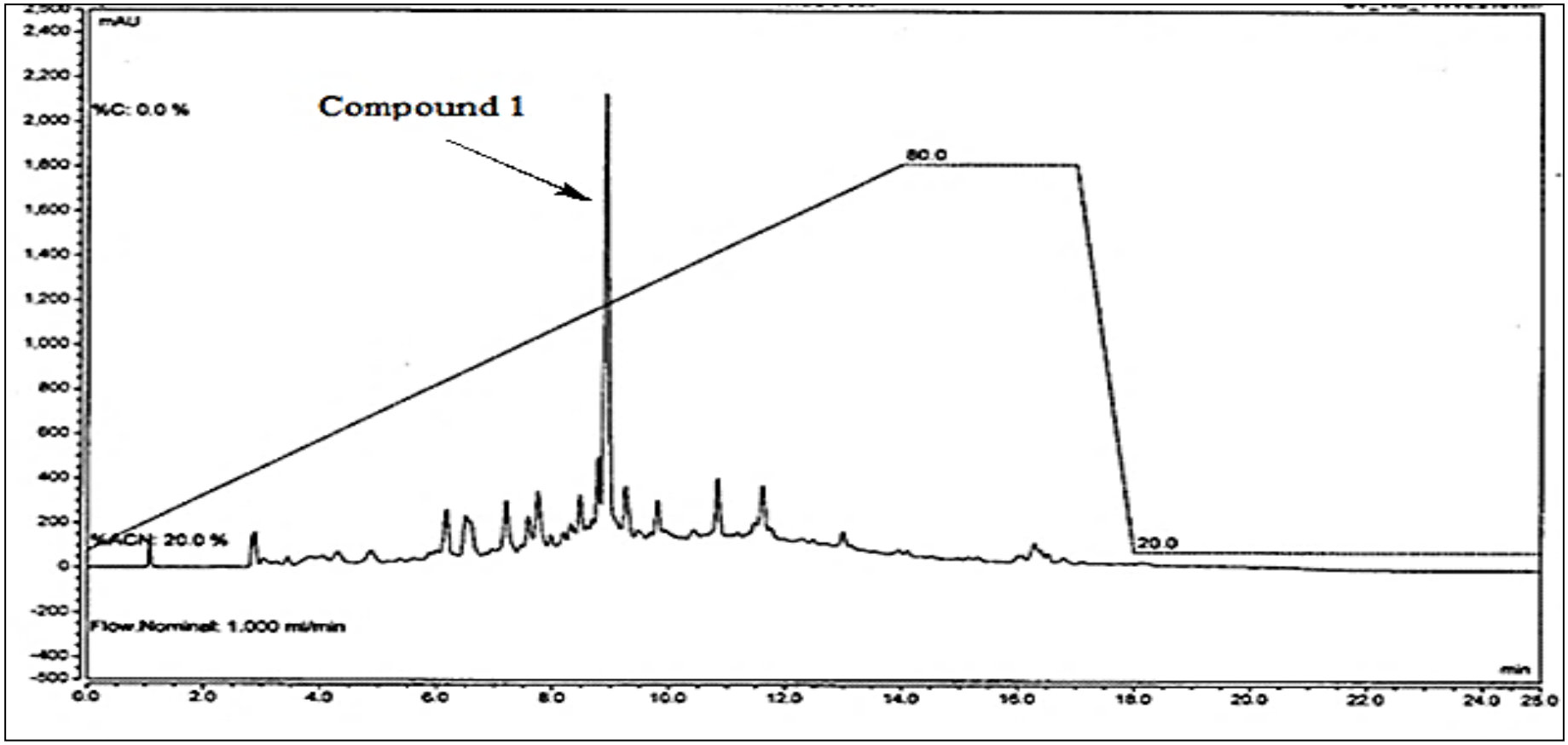


Figure 7.1. High-performance liquid chromatography (HPLC) profile of the chloroform extract of *C. costatus* stems (0-25 min) at 210 nm. The peak of compound (1) is indicated

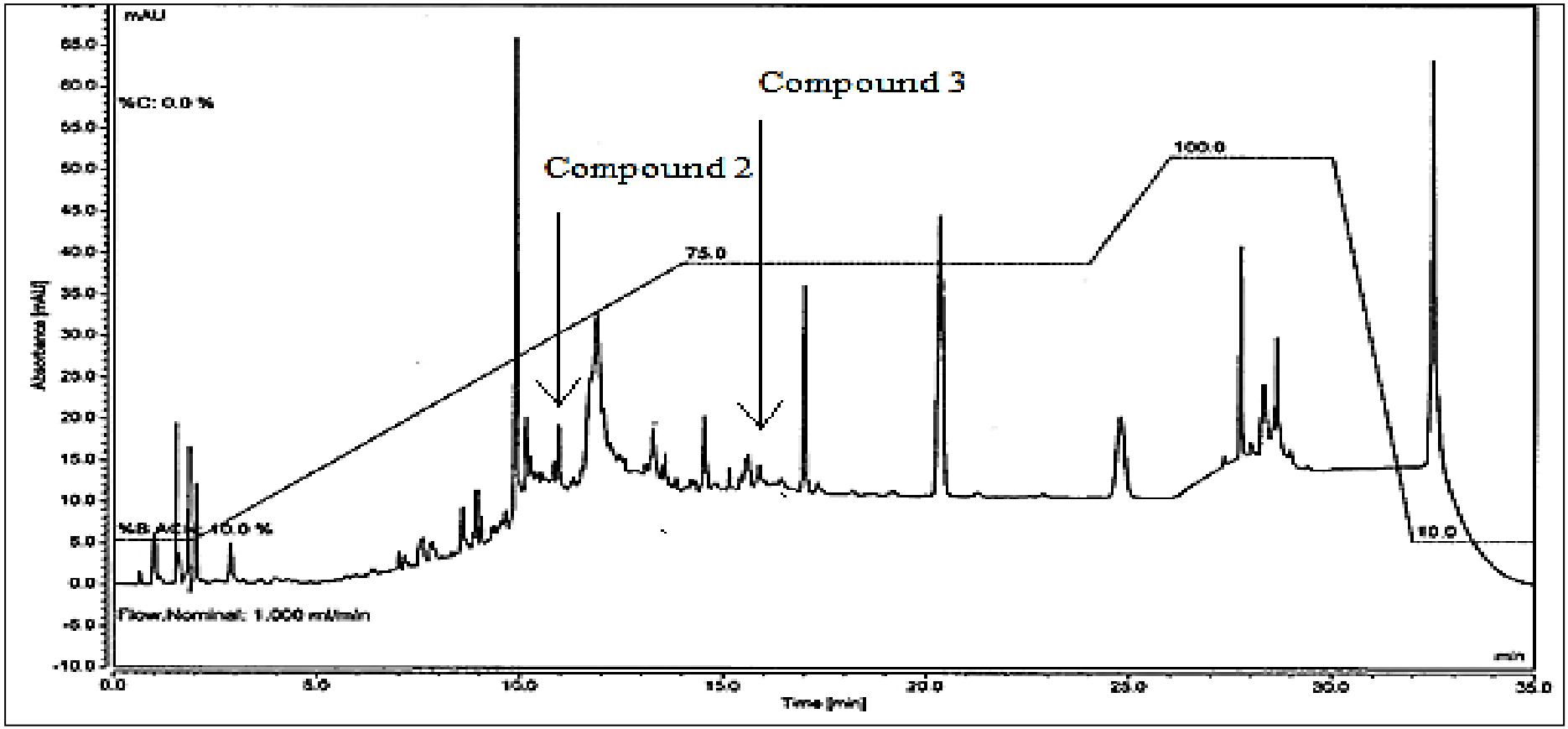


Figure 7.2. High-performance liquid chromatography (HPLC) profile of the chloroform extract of leaves of *U. grandifolia* (0 – 35 min) at 210 nm. The peaks of compounds (2) and (3) are indicated

7.3.3. Structure elucidation and identification of compounds (1), (2) and (3)

Structures of the isolated compounds were elucidated by nuclear magnetic resonance (NMR), mass spectroscopy (MS), as well as comparison with data reported in the literature. The interpretation of ^{1}H NMR and comparison with data reported in the literature were done on compounds (**1**), (**2**), and (**3**), resulting in the identification of pinoresinol (**1**), zeylenol (**2**), and ferrudiol (**3**). Although pinoresinol, zeylenol, and ferrudiol are known in other plants, it is however the first report on the occurrence of pinoresinol in *Chilocarpus costatus* Miq. and ferrudiol in *U. grandiflora*. Zeylenol is a known constituent of *U. grandiflora* (Seangphakdee *et al.,* 2013). These findings are of critical importance in chemotaxonomy.

7.3.3.1. Pinoresinol (1)

Pinoresinol is a lignan and knowledge regarding its biological activity remains limited. For example, it inhibited α-glucosidase in rat (IC_{50} value of 34.3l M) (Wikul *et al.,* 2012). At the same time, pinoresinol functions as a defensive agent in plants (Schroeder *et al.,* 2006).

In this study, compound **1** was identified as pinoresinol. In brief, compound **1** (pinoresinol) is a colorless amorphous solid with m.p. 120-121 ºC (Guz *et al.,* 2000; Diep *et al.,* 2007). Its molecular formula of $C_{20}H_{22}O_6$ was in agreement with the mass spectral data of this study *m/z* 341 [M-H_2O+H^+]. The ^{1}H-NMR spectrum analysis (acetone-d_6, 400 MHz) revealed that there were

peaks attributed to protons of the two aromatic rings at 7.0, 6.85, and 6.80 ppm which suggests that each aromatic ring of the structure has three substituted positions. Oxymethine protons 2 and 6 were doubled at 4.69 ppm while methylene protons adjacent to the oxygen atom were assigned to the doublet of doublets at 4.21 and 3.83 ppm. Each proton of these methylene protons had different chemical shifts because rotation around the bond was restricted due to ring strain which resulted in different chemical environments for each proton of the methylene protons.

The methine protons evoked in a multiplet at 3.10 ppm while methoxy groups were assigned the singlet at 3.86 ppm and this relatively higher chemical shift for the methoxy protons was due to the deshielding effect of the adjacent highly electronegative oxygen atom. Finally, the spectrum also showed the presence of a broad peak at 7.61 ppm and this broadness indicated that the peak belonged to the hydroxyl groups of the structure. Based on these findings, the structure of **1** was proposed to be as shown in Figure 7.3. Moreover, these analytical data are in good agreement with those reported in the literature confirming the suggested structure of **1** and its identity as pinoresinol (Guz *et al.,* 2000; Diep *et al.,* 2007). The other IUPAC name for pinoresinol was (±)2,6-bis(4’-hydroxy-3’-methoxy-phenyl)-3,7-dioxabicyclo [3.3.0] octane.

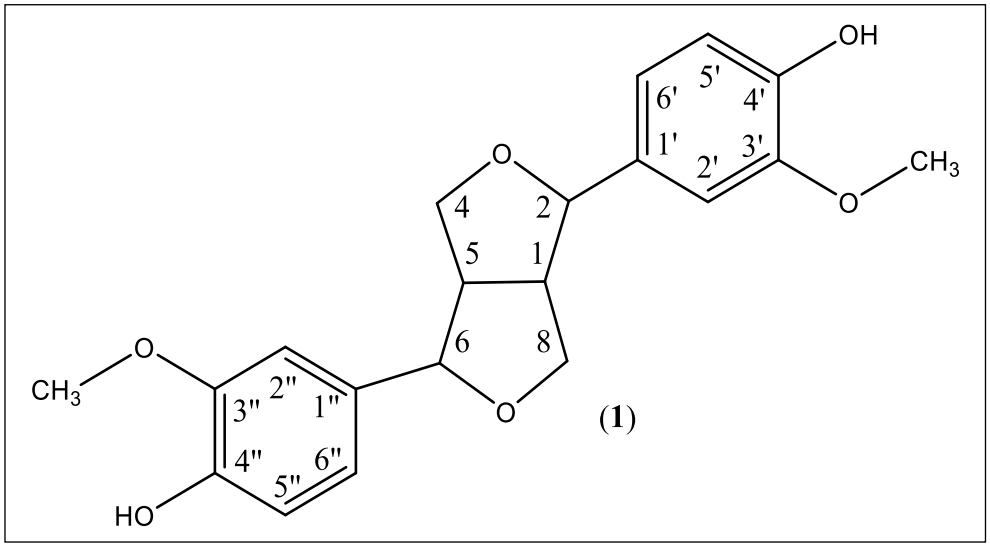


Figure 7.3. The structure of pinoresinol (1)

Table 7.1. ^{1}H NMR spectral data ($CDCl_3$, 500 MHz) of pinoresinol (1)

NMR shifts			
Position	**δ_H**	**δ_H [a]**	**δ_H [b]**
1,5	3.10m	3.10m	3.10m
2,6	4.69d	4.74d	4.73d
4,8	4.21dd	4.25dd	4.24dd
	3.83dd	3.88dd	3.88dd
2',2''	7.0d	6.88d	6.89d
5',5''	6.80d	6.88d	6.88d
6',6''	6.85dd	6.82dd	6.82dd
3',3''-OCH_3	3.86s	3.91s	3.90s
OH	7.61s	5.59s	-

*** [a]Guz *et al.* (2000); [b]Diep *et al.* (2007).**

7.3.3.2. Zeylenol (2)

Zeylenol also known as uvaribonol B belongs to the benzocylclohexane oxide derivatives class (HMDB, 2018). Zeylenol is a colorless glassy solid insoluble in water (Jolad *et al.,* 1981). Historically, zeylenol was isolated from *Uvaria zeylanica* by Cole and Bates in 1981 and its structures were confirmed by chemical correlation and circular dichroism spectroscopy. Plants containing zeylenol showed biological activities, such as selective cytotoxicity for HL-60 leukaemia cells and moderate cytotoxicity for MDA-MB231 human breast cancer cells, as well as good antitumor activity (Tang *et al.,* 2011), antifeedant (Nyandoro *et al.,* 2016), and anti-inflammatory activities (Vinaykumar *et al.,* 2017).

Compound **2** was identified as zeylenol. In short, compound **2** (zeylenol) is a colorless crystal with m.p 110-112 (Jolad *et al.,* 1981; Pan *et al.,* 1995; Seangphakdee *et al.,* 2013; Tang *et al.,* 2011). Its molecular formula of $C_{21}H_{20}O_7$ was in agreement with the mass spectral data of this study *m/z* 407 [M+Na]. Some of the compound's NMR chemical shifts were slightly different from the chemical shifts found in other journals (Takeuchi *et al.,* 2001; Tang *et al.,* 2011), probably due to slight of impurities. The structure contains secondary and tertiary hydroxyl groups, alkene group and benzoyl groups (Vinaykumar *et al.,* 2017). The ^{1}H-NMR spectrum ($CDCl_3$, 600 MHz) showed a pair of doublets at 5.80 and 5.99 ppm assigned to the alkene group protons, H-4 and H-5, respectively. The peaks at 4.22, 4.32, and 5.70 ppm have been assigned to protons 2, 6 and 3, respectively, and these protons absorbed further the downfield

due to the deshielding effect exerted by the adjacent highly electronegative oxygen atoms.

The presence of two benzoyl groups was confirmed by the aromatic proton signal. These aromatic protons of the benzoyl groups were attributed by doubling the doublet peaks between 7.30 and 8 ppm. Based on these findings, the structure of **2** was proposed as shown in Figure 7.3. Moreover, these analytical data are in agreement with those reported in the literature that confirm the suggested structure of **2** and its identity as zeylenol (Takeuchi *et al.,* 2001; Tang *et al.,* 2011).

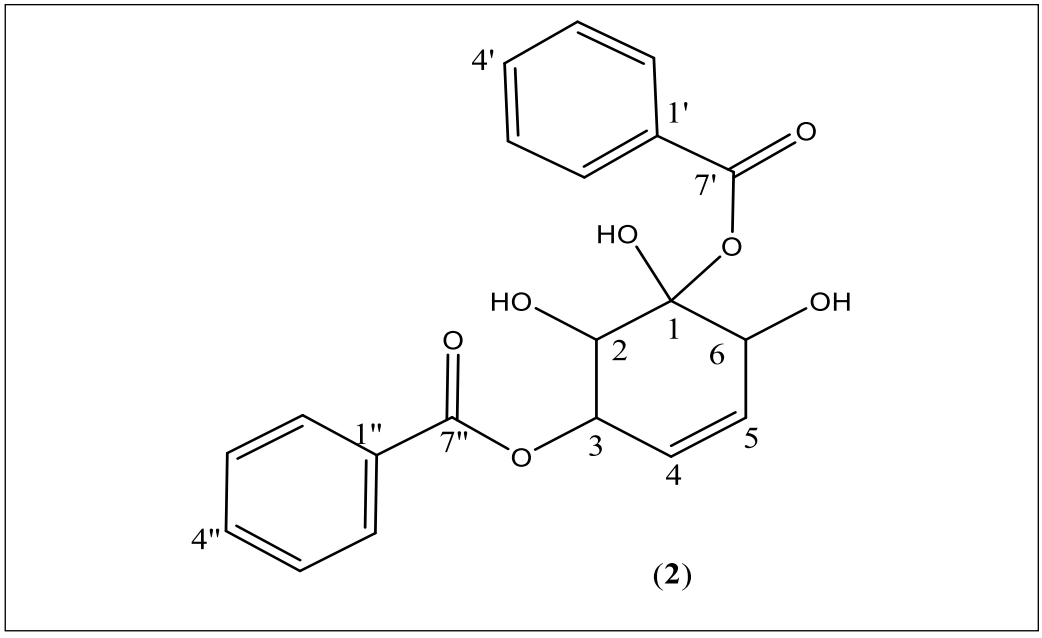


Figure 7.4. The structure of zeylenol (2)

Table 7.2. ^{1}H NMR spectral data ($CDCl_3$, 600 MHz) of zeylenol (2)

NMR shifts			
Position	**δ_H**	**δ_H [a]**	**δ_H [b]**
2	4.22d	4.22d	4.23dd
3	5.70d	5.69dd	5.70dd
4	5.80dd	5.81dd	5.68dd
5	5.99dd	5.94dd	5.81dd
6	4.32d	4.34d	4.13d
2',6'	8.0dd	7.99dd	7.99dd
3',5'	7.30dd	7.42m	7.34dd
4'	7.47dd	7.56m	7.47dd
2'',6''	8.0dd	8.03d	7.80dd
3'',5''	7.30dd	7.42m	7.34dd
4''	7.47dd	7.56m	7.47dd

* **[a]Tang *et al.* (2011); [b]Takeuchi *et al.* (2001).**

7.3.3.3. Ferrudiol (3)

Ferrudiol consists of 3 benzoyl ester groups, a secondary alcohol, and a chemically tertiary alcohol group (Chem Essen Inc., 2018). In this study, compound **3** was identified as ferrudiol. In brief, compound **3** (ferrudiol) is a colorless glassy solid with m.p. 192-195^{o}C. Its molecular formula $C_{28}H_{24}O_8$ was in agreement with the mass spectral data of this study *m/z* 512 [$M+Na^{+}+H^{+}$]. Some of the NMR chemical shifts of the compound were slightly different from the chemical shifts found in other journals (Gary *et al.,* 1982) for the same

compound, probably due to the presence of traces of impurities. The ^{1}H-NMR spectrum of compound **3** was found to be very similar to that of compound **2**. However, there is a notable increase in the number of peaks in the spectrum of **3** relative to that of compound **2** between 7.30 and 8 ppm. These additional peaks in the spectrum of **3** were assigned to the aromatic protons of the additional C-6 benzoyl group of compound **3** and further confirm that compound **3** is ferrudiol. In fact, the analytical data are in good agreement with those reported in the literature confirming the suggested structure of **3** and its identity as ferrudiol (Gary *et al.*, 1982).

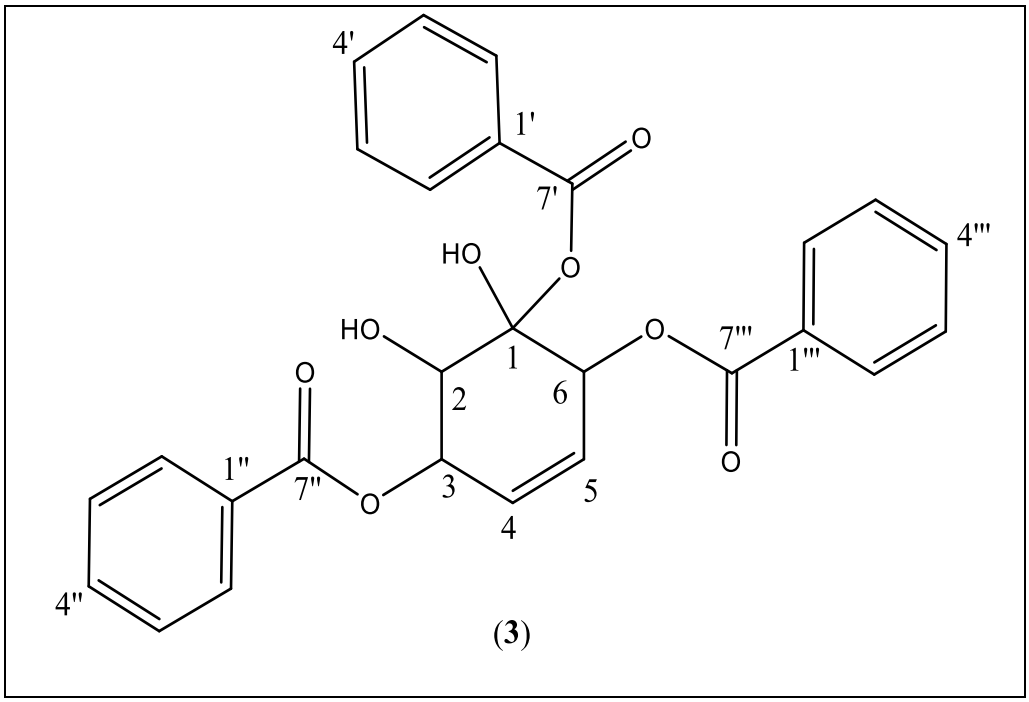


Figure 7.5. The structure of ferrudiol (3)

Table 7.3. ^{1}H NMR spectral data ($CDCl_3$, 600 MHz) of ferrudiol (3)

NMR shifts		
Position	**δ_H**	**δ_H [c]**
2	4.22d	4.32d
3	5.70dd	5.83dd
4	5.80dd	5.80dd
5	5.99dd	5.98dd
6	4.32d	5.85d
2',6'	8.0dd	-
3',5'	7.30dd	-
4'	7.47dd	-
2'',6''	8.0dd	-
3'',5''	7.30dd	-
4''	7.47dd	-
2''',6'''	8.0dd	-
3''',5'''	7.30dd	-
4'''	7.47dd	-

*[a]**Gary *et al.* (1982)**

7.4. Conclusion

All compounds were isolated from *C. costatus* Miq. and *U. grandiflora* Roxb. ex Hornem. One lignan (compound **1**) and two benzocylclohexane oxide derivatives (compound **2** and compound **3**) were isolated via HPLC and identified. To the best of our knowledge, although pinoresinol, zeylenol, and

ferrudiol are known in other plants, however, it is the first report on the occurrence of pinoresinol in the genus *Chilocarpus* and ferrudiol in the *U. grandiflora*. In natural product chemistry, botany, and chemotaxonomy, report of known compounds in rare plants are of critical importance (Wiart, 2007). It helps botanists to update plant clarification, assists pharmacologists to understand the pharmacological activity of medicinal plants, and assist biochemists to place extra knowledge on biosynthesis in plants.

Chapter 8: Bioactivities of isolated compounds from *C. costatus* Miq. and *U. grandiflora* Roxb. ex Hornem.

8.1. Introduction

Medicinal plants have long been used as a source of drugs in China, India, East, and Western Europe (Hamburger and Hostettmann, 1991). As mentioned in Chapter 1, about 25% of prescribed medicines available today in pharmaceutical industries are derived from higher plants (Farnsworth, 1985; Payne *et al.*, 1992; Rao and Ravishankar, 2002). Over time, technologies have been developed allowing the isolation of active principles (e.g. morphine, strychnine, and quinine) (Hamburger and Hostettmann, 1991).

This development marks a new era for synthetic medicines and modern research in medicinal plants. Leading pharmaceutical companies are showing low interest in higher plants as a source for new lead structures. Only a small percentage of plants has been investigated for their phytochemical, biological and pharmacological screening, whereas the remainder has not been explored. From 1957 to 1981, the United States National Cancer Institute (NCI) screened 35000 plant species for antitumor activities (Hamburger and Hostettmann, 1991).

8.1.1. Investigation of active metabolites activity in plants

The plant kingdom offers thousand of natural products. The isolation of plant compounds is tedious. When looking for a bioactive constituent, bioassays are done with extracts because it would be time consuming and costly to isolate all compounds first and test them all (Hamburger and Hostettmann, 1991). These tests should be simple, sensitive, reliable, fast, reproducible, and inexpensive (Hamburger and Hostettmann, 1991). For example, researchers use the binding site of platelet-activating factor (PAF) in human cells to investigate the PAF antagonistic activities (Küster and Frölich, 1986). The application of cell culture has become vital in the search for therapeutic agents. Common cell lines, such as VERO, MRC-5, and HCT-116 are widely used for primary screening of extracts.

8.2. Materials and methods

8.2.1. Chemicals and reagents

Chemicals and reagents were used for the experiment as per Chapter 4 (Antiparasitic activities of the plant chloroform extracts) and Chapter 5 (Antibacterial activities of the plant chloroform extracts).

8.2.2. Cell lines

Cell lines were used for the experiment as per Chapter 4 (Antiparasitic activities of the plant chloroform extracts) and Chapter 5 (Antibacterial activities of the plant chloroform extracts).

8.2.3. Equipment and software

Similar equipment and software were used as per Chapter 4 (Antiparasitic activities of the plant chloroform extracts) and Chapter 5 (Antibacterial activities of the plant chloroform extracts).

8.2.4. Maintenance of the cell cultures

Maintenance of cell cultures was used as per Chapter 4 (Antiparasitic activities of the plant chloroform extracts) and Chapter 5 (Antibacterial activities of the plant chloroform extracts).

8.2.5. Bioactivities of the isolated compounds:

8.2.5.1. Antiplasmodial assay

The toxicity of pinoresinol against *P. falciparum* K1 chloroquine-resistant was determined using malaria SYBR Green I-based fluorescence (MSF) assay (Smilkstein *et al.,* 2004). Ring stage synchronized parasites at 1% parasitemia,

and 2% hematocrit were placed into the individual well of 96 well plates, while the 2% hematocrit non-parasitized erythrocytes served as reference controls. Pinoresinol was serially prepared diluted in complete medium and dispensed into the test wells.

The plates were incubated at 37°C for 48 hours. At the end of the incubation period, SYBR Green I solution was added to each well and mixed by a microplate mixer. After 1 hour incubation in the dark at room temperature, the fluorescence signal was measured with a Spectramax M5 Multi-Mode microplate reader, Molecular Devices (California, USA) with 485 nm centred excitation bands and emission wavelength. Chloroquine and dihydroartemisinin were used as positive antiplasmodial control drugs. All experiments were performed in triplicate. Results were expressed as the concentration reducing the number of live parasites by 50% (IC_{50}).

8.2.5.2. Antileishmanial assay

The antileishmanial assay was performed as per Chapter 4 (Antiparasitic activities of the plant chloroform extracts).

8.2.5.3. Cytotoxicity assay

Cytotoxicity assay was performed as per Chapter 4 (Antiparasitic activities of the plant chloroform extracts).

8.2.5.4. Antibacterial assay

Antibacterial assay was performed for the experiment as per Chapter 5 (Antibacterial activities of the plant chloroform extracts).

8.2.6. Statistical analysis

Statistical analysis of experimental data was performed using data analysis software mentioned in Chapter 4 and Chapter 5. Data were presented as mean ± standard deviation, which was obtained from the three independent experiments.

8.3. Results and discussion

8.3.1. Antiplasmodial assay

Mahmoudi *et al.* (2006) classify antiplasmodial activity of pure compounds as follow: IC_{50}< 0.06 µM (very active), IC_{50} between 0.06 and 5 µM (active), and IC_{50}> 5 µM (inactive). Pinoresinol demonstrated IC_{50} of 180.8 ± 5.7 µM for chloroquine-resistant *P. falciparum* K1 strains (Table 8.1). Accordingly, pinoresinol is inactive for chloroquine-sensitive *P falciparum* K1 strains. However, the results obtained contradict Apisantiyakom *et al.* (2004), who reported that pinoresinol inhibited P. *falciparum* K1 with an IC_{50} of 3.4 µg/mL. It can also be said that an IC_{50} of 180.8 µM is not a sign of complete inactivity, but very weak activity. It is reasonable to infer that some minor antiplasmodial constituents are present in the extract.

In natural product pharmacology, it is not suprising to have different IC_{50} results for parasites or cancer cells. Regarding pinoresinol, the sole report was from a non-peer reviewed publication, written in Thai in a non-Scopus or non-Pubmed journal from local publication. Since there is only one report from a local non-peer reviewed journal, it is reasonable to infer that our results published in a high-impact, peer reviewed paper are accurate.

8.3.2. Antileishmanial assay

Mbongo *et al.* (1997) state that antileishmanial activity for pure compounds is defined with an IC_{50} < 50 µM. Meanwhile, Liu *et al.* (2003) classify antileishmanial activity of pure compounds as follows: EC_{50} value < 5 µM (very good activity), 5-10 µM (good activity), EC_{50} 10-20 µM (moderate activity), and EC_{50}> 20 µM (weak activity). Chollet *et al.* (2008) define pure compounds with IC_{50} value of 50 µM as moderately active compounds. Based on the results obtained in Table 8.1, *L donovani* was less susceptible to pinoresinol, ferrudiol, and zeylenol with of IC_{50} of 213.4 ± 0.2, 101.1 ± 0.3, and 110.0 ± 0.1 µM, respectively.

In short, pinoresinol had weak activity against *L. donovani* and this in agreement with the previous study who stated that pinoresinol was inactive against *L. major* (Glaser *et al.*, 2015). Although both zeylenol and ferrudiol were found weakly active against *L. donovani*, the results differ from Roumy *et al.* (2009) for *L. amazonesis*. Roumy *et al.* (2009) stated that a mixture of 4,6,20 -

trihydroxy-6- [10’ (Z)-heptadecenyl]-1-cyclohexen-2-one and 1,4,6-trihydroxy-1,20 -epoxy-6- [10’ (Z)-heptadecenyl]-2-cyclohexene from *Tapiria guianense* Aubl. (Anacardiaceae) were active against *L. amazonensis* with IC_{50} values below 5 µM. One reason for this difference, could because of the strain and species studied. Our strain and species are different from the above studies. It is well known that strains present in the Indian subcontinent have developed tolerance against antileishmanial drugs because of the drug pressure. In this research, we are providing for the first time evidence of weak activities of pinoresinol, ferrudiol, and zeylenol against *L. donovani*.

Table 8.1. Cytotoxicity, antileishmanial, and antiplasmodial activities of compounds (µM)

Extract	Cytotoxicity	Antileishmanial activity	Antiplasmodial activity
	MRC-5 [b] (CC_{50})	***L. donovani* [b] (IC_{50})**	***P. falciparum* [b] (IC_{50})**
Pinoresinol	>27.9	213.4 ± 0.2	180.8 ± 5.7
Zeylenol	>26.0	110.0 ± 0.1	N.T.
Ferrudiol	>20.4	101.1 ± 0.3	N.T.
Vincristine[a]	0.3 ± 1.5	N.T.	N.T.
Miltefosine[a]	N.T.	4.6 ± 2.5	N.T.
Chloroquine[a]	N.T.	N.T.	0.1 ± 14.6
Dihydroartemisinin[a]	N.T.	N.T.	0.005 ± 0.6

Each value represents mean ± standard deviation of 3 independent experiment. [a]Positive control drug. [b] MRC-5: Normal human lung cell; *L. donovani*; *Leishmania donovani* BHU-1251 strain; *P. falciparum: Plasmodium falciparum* K1 chloroquine-resistant strain. SI: Selectivity Index

NT: Not tested

8.3.3. Cytotoxicity assay

According to the American National Cancer Institute, the threshold for cytotoxicity of pure compounds is defined at a CC_{50} value of 10 µM (Efferth and Kuete, 2010). Investigation on the toxicity of pinoresinol, ferrudiol, and zeylenol to MRC-5 cells was examined (Table 8.1). Findings obtained showed that all the compounds demonstrated CC_{50} above 20 µM. Therefore, pinoresinol, zeylenol and ferrudiol are non-toxic for MRC-5 cells.

8.3.4. Antibacterial assay

Testing of pinoresinol against bacterial activity was performed on seven types of bacteria namely: MRSA (clinical isolate), *S. aureus* ATCC®25923™, *S. epidermidis* ATCC®12228™, *E. faecalis* ATCC®29212™, *K. pneumoniae* (clinical isolate), *E. coli* ATCC®25922™, and *P. aeruginosa* ATCC®15442™ using broth microdilution technique. Based on the results obtained, pinoresinol inhibited *S. epidermidis* with MIC value of 750 ± 0.5 µg/mL and demonstrated MIC values above 1,500 µg/mL against the remaining 6 bacteria tested (Table 8.2). Examination on the synergistic effect of pinoresinol with vancomycin or cefotaxime for *S. epidermidis* was also performed (Table 8.3). Pinoresinol increased sensitivity of *S. epidermidis* to cefotaxime. No synergistic effects were observed with vancomycin.

Table 8.2. Minimum inhibiting concentration (MIC) of pinoresinol against bacteria (µg/mL)

Extract	Mean MIC (µg/mL)						
	Gram-positive bacteria				Gram-negative bacteria		
	MRSA[b]	***S. aureus*[b]**	***S. epidermidis*[b]**	***E. faecalis*[b]**	***E. coli*[b]**	***P. aeruginosa*[b]**	***K. pneumoniae*[b]**
Pinoresinol	>1500	>1500	**750 ± 0.5**	>1500	>1500	>1500	>1500
Vancomycin[a]	1500 ± 0.03	0.7±0.0	1.5±0.0	11.7	N.T.	N.T.	N.T.
Cefotaxime[a]	11.7±0.3	11.7±0.3	11.7±0.1	11.7±0.3	11.7±0.1	375±0.3	11.7±0.3

Each value represents mean ± standard deviation of 3 independent experiments. [a]Antibiotic standard. [b]*Staphylococcus aureus* (ATCC®25923™); MRSA: Methicillin-resistant *Staphylococcus aureus* (clinical isolate); *Staphylococcus epidermidis* (ATCC®12228™); *Enterococcus faecalis* (ATCC®29212™); *Escherichia coli* (ATCC®25922™); *Pseudomonas aeruginosa* (ATCC®15442™); *Klebsiella pneumoniae* (clinical isolate). N.T.: Not tested

Table 8.3. Fractional inhibitory concentration index (FICI) of pinoresinol with standard antibiotics against *S. epidermidis*

Extract	Antibiotics	FICI[a]	Effect
Pinoresinol	Cefotaxime	**0.5**	Synergistic
	Vancomycin	2.2	Indifferent

*FICI: fractional inhibition concentration index.

*Values are means of triplicates.

[a]FICI ≤ 0.5 represent synergistic effect; > 0.5 and ≤4.0 represent indifference; > 4.0 represent antagonistic effect.

8.4. Conclusion

To the best of our knowledge, this is the first report of the activities of pinoresinol against *L. donovani*. Also, we provide the evidence that pinoresinol increases the antibiotic activities of cefotaxime against *S. epidermidis.* This is of critical importance in this current age of bacterial resistance. We also demonstrate for the first time the activities of zeylenol and ferrudiol against *L. donovani.*

Chapter 9: General conclusions and future recommendations

9.1. General conclusions

Objective 1: To collect and determine the extraction yields of chloroform extracts of leaves and stems in plants.

a) Sampling of plants in Perak afforded the collection of 6 medicinal plants from three different families of the Class Magnoliopsida (Dicotyledons): Annonaceae, Apocynaceae, and Ebenaceae (Takhtajan, 2009). *C. costatus* Miq., *L. eugeniifolia* (Wall. ex. G. Don) A. DC., and *T. peduncularis* Wall belong to the Apocynaceae Juss. (1789). *U. grandifolia* Roxb. ex Hornem. and *A. suaveolens* (Blume) Blume belong to the Annonaceae Juss. (1789). *D. wallichii* King & Gamble belongs to the Ebenaceae Gürke (1890).

b) Extraction of plant leaves and stems extracts were done with sequential macerations in solvents of increasing polarity: hexane, chloroform, and methanol. The results obtained showed that yields varied between plant species and part of plants. Most methanol extracts had the highest extraction yield, followed by chloroform, and hexane extracts. The lowest extraction yield was 0.01% (*C. costatus* stem hexane extract) and the highest extraction yield was 6.44% (*A. suaveolens* stem methanol extract).

c) Chloroform extracts were used for the total experiments because we targeted mid-polar molecules.

Objective 2: To determine the antiparasitic activities of the chloroform extracts of stems and leaves by *in vitro* antileishmanial, antiplasmodial, and cytotoxicity assays.

a) Chloroform extracts elicited antileishmanial activity against *L. donovani* BHU-1251 promastigotes in the ascending order (from least active to most active): leaves *C. costatus* > stems *L. eugeniifolia* > stems *U. grandiflora* > stems *T. peduncularis* > stems *D. wallichii* > leaves *A. suaveolens* > leaves *U. grandiflora* > leaves *T. peduncularis* > leaves *L. eugeniifolia* > stems *A. suaveolens* > stems *C. costatus* > leaves *D. wallichii.* The readings of IC_{50} for the chloroform extracts in the ascending order were 94.25, 90.6, 74.74, 66.36, 59.37, 49.94 ,40.51, 27.30, 21.76, 17.33, 17.32, and 7.6 µg/mL, respectively.

b) Chloroform extracts exhibited antiplasmodial activity against chloroquine-resistant *P. falciparum* K1 strains in the ascending order (inactive to most active): stems *L. eugeniifolia* > leaves *T. peduncularis* > stems *A. suaveolens* > stems *U. grandiflora* > stems *D. wallichii* > leaves *A. suaveolens* > leaves *U. grandiflora* > leaves *L. eugeniifolia* > leaves *D. wallichii* > stems *T. peduncularis* > stems *C. costatus* > leaves *C. costatus.* The readings of IC_{50} for the chloroform extracts in the ascending order were > 15.70, 5.41, 4.66, 4.54, 4.47, 4.36, 3.78, 3.03, 1.73, 1.32, 1.15, and 0.85 µg/mL, respectively.

c) The cytotoxicity of the chloroform extracts for normal human lung epithelial cells was examined. The results revealed that 4 of the 12 chloroform

extracts tested were toxic to human cells, which were stems *T. peduncularis*, stems *L. eugeniifolia*, stems *D. wallichii,* and stems *U. grandiflora*. In fact, stems for *L. eugeniifolia, D. wallichii,* and *U. grandiflora* were dropped for both antileishmanial and antiplasmodial activities, as they had SI values below 5.

Objective 3: To investigate the antibacterial activities of the chloroform extracts of leaves and stems *in vitro*.

a) In disc diffusion assay, there was no recorded zone of inhibition for all extracts against *E. faecalis* strains. Stems *L. eugeniifolia* and leaves *U. grandiflora* had an inhibition zone of of 8.0 and 7.7 mm against MRSA. Stems of *T. peduncularis, A. suaveolens,* and *L. eugeniifolia* had the zone of inhibition of 9.0, 9.3, and 6.7 mm against *S. aureus*. Stems *U. grandiflora* had the zone of inhibition of 7.3 mm against *S. epidermidis*. Stems *C. costatus,* stems *A. suaveolens,* and leaves *A. suaveolens* had an inhibition zone of 7.3, 7.0, and 7.0 mm against *E. coli.* Both leaves and stems *A. suaveolens,* both leaves and stems *L. eugeniifolia,* leaves *D. wallichii*, and stems *U. grandiflora* presented an inhibition zone of 7.0, 7.0, 7.0, 7.0, 8.0, and 7.7 mm, respectively against *P. aeruginosa*. Stems *T. peduncularis* and leaves *D. wallichii* showed positive antibacterial activities with zone of inhibition of 6.7 mm against *K. pneumoniae*.

b) Chloroform extract of *C. costatus* Miq. stem had good antibacterial activity against the specific nosocomial bacterial strains *Staphylococcus epidermidis* ATCC®12228™ in MIC assay with MIC value of 187.5 µg/mL. in all experiment tested with MIC values above 1,500 µg/mL.

c) The stem chloroform *C. costatus* extracts was bactericidal as demonstrated by MBC/MIC ratio and killing kinetics against *S. epidermidis.* It was more bactericidal than cefotaxime and/or vancomycin.

Objective 4: To evaluate the antioxidant properties of the chloroform extracts of the leaves and stems.

a) Chloroform extracts exhibited total phenolic content in ascending order (lowest to highest) stems *C. costatus* > leaves *T. peduncularis* > stems *U. grandiflora* > stems *T. peduncularis* > stems *L. eugeniifolia* > stems *D. wallichii* > leaves *C. costatus* > leaves *D. wallichii* > stems *A. suaveolens* > leaves *U. grandiflora* > leaves *L. eugeniifolia* > leaves *A. suaveolens.* The readings of the total phenolic content of the chloroform extracts in ascending order are 0.030, 0.030, 0.030, 0.031, 0.031, 0.031, 0.032, 0.032, 0.033, 0.033, 0.34, and 0.044 mg GAE/ 10 g FW, respectively.

b) Chloroform extracts exhibited FRAP in the ascending order (lowest to highest): stems *C. costatus* > stems *L. eugeniifolia* > leaves *A. suaveolens* > stems *T. peduncularis* > stems *D. wallichii* > stems *A. suaveolens* > leaves *D. wallichii* > stems *U. grandiflora* > leaves *L. eugeniifolia* > leaves *T. peduncularis* > leaves *C. costatus* > leaves *U. grandiflora.* The readings of the total flavonoid content for the chloroform extracts in the ascending order are 0.0123, 0.0123, 0.0127, 0.0137, 0.0142, 0.0147, 0.0153, 0.0158, 0.0162, 0.0164, 0.0165, and 0.0193 mg $FeSO_4.7H_2O$ / 10 g FW, respectively.

c) All chloroform extracts had low antioxidant properties based on the total phenolic and FRAP content. The low positive correlation of the regression curve obtained from the TPC and FRAP assay supports the assumption.

Objective 5: To identify and test compound with good drugability from the most potent plant extracts on the *in vitro* pharmacological tests.

a) Isolation of *C. costatus* stem chloroform extracts and *U. grandiflora* leaves chloroform using HPLC yielded compound 1, 2, and 3. After comparing the NMR spectroscopic data with data published in (mentioned in Chapter 7), these compounds were identified as pinoresinol, zeylenol, and ferrudiol, respectively.

b) All isolated compounds from *C. costatus* and *U. grandiflora* were then subjected to a range of bioactivities.

c) The cytotoxicity of the isolated compounds against normal human lung cells, MRC-5 revealed that pinoresinol, zeylenol, and ferrudiol were non-toxic.

d) A study on antileishmanial activities of the isolated compounds showed that pinoresinol, zeylenol, and ferrudiol were weakly active towards *L. donovani.*

e) Pinoresinol was weakly active against chloroquine-resistant *P. falciparum* K1.

f) Pinoresinol had weakly activity on *S. epidermidis* and acted synergistically against *S. epidermidis* when combined with cefotaxime.

9.2. Limitation of the study

There were some limitations, although carrying the research study as part of the experiment was postponed from the real time plan. The reasons for delaying the process are due to the following:

1) The process of obtaining parasitic cells, bacterial cells, and normal cells depends on the availability of stock.

2) Parasitic cells are sensitive to the environment and difficult to maintain for a long-time of subculture. They need to be cryopreserved quickly each time and injected to virulent animals for sustenance.

3) Bacterial cells are easily contaminated and need a sterile environment to prevent contamination.

9.3. Future recommendations

Pinoresinol could be used as a starting material for the hemisynthesis of derivatives that could be tested further as antibiotic potentiator *in vivo*. Further pharmacological studies and clinical trials need to be done to ascertain the

efficacy, safety, and mechanism of action of pinoresinol derivatives prior to clinical application.

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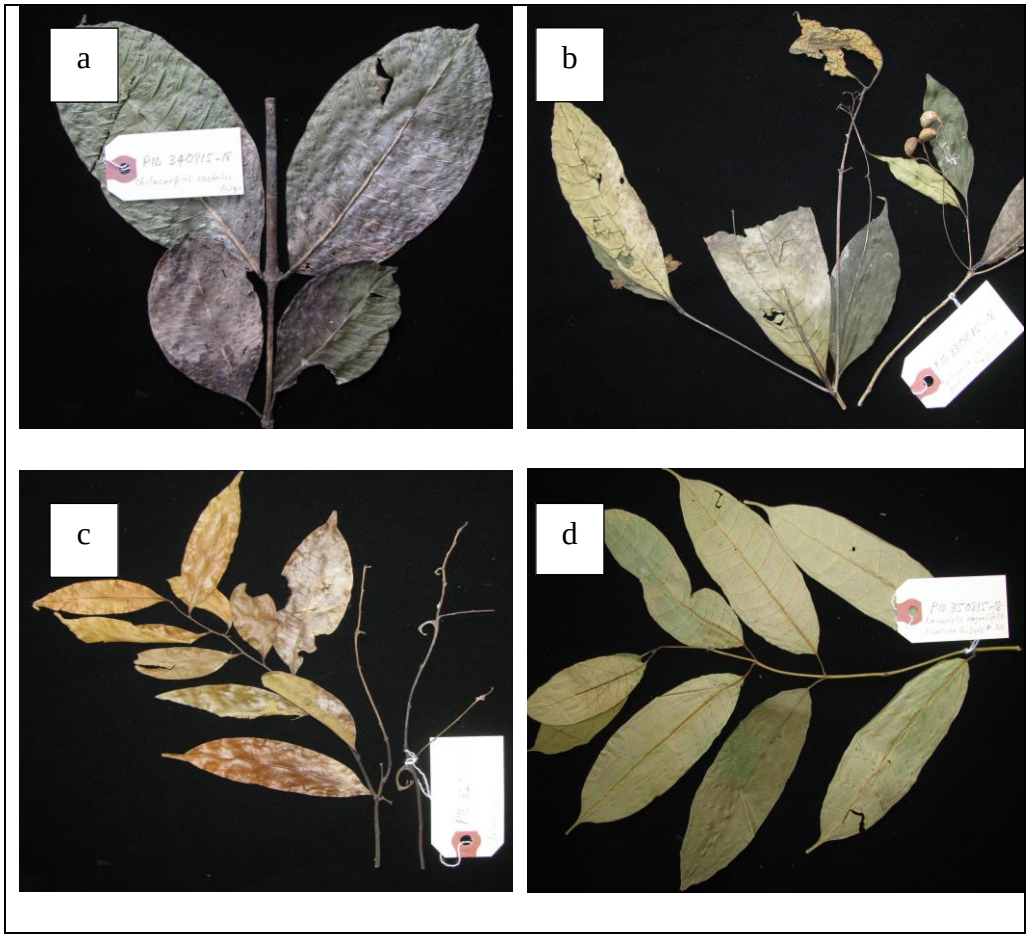
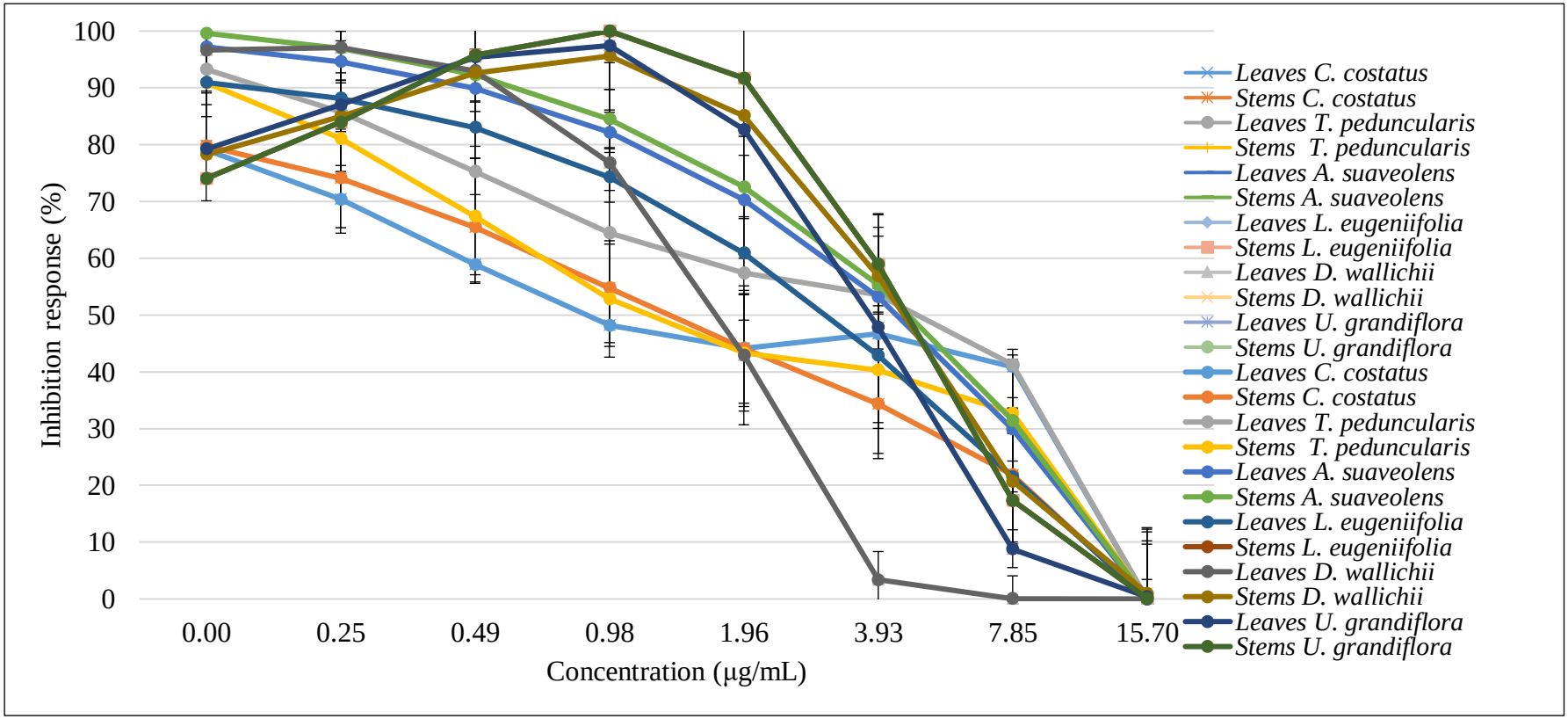
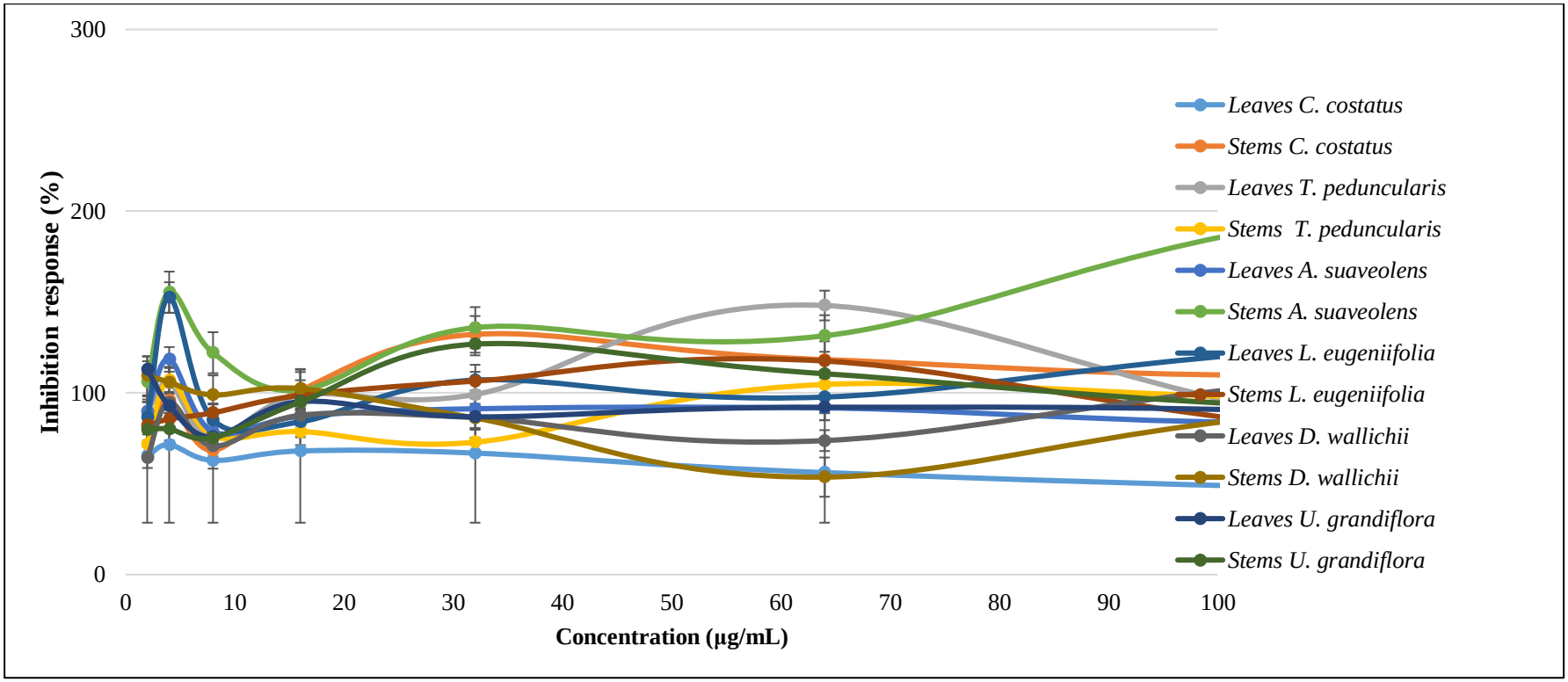
Appendices



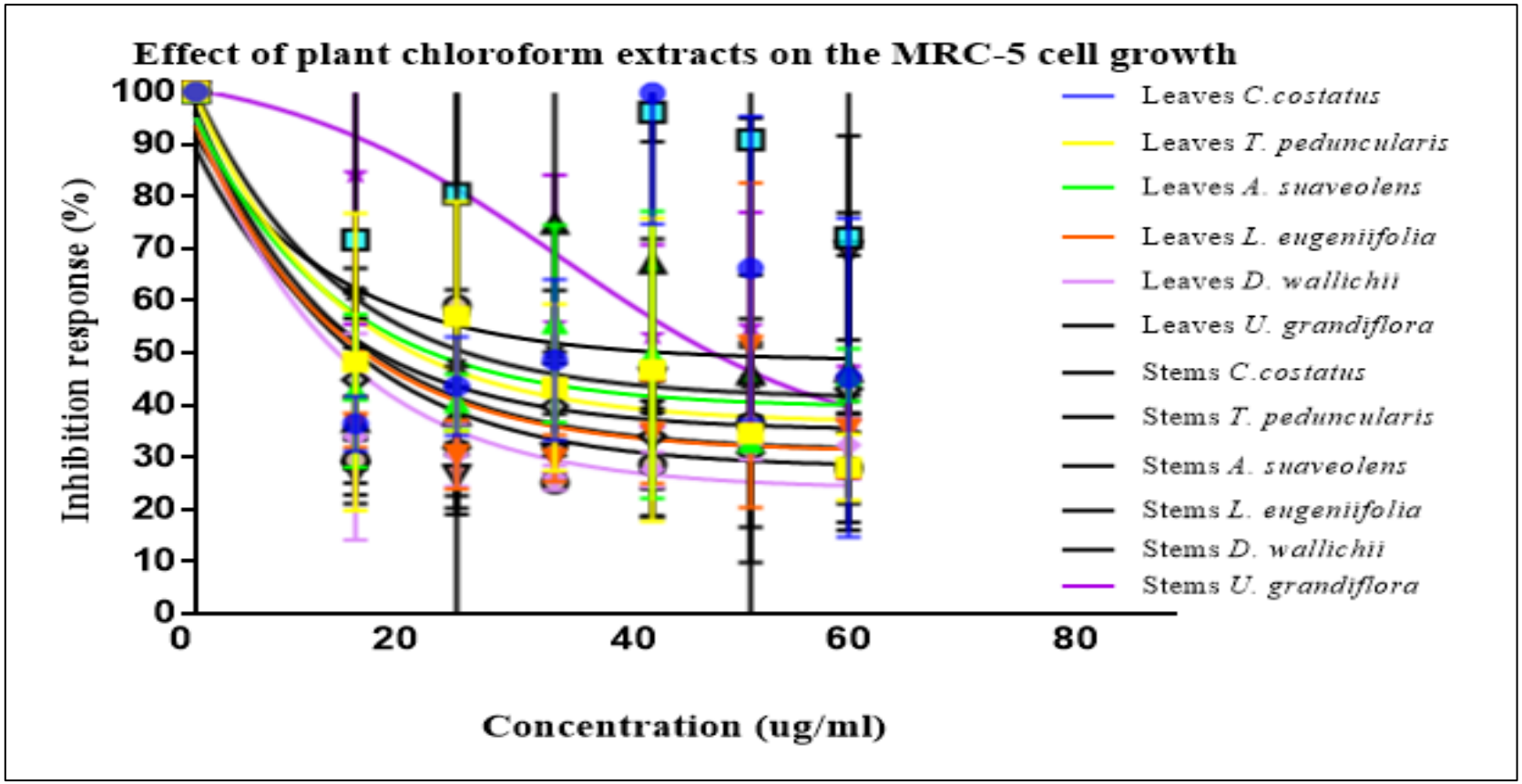
Appendix 1.1. Plant specimens: (a) *C. costatus* Miq. (b) *T. peduncularis* Wall. (c) *A. suaveolens* (Blume) Blume. (d) *L. eugeniifolia* (Wall. ex. G. Don) A. DC.



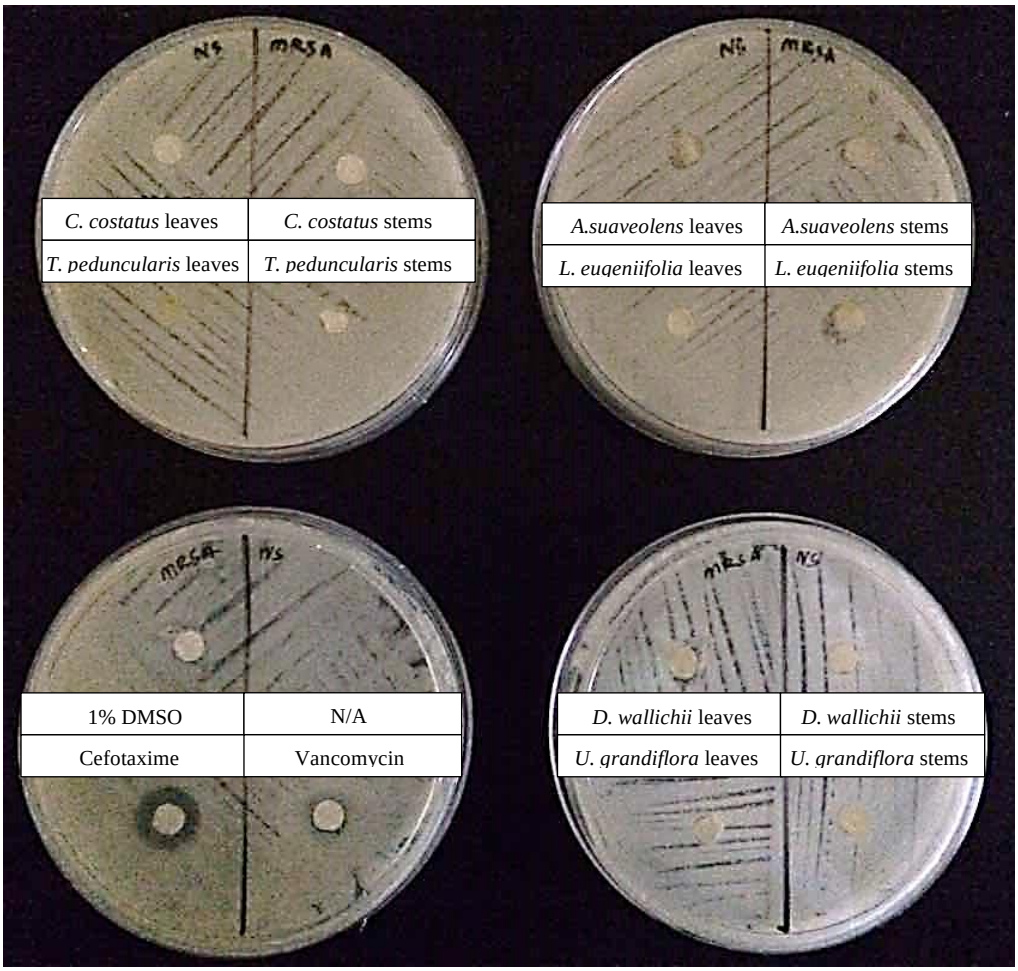
Appendix 1.2 Antiplasmodial effect of chloroform extracts against *P. falciparum* K1 chloroquine-resistant



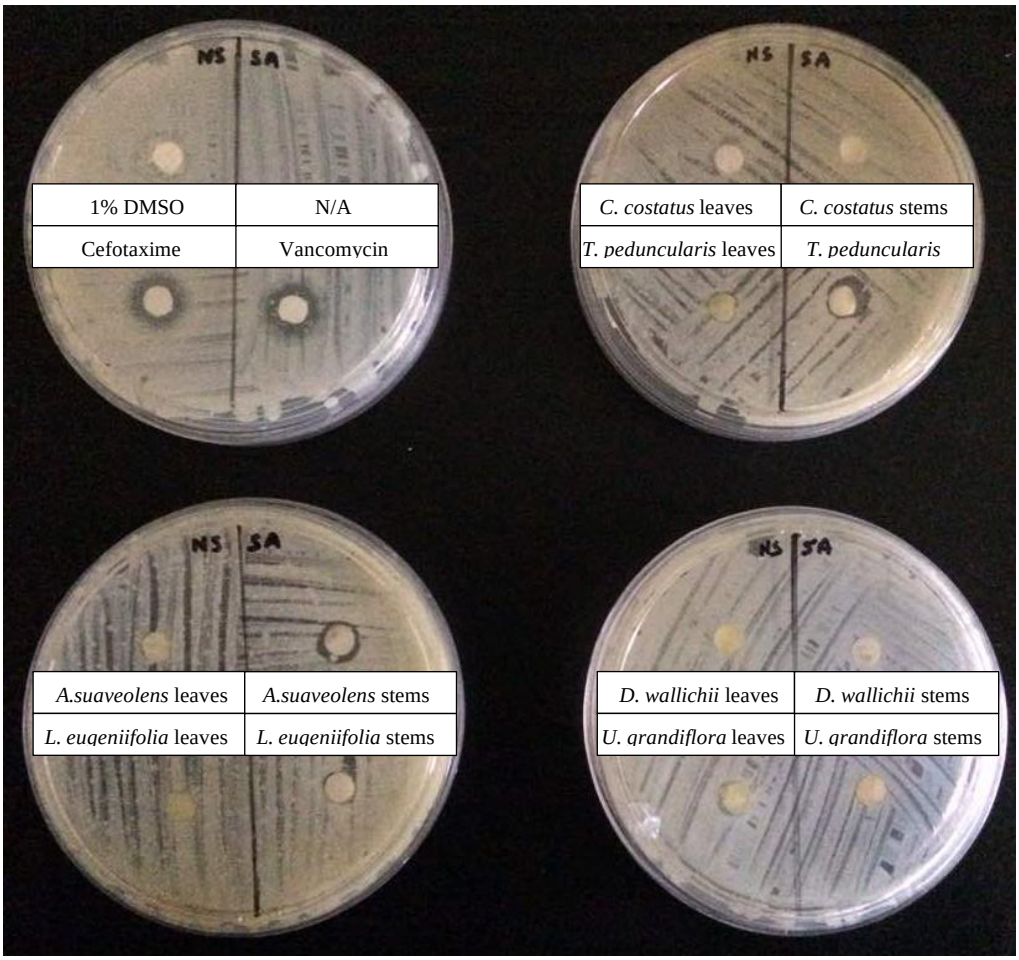
Appendix 1.3. Antileishmanial effect of chloroform extracts against *L. donovani* BHU-1251



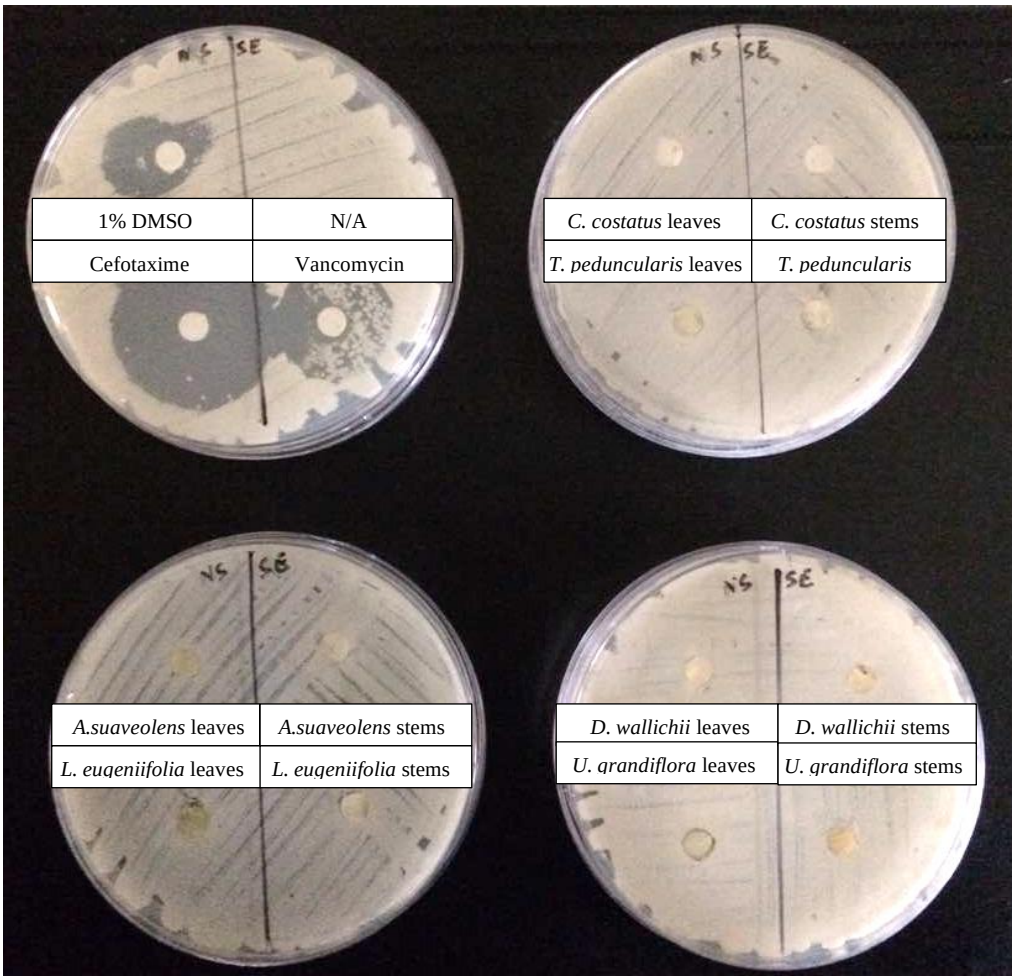
Appendix 1.4. Effects of chloroform extracts on the MRC-5 cells



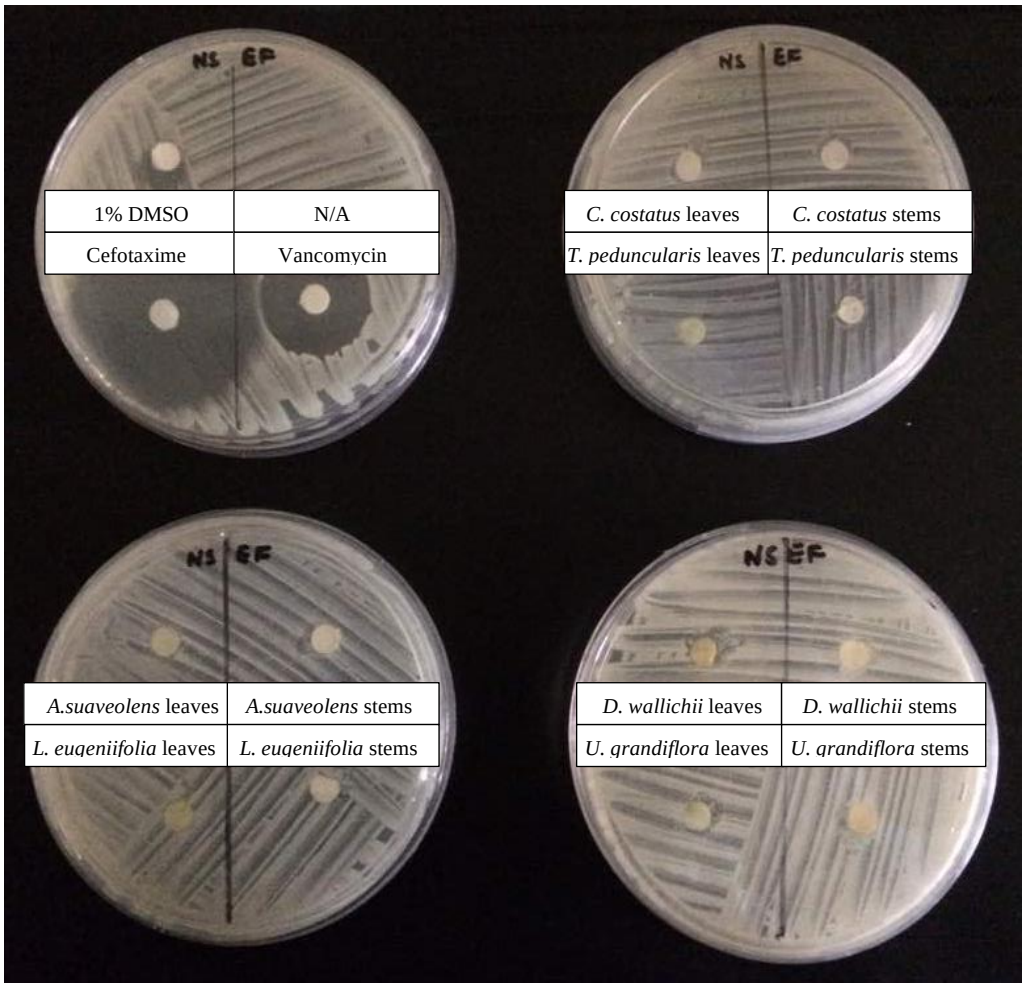
Appendix 1.5. Disc diffusion activity of chloroform extracts against MRSA



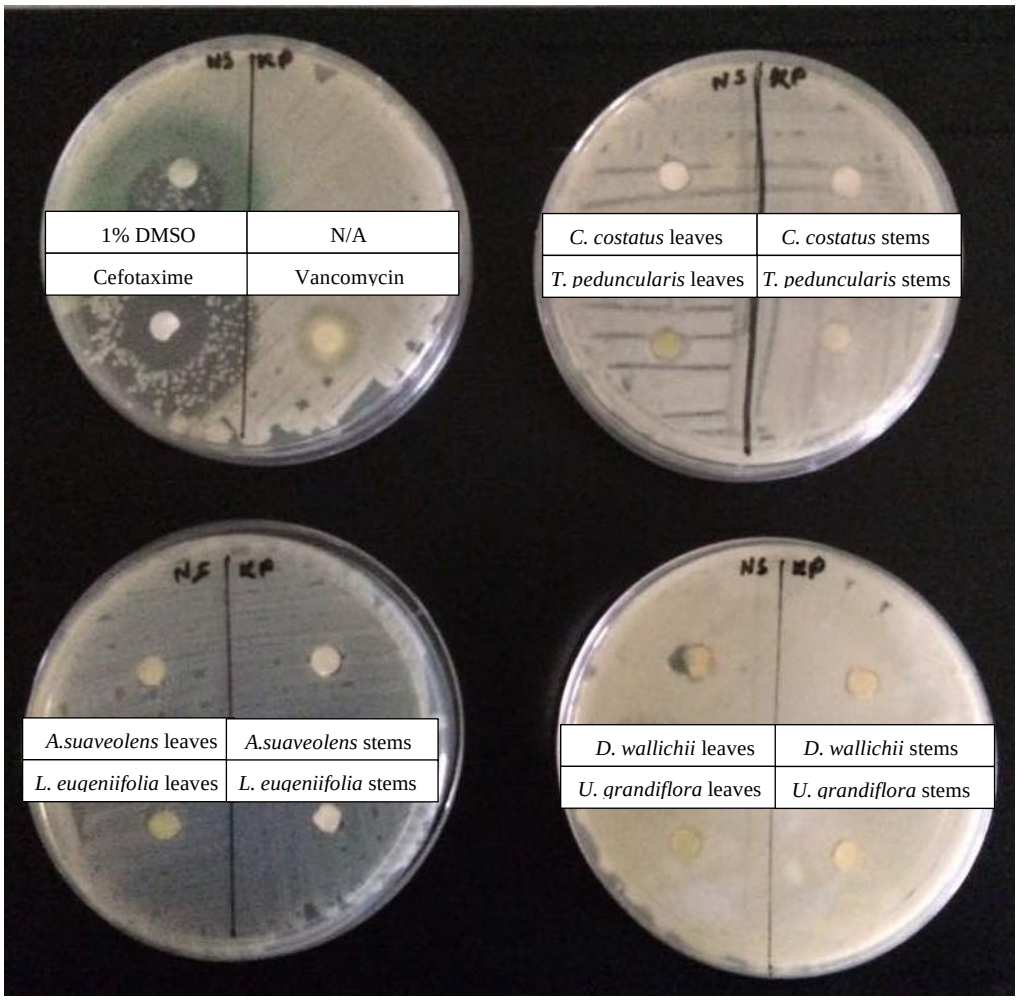
Appendix 1.6. Disc diffusion activity of chloroform extracts against *S. aureus*



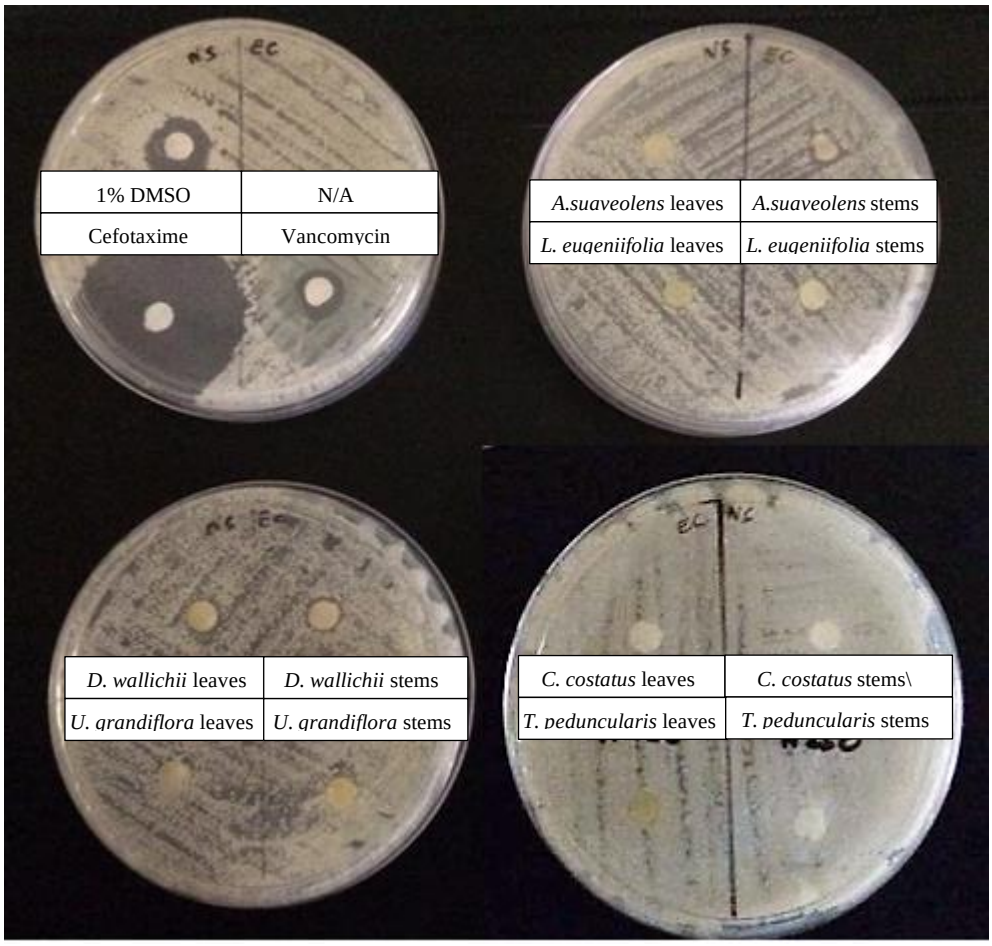
Appendix 1.7. Disc diffusion activity of chloroform extracts against *S. epidermidis*



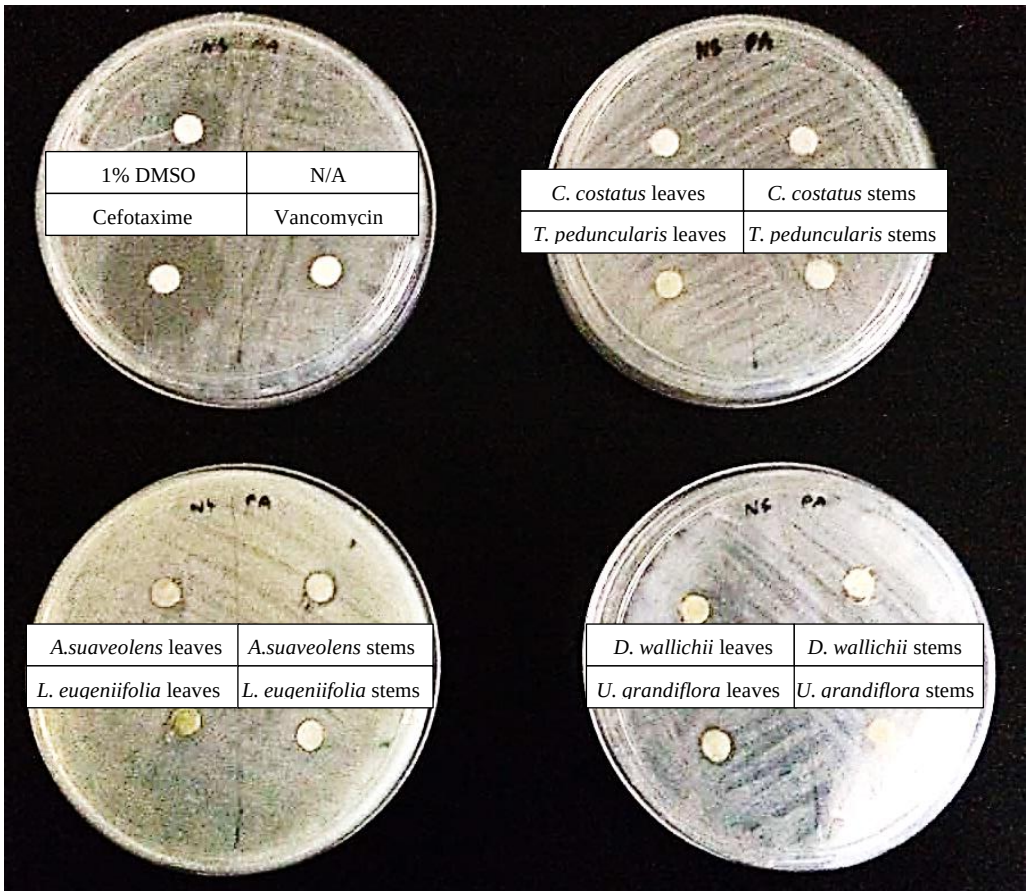
Appendix 1.8. Disc diffusion activity of chloroform extracts against *E. faecalis*



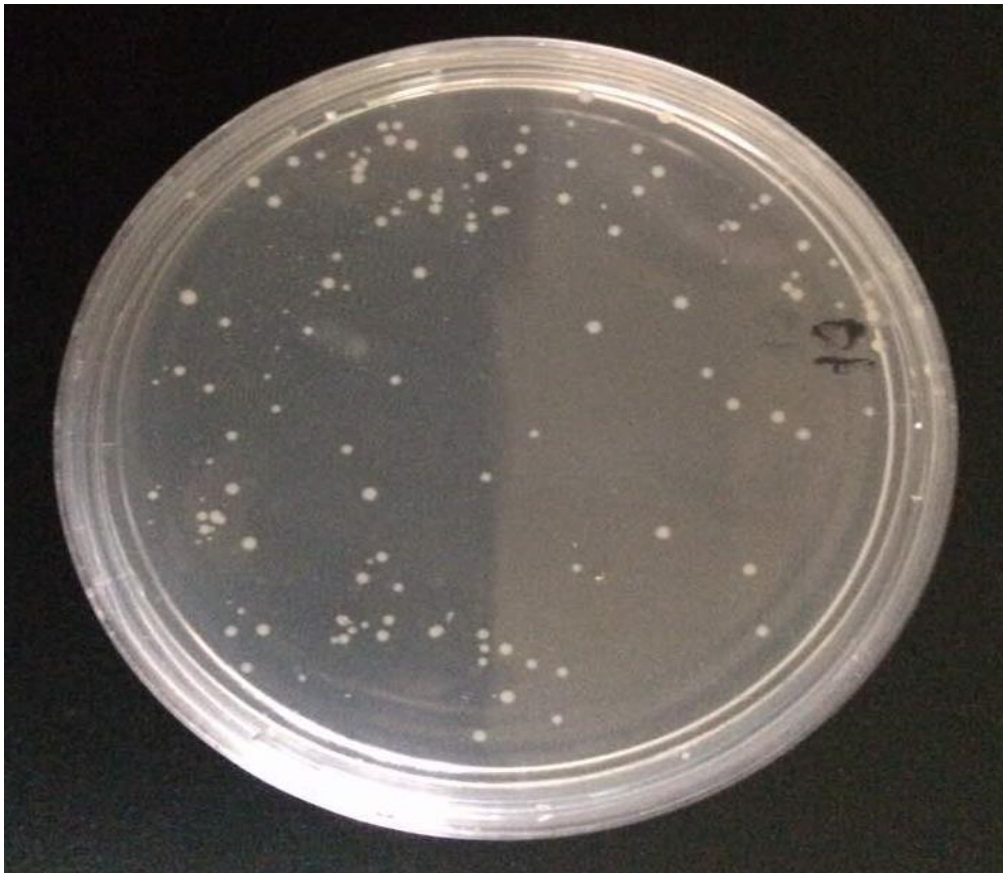
Appendix 1.9. Disc diffusion activity of chloroform extracts against *K. pneumoniae*



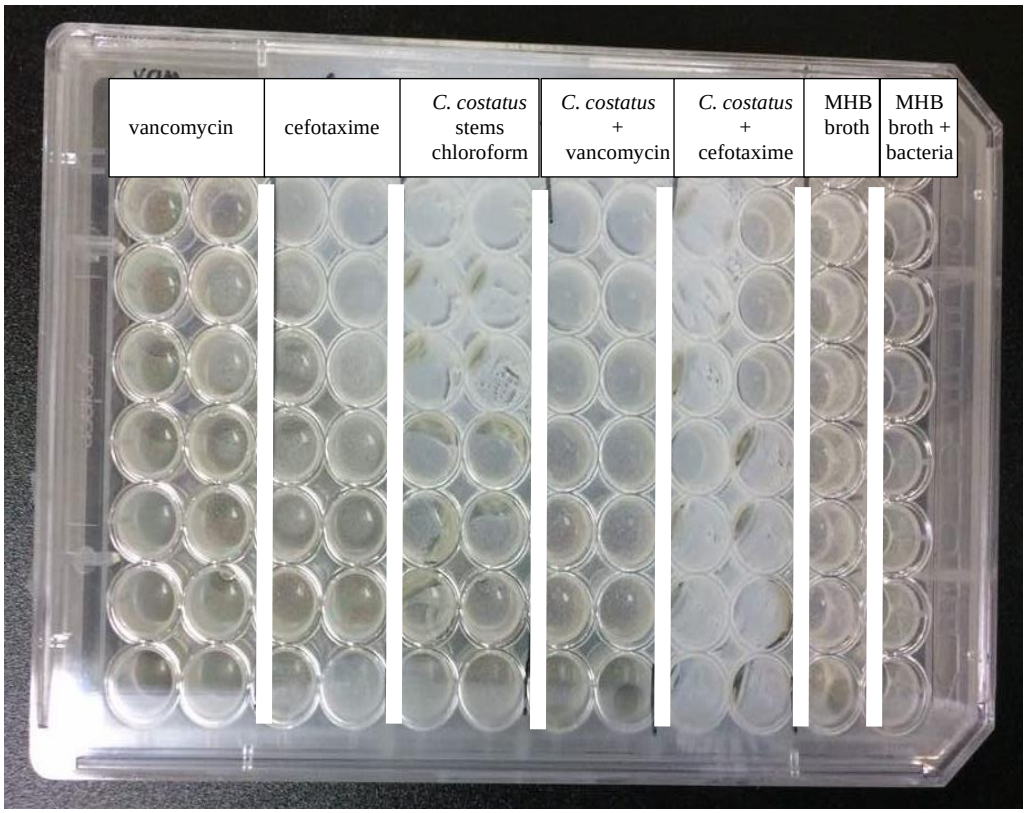
Appendix 2.0. Disc diffusion activity of chloroform extracts against *E. coli*



Appendix 2.1. Disc diffusion activity of chloroform extracts against *P. aeruginosa*

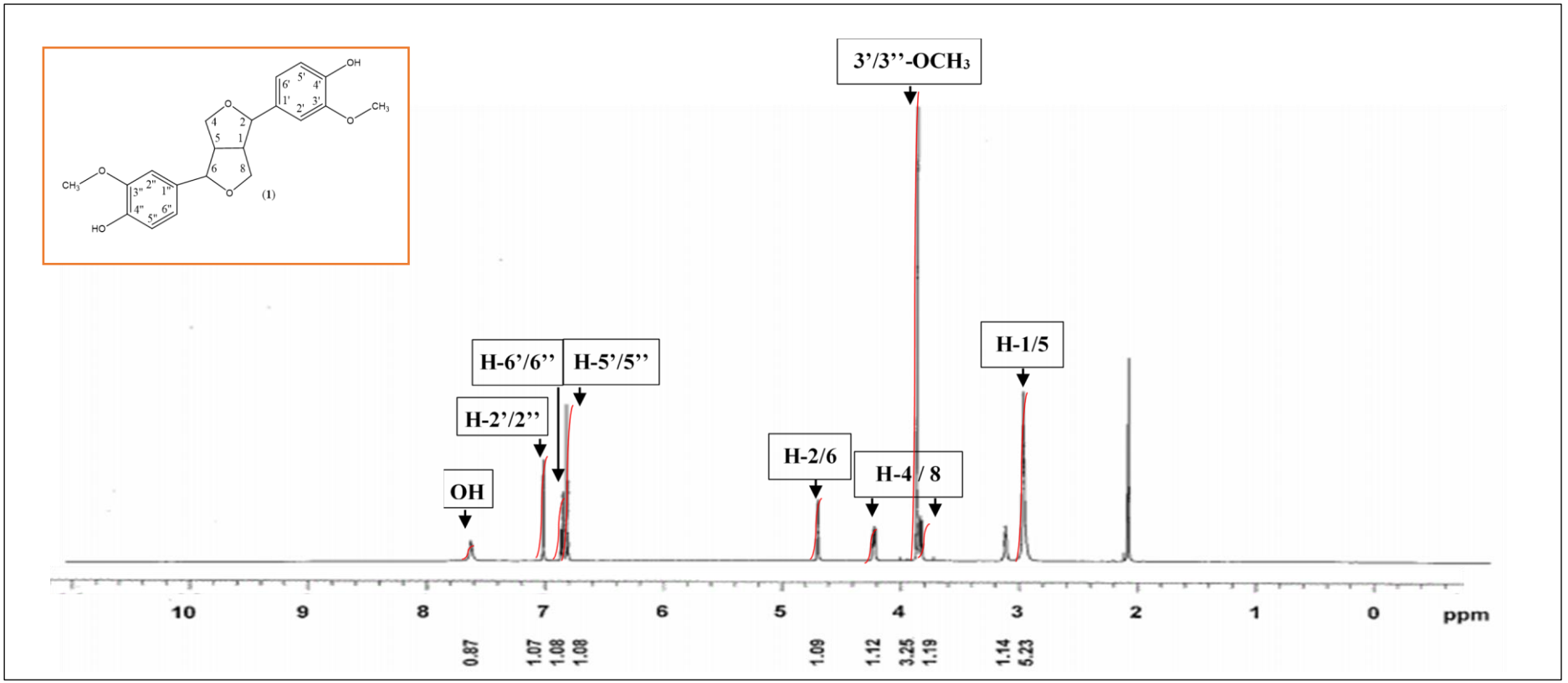


Appendix 2.2. Result of MBC/MIC ratio showing bactericidal chloroform extract of C. costatus stem against *S. epidermidis*

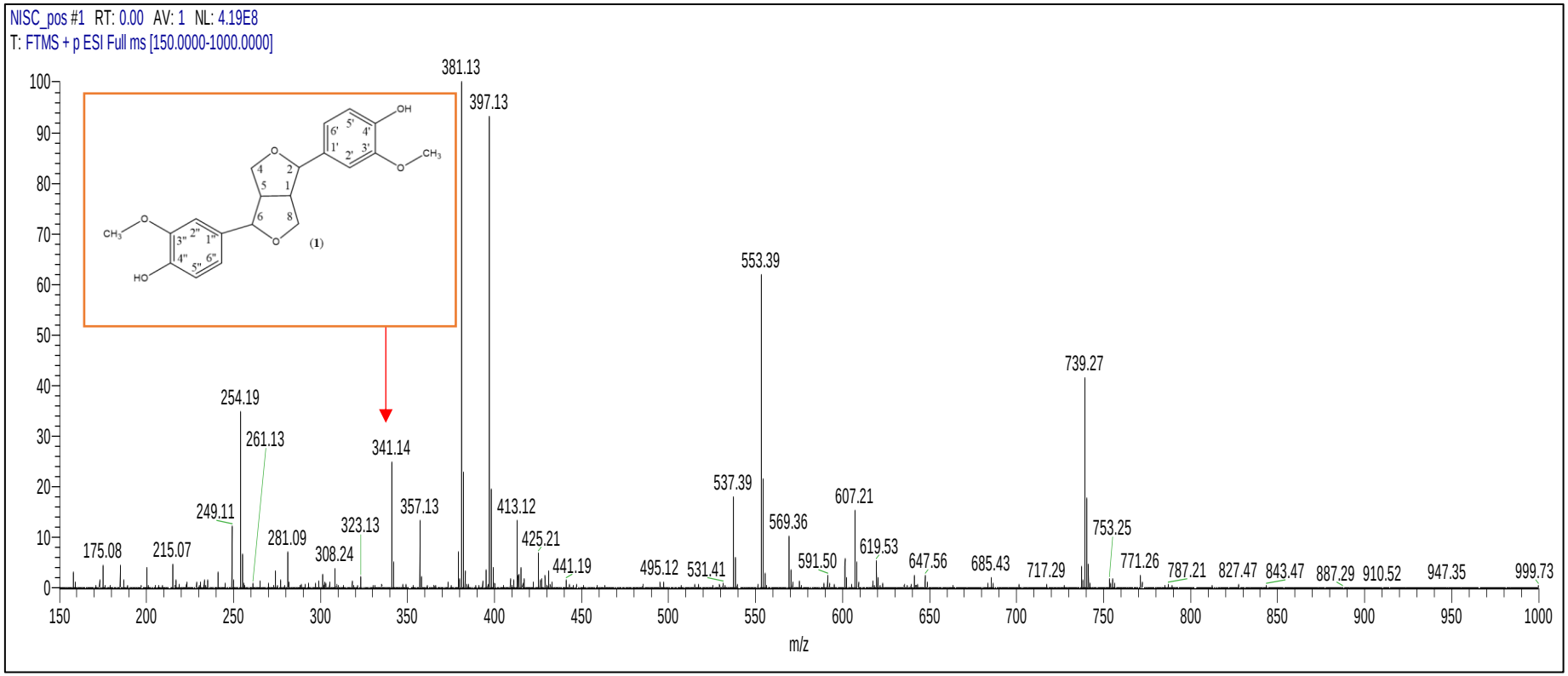


Appendix 2.3. Synergistic assay of chloroform extract of *C. costatus* stem with vancomycin/ cefotaxime

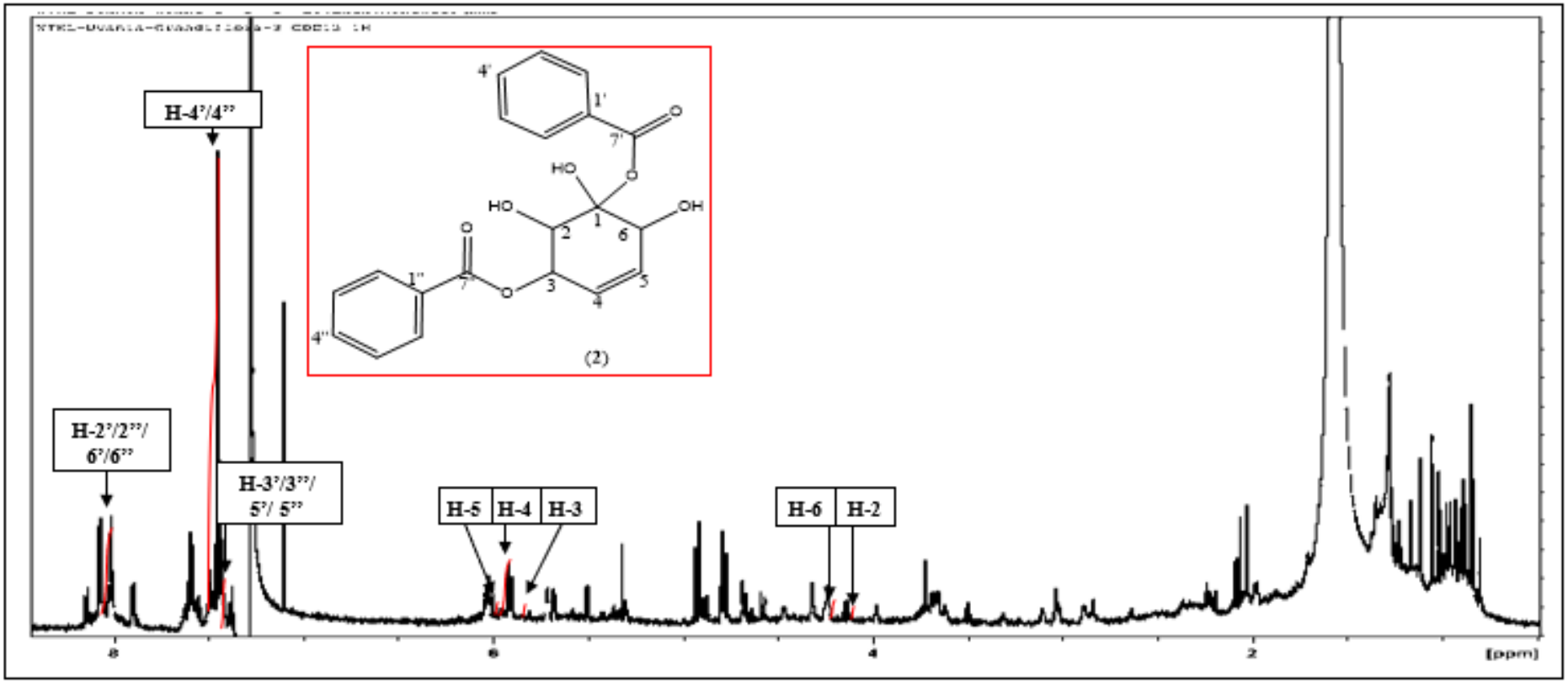
Appendix 2.4. Results of the time-killing assay of C2-cefotaxime, V2-vancomycin and S2-C. costatus stem chloroform against *S. epidermidis* at 1, 2, 3 and 4 hours



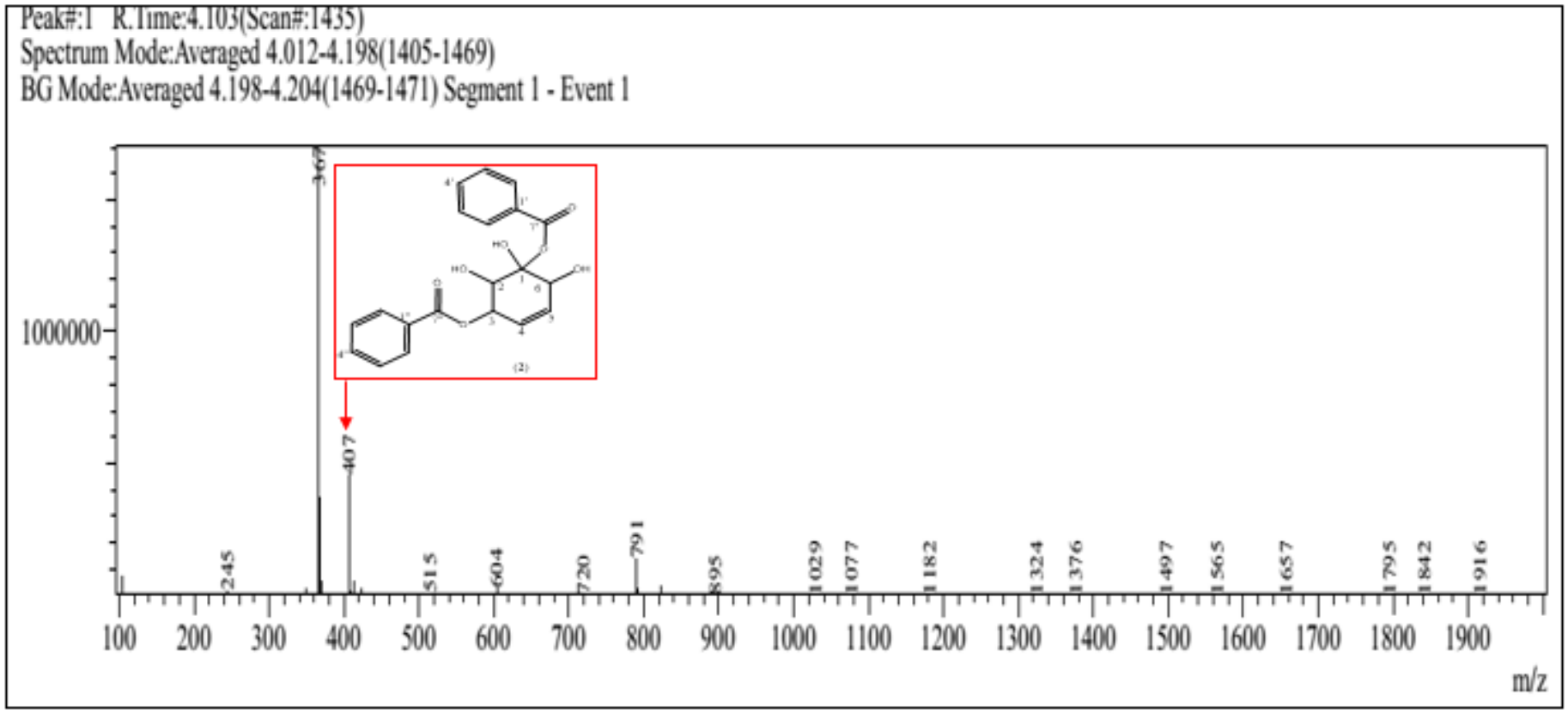
Appendix 2.5. ^{1}H NMR spectrum of compound 1 (pinoresinol)



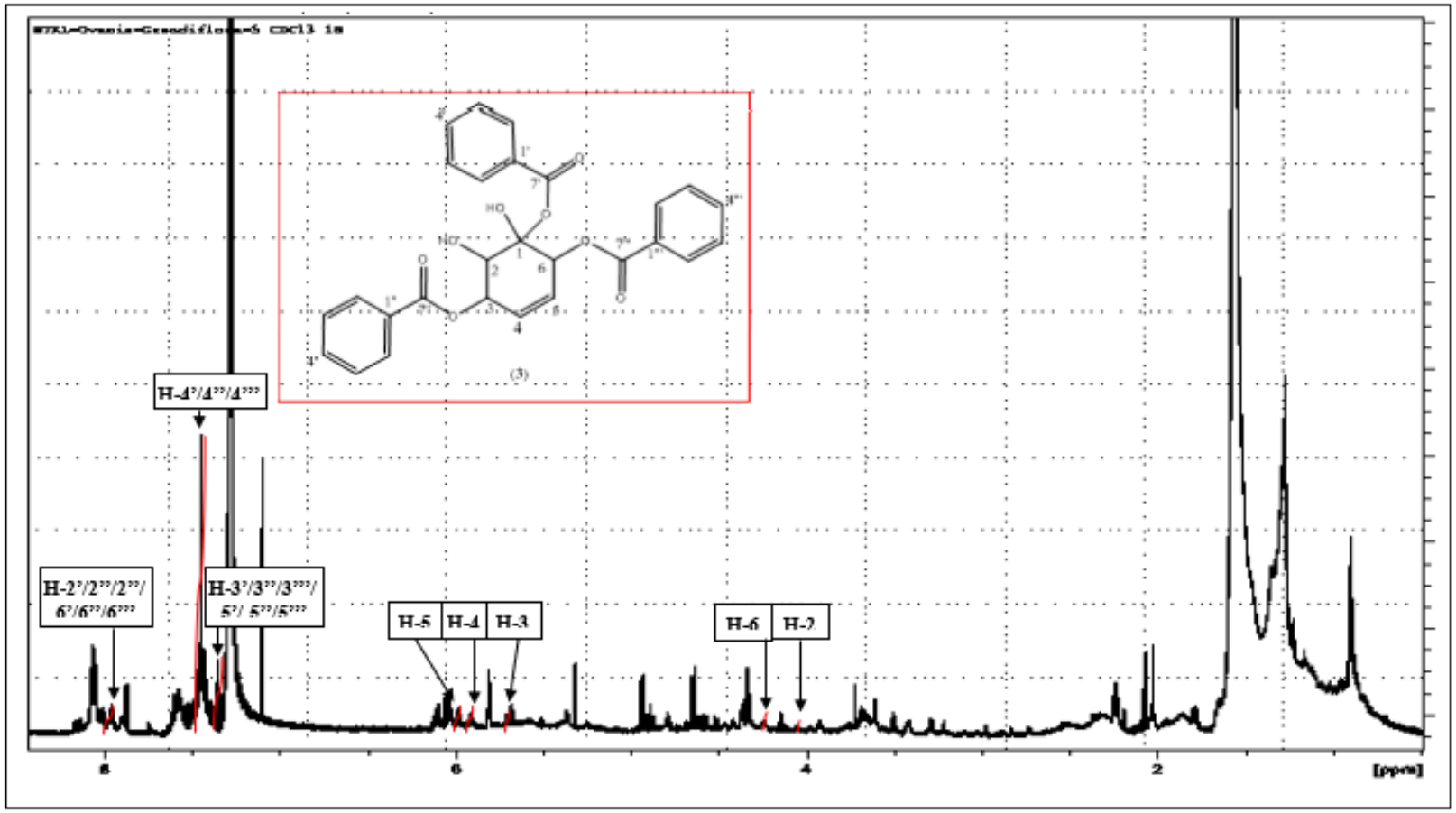
Appendix 2.6. HR-ESI-MS spectra of compound 1 (pinoresinol) with molecular formula, $C_{20}H_{22}O_6$ and *m/z* 341 [M-H_2O+H^+]



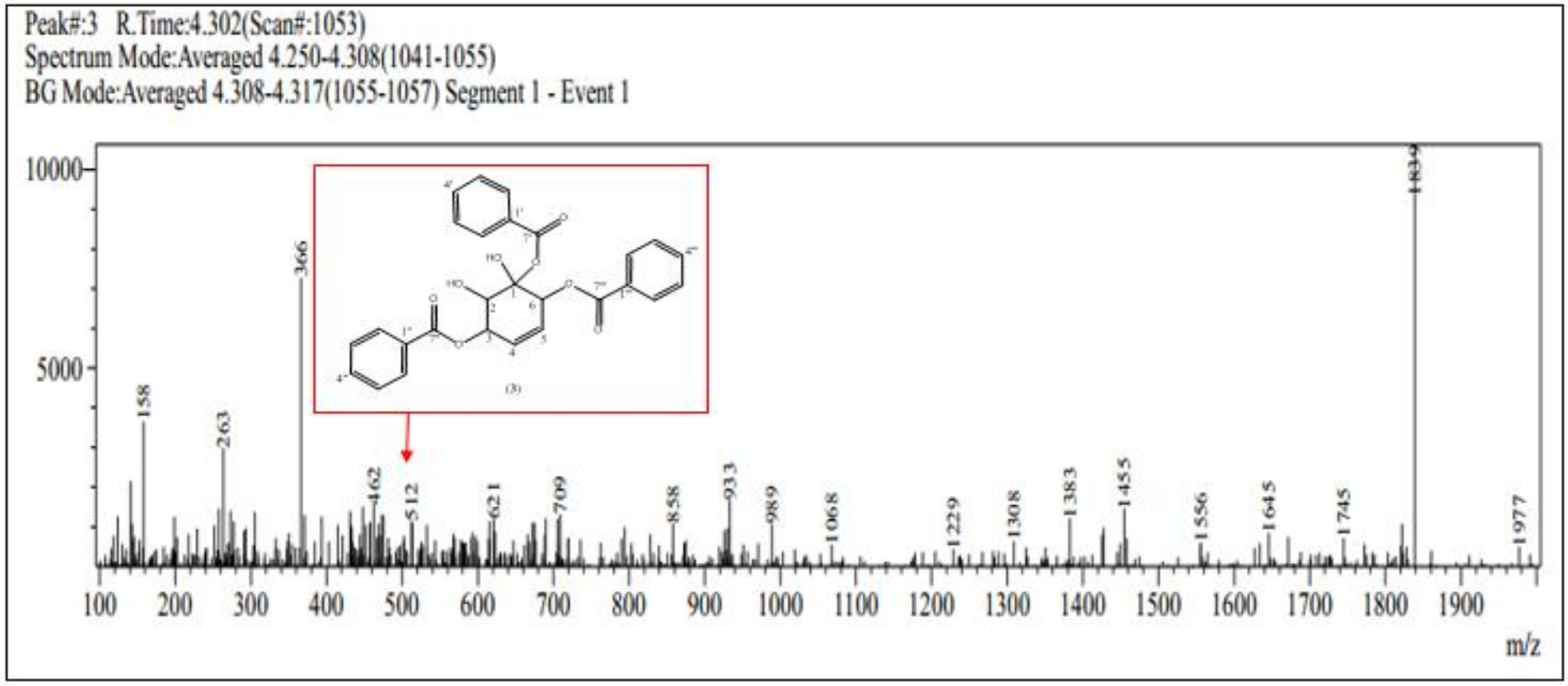
Appendix 2.7. ^{1}H NMR spectrum of compound 2 (zeylenol)



Appendix 2.8. HR-ESI-MS spectra showed compound 2 (zeylenol) with molecular formula $C_{21}H_{20}O_7$ and *m/z* 407 [M+Na]



Appendix 2.9. ^{1}H NMR spectrum of compound 3 (ferrudiol)



Appendix 3.0. HR-ESI-MS spectra of compound 3 (ferrudiol) with molecular formula, $C_{28}H_{24}O_8$ and *m/z* 512 [M+Na++H^+]