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Bioactivity of Several *Etlingera* Gingers Endemic to Borneo

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Abstract

The *Etlingera* genus consists of 100 species of ginger plants, in which many are used in traditional medicine. The present study investigated the antioxidant and antimicrobial activities of *Etlingera coccinea*, *Etlingera pubescens*, and *Etlingera sessilanthera*. The wound healing property of *E. coccinea* was also evaluated, since it is traditionally used to treat skin wounds.

The methanol extracts of the leaves, stems, and rhizomes of *E. coccinea*, *E. pubescens*, and *E. sessilanthera* were screened for their total phenolic content (TPC), ascorbic acid equivalent antioxidant capacity (AEAC), and oxygen radical absorbance capacity (ORAC). Among all crude extracts tested, the leaves of *E. coccinea* showed highest TPC, AEAC, and ORAC values (1246 ± 28 mg gallic acid equivalent/ 100 g fresh weight, 2702 ± 89 mg ascorbic acid equivalent/ 100 g fresh weight, and 423.9 ± 18.2 µmol Trolox equivalent/ g fresh weight, respectively).

The antioxidant properties of *E. pubescens* have never been reported in previous studies, therefore, this species was subjected to compound isolation work. From its leaf methanol extract, a diarylheptanoid, 1,7-bis(3,4-dihydroxyphenyl)heptan-3-yl acetate (etlingerin), was purified and identified. This study is the first to report the bioactivity of etlingerin. Results showed that etlingerin exhibited comparable free radical scavenging activities with curcumin (which is also a diarylheptanoid), with DPPH free radical scavenging (FRS) IC_{50} values determined as 9.91 ± 0.06 and 26.1 ± 0.2 µM, respectively, while ORAC EC_{50} values determined as 2.73 ± 0.02 and 2.59 ± 0.03 µM, respectively. However, when cell-based antioxidant assay was employed, etlingerin displayed superior protection on the intracellular fluorescent probe, 2,7-dichlorodihydrofluorescein (DCFH): at the highest concentration tested (20 µg/mL), etlingerin and

curcumin showed percentage protection values of 67.6 ± 2.2 % and 55.9 ± 4.2 %, respectively. This was supported by the cytoprotective study, in which etlingerin protected HaCaT cells that were challenged with hydrogen peroxide, while no protection was observed for curcumin.

This study also investigated the antimicrobial activities of the sequential solvent extracts of *E. coccinea* and *E. sessilanthera*, as well as the methanol extracts of *E. pubescens*. The leaf hexane extract of *E. coccinea*, rhizome hexane extract of *E. sessilanthera*, and leaf methanol extract of *E. pubescens* were found to be the most active extract for each species, with minimum inhibitory concentration (MIC) values determined as low as 0.016, 0.125, and 0.125 mg/mL, respectively, against ten gram-positive bacterial strains. From these active extracts, *trans*-2-dodecenal, etlingerin, and 8(17),12-labdadiene-15,16-dial, were isolated, and identified as the major antimicrobials from *E. coccinea*, *E. pubescens*, and *E. sessilanthera*, respectively. *Trans*-2-dodecenal and 8(17),12-labdadiene-15,16-dial inhibited *Staphylococcus aureus* ATCC 43300, *Staphylococcus aureus* ATCC 6538P, *Bacillus cereus* ATCC 14579 and *Bacillus subtilis* ATCC 8188, with MIC values determined ranging from 4 to 8 µg/mL. Etlingerin also inhibited the aforementioned bacterial strains, with MIC values ranging from 0.063 to 0.125 mg/mL. In addition, this compound was found to be bactericidal. Mechanistic study revealed that it could exert rapid killing effects (as fast as 60 minutes), while also caused an increase in propidium iodide dye uptake, as well as resulted in leakage of intracellular materials. Thereafter, current findings suggest alteration in membrane permeability as its antimicrobial mechanism. Future work should investigate the interaction between etlingerin and the bacterial membrane.

This study also evaluated the wound healing property of the young shoots of *E. coccinea*. Its water extracts enhanced proliferation and migration of BEAS-2B, HaCaT, and NIH/3T3 cells, with cell viabilities and improvements in wound area reduction determined as high as 158.7 ± 3.3 and 82.0 ± 22.5 %, respectively. Based on the LCMS and tandem mass spectrometry analyses, procyanidin B2 and C1 (tannins), quercetin-3-glucuronide, as well as other phenolic compounds, were identified as the major phytochemicals present in the extract. *E. coccinea*'s water extracts were also shown to exhibit antioxidant and antimicrobial activities, which can be beneficial to the wound healing process. Particularly, the tannin-rich fraction inhibited the gram-positive bacteria (*S. aureus* ATCC 43300, *S. aureus* ATCC 6538P, and *B. cereus* ATCC 14579) with MIC values ranging from 0.063 to 1 mg/mL, while also showed low DPPH FRS IC_{50} and ORAC EC_{50} values (4.171 ± 0.036 µg/mL and 0.1961 ± 0.0925 µg/mL, respectively). These results suggest that the tannins play major roles in the antimicrobial and antioxidant activities of the water extract of *E. coccinea*'s young shoots.

In summary, the present work showed that *E. coccinea*, *E. pubescens*, and *E. sessilanthera* have good antioxidant and antimicrobial properties. The three compounds isolated should be developed as antimicrobial agents, and etlingerin also has a potential to be used in treating oxidative stress-related diseases. Lastly, the basis that the young shoots of *E. coccinea* displayed positive effects that can aid the wound healing process supports its traditional use in treating skin wounds, as well as highlights its potential to be developed as a wound healing agent.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

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^{13}C NMR = carbon-13 nuclear magnetic resonance

^{1}H NMR = proton nuclear magnetic resonance

AAE = ascorbic acid equivalent

AAPH = 2,2'-azobis(2-amidinopropane) dihydrochloride

AEAC = ascorbic acid equivalent antioxidant capacity

ANOVA = analysis of variance

ATCC = American Type Culture Collection

CAA = cellular antioxidant activity

CDC = Centers for Diseases Control and Prevention

CEMD = Conservation and Environmental Management Division

CLSI = Clinical and Laboratory Standards Institute

DCFH-DA = 2,7-dichlorodihydrofluorescein diacetate

DCM = dichloromethane

DE = dried water extract

DMEM = Dulbecco's Modified Eagle Medium

DMSO = dimethyl sulfoxide

DNA = deoxyribonucleic acid

DPPH = 1,1-diphenyl-1-picryl-hydrazyl

DVCA = Danum Valley Conservation Area

EA = ethyl acetate

EC_{50} = 50 % effective concentration

ECM = extracellular matrix

EDTA = Ethylenediaminetetraacetic acid

EGCG = epigallocatechin gallate

EGF = epidermal growth factor

EGFR = epidermal growth factor receptor

ESI-MS = electron spray ionization – mass spectrometry

FAO = Food and Agriculture Organization of the United Nations

FBS = fetal bovine serum

FC = Folin Ciocalteu

FE = fresh water extract

FRC = Forest Research Center

FRS = free radical scavenging

GAE = gallic acid equivalent

GPS = Global Positioning System

H_2O = water

HBSS = Hank's Balanced Salt Solution

HEX = hexane

HSD = Honest Significant Difference

IC_{50} = 50 % inhibitory concentration

IUCN = International Union for Conservation of Nature

LCMS = liquid chromatography mass spectrometry

LPS = lipopolysaccharide

MBC = minimum bacteridical concentration

MeOH = methanol

MHA = Muller Hinton agar

MIC = minimum inhibitory concentration

MS^2 = tandem mass spectrometry

MT*T* = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

NMR = nuclear magnetic resonance

ORAC = oxygen radical absorbance capacity

PI = propidium iodide

PTFE = polytetrafluoroethylene

Q-TOF = quadrupole time-of-flight

RNS = reactive nitrogen species

ROS = reactive oxygen species

SaBC = Sabah Biodiversity Center

SE = standard error

SPSS = Statistical Package for the Social Sciences

SSE = sequential solvent extraction

TE = Trolox equivalent

TLC = thin layer chromatography

TPC = total phenolic content

TSA = tryptic soy agar

TSB = tryptic soy broth

UPLC = ultra-performance liquid chromatography

USD = United States dollars

WHO = World Health Organization

CHAPTER 1

INTRODUCTION

Chapter 1 - Introduction

1.1 Overview of the Zingiberaceae family and the *Etlingera* genus

The family Zingiberaceae consists of 1548 species of ginger plants that are categorized under 50 different genera (The-Plant-List, 2013). Zingiberaceae can be further classified into four tribes (Hedychieae, Alpinieae, Zingibereae, and Globbeae), based on the plants' vegetative and floral characteristics (Kress et al., 2002). Most of these ginger plants are easily recognized through their aromatic leaves and fleshy rhizomes, as well as elliptic to elliptic-oblong shaped leaves arranged in two ranks on the leaf-shoot (Habsah et al., 2000).

The genus *Etlingera* is part of the Alpinieae tribe, which also consists of eight other genera; *Alpinia*, *Hornstedtia*, *Amomum*, *Elettariopsis*, *Elettaria*, *Geocharis*, *Plagiostachys*, and *Geostachys* (Kress et al., 2002). Distribution wise, *Etlingera* is primarily found in the Southeast Asia, with Borneo harboring most of its species (Poulsen, 2006). In Borneo, these ginger plants are important in the daily lives of the local indigenous people. They are commonly eaten, used as ornamentals and utilized as building materials (Poulsen, 2006). Most interestingly, many *Etlingera* plants are traditionally used to treat illnesses. In this study, the bioactivity of *E. coccinea*, *E. pubescens*, and *E. sessilanthera*, were investigated.

1.2 Background on *E. coccinea, E. pubescens,* and *E. sessilanthera*

E. coccinea (see Figure 1) is a species of ginger plant endemic to Borneo. Locally known as 'tuhau' in Sabah, *E. coccinea* is commonly eaten in the form of pickled vegetable, which is a famous local traditional delicacy made by the Kadazan/Dusun indigenous community (Poulsen, 2006). It can grow to an average height of about two meters, and easily identified by its strong coriander-like

smell (Daniel-Jambun et al., 2017; Poulsen, 2006). Its reported medicinal uses include treatment of stomach ache, gastric problems, food poisoning, and wounds (Poulsen, 2006; Vairappan et al., 2012; Wong and Guntavid, 2000). Literature review revealed that its antimicrobial and antioxidant properties were previously examined; however, the active compounds were not identified (Shahid-Ud-Daula et al., 2015; Vairappan et al., 2012).

Figure 1: Images of *Etlingera coccinea* (left), *Etlingera pubescens* (middle), and *Etlingera sessilanthera* (right), in their natural habitat.

E. pubescens (see Figure 1) is commonly known by the indigenous communities in Borneo through its various names: 'tubu apo', 'tubu bunga', 'tubu kerinang', and 'tubu labo' (Poulsen, 2006). It has a relatively low abundance in nature, thus classified as 'Near Threatened'. According to the International Union for Conservation of Nature (IUCN), "a taxon is Near Threatened when it has been evaluated against the criteria but does not qualify for Critically Endangered, Endangered or Vulnerable now, but is close to qualifying for or is likely to qualify for a threatened category in the near future" (IUCN, 2017; Poulsen, 2006). The leaves of *E. pubescens* are edible, while also

used as roofing by the indigenous community (Poulsen, 2006). Based on literature review, no publications were found on the bioactivity of *E. pubescens*.

E. sessilanthera (see Figure 1) is locally known as 'tubu pelapad' by the Kelabit community and this species of ginger plant can grow up to three meters tall. One important characteristic of this ginger plant is that there are purple tinges on the edge of the underside of its leaves, though full identification is always achieved by assessing its inflorescence (Poulsen, 2006). The stems of *E. sessilanthera* are usually eaten either raw or cooked. Like *E. pubescens*, *E. sessilanthera* is also categorized as a 'Near Threatened' species (Poulsen, 2006). Literature review revealed no published articles on the bioactivity of *E. sessilanthera*.

Armed with the knowledge that *Etlingera* plants are commonly used in traditional medicine, as well as some preliminary evidence from scientific publications, *E. coccinea*, *E. pubescens*, and *E. sessilanthera* are strong candidates for drug discovery. This study focuses on the antimicrobial and antioxidant properties of these ginger plants. The wound healing property of *E. coccinea* was also investigated, since it has been used by the indigenous community in Sabah to treat skin wounds.

1.3 Antimicrobial resistance and the need for more antimicrobials

Microorganisms can be found in almost all habitats present in nature, and they are estimated to form about 60 % of the Earth's biomass (Fraser et al., 2000; Radulovic et al., 2013). Notably, their ability to cause infections makes them a threat to human health. This issue was successfully addressed through the discovery of penicillin by Alexander Fleming in 1928 (Gaynes, 2017). However, throughout the decades, the world has witnessed the downfall of antimicrobials due to

the emergence of resistant microorganisms, and this was mainly due to the abuse and misuse of antimicrobial agents (Roca et al., 2015). Antimicrobial resistance is no longer just a medical issue, but it had become a global health threat. In 2013, the Centers for Disease Control and Prevention (CDC) reported that in the United States alone, at least 2 million people are infected with antimicrobial resistant bacteria, with at least 23 thousand deaths annually (CDC, 2013). This highlights the urgent need to discover new antimicrobials that can target the resistant microorganisms.

Plants are rich in secondary metabolites such as tannins, terpenoids, alkaloids, and flavonoids, which are known to possess antimicrobial properties (Sibanda and Okoh, 2007). In vitro, in vivo, and clinical trials have shown the efficacy of several plants and their bioactive constituents in treating bacterial infections, thereby proving that plants can be good sources of antimicrobials (Nabavi et al., 2015; Radha and Laxmipriya, 2015; Sharifi-Rad et al., 2017). For example, *Aloe vera* contains *Aloe* protein (14 kDa), anthraquinones, polysaccharides, and pyrocathecol, which are secondary metabolites that have antimicrobial activities (Radha et al., 2015).

1.4 Oxidative stress and antioxidants

Antioxidants are compounds that can delay or prevent oxidation of other compounds when present at low concentrations (Kasote et al., 2015). These compounds have numerous beneficial effects to human health, including anti-inflammatory, anti-carcinogenic, and anti-atherosclerotic, as a result of their antioxidant activities (Krishnaiah et al., 2011). Plants are excellent sources of antioxidants as they produce these compounds in significant amounts to protect themselves from free radical damage (Kasote et al., 2015).

Free radicals are defined as molecules or molecular fragments that contain one or more unpaired electrons (Poprac et al., 2017). In the field of biology and medicine, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are the most common form of free radicals, and they play significant roles in human health (Poprac et al., 2017). At appropriate amounts, these free radicals play several key roles which are critical to the normal cell functions. These include 1) defense against invading pathogens, and 2) act as signaling molecules to modulate cellular processes such as proliferation, metabolism, differentiation, and survival (Auf Dem Keller et al., 2006; Ray et al., 2012). The overproduction of ROS and/or RNS, as well as reduced efficiency of the cellular antioxidant system, can result in excessive levels of these free radicals; this is termed as oxidative stress (Poprac et al., 2017). This occurrence is undesirable as high ROS/RNS levels could impair cellular functions, or worse, cell deaths, due to oxidation of cellular macromolecules such as DNA, protein, and lipid (Schäfer and Werner, 2008). Consequently, this can lead to the development/ progression of many health complications including cancer, neurodegenerative disorders (Alzheimer's and Parkinson's disease), and diabetes (Newsholme et al., 2016; Poprac et al., 2017).

In the search for remedies, plant phenolics have gained a significant attention. This is due to their aromatic feature and highly conjugated system containing multiple hydroxyl groups that make these compounds excellent antioxidants (Zhang and Tsao, 2016; Zhang et al., 2015). In addition, high intake of food (and food products) rich in antioxidants have been associated with reduced risks of diseases linked to oxidative stress (Zhang and Tsao, 2016). A recent review by Kovacic and Weston (2017) provides evidence that curcumin (from *Curcuma longa*), sesamol (from *Sesamum indicum*), and piperine (from *Piper nigrum*), have beneficial effects on Alzheimer's disease, thus highlighting the potential to discover useful antioxidants from plants.

1.5 Challenges in wound healing

Skin is an important organ that primarily functions as a protective barrier against environmental insult (Dreifke et al., 2015). When wounded, its normal anatomical structure and function are disrupted, hence pose serious risks of morbidity and mortality (Dreifke et al., 2015). Although so, the human body possesses an intrinsic mechanism to restore the damage caused (Diegelmann and Evans, 2004). When this mechanism is adversely affected, nonhealing wounds can develop into chronic wounds. The latter are frequently described as an epidemic as they are diagnosed at an alarming rate (Dreifke et al., 2015). They often become a burden to patients due to the long treatment durations and high treatment costs (Fife and Carter, 2012; Phillips et al., 2016; Sen et al., 2009). Moreover, nonhealing wounds can also result in functional limitations (gait changes and difficulty ambulating), infections, and malignant transformations, which in turn cause physical burdens (Menke et al., 2007).

Plants are widely used as herbal remedies to treat wounds (Agyare et al., 2016; Jarić et al., 2017). Some well-known examples include *A. vera*, *Calendula officinalis*, and *Centella asiatica*. Apart from being traditionally used, these plants have also been proven effective through in vitro, in vivo, as well as clinical studies (Bylka et al., 2014; Das et al., 2016; Duran et al., 2005; Feily and Namazi, 2009; Fronza et al., 2009; Leach, 2008; Manoj et al., 2009; Preethi and Kuttan, 2009; Surjushe et al., 2008). The large body of evidence available on the wound healing property of plants highly suggest that they can be good sources of wound healing agents.

A wound healing agent should have the ability to enhance cell proliferation (the increase in their numbers by means of cell division) and migration (movement of cells), as this will lead to

restoration of the anatomical structures and functions of the affected tissues (Dreifke et al., 2015). However, a good wound healing agent should also possess antimicrobial properties as bacterial infection can negatively affect the wound healing mechanism. When an open wound occurs, bacterial cells that normally inhabit the skin surface can enter the underlying tissues (Guo and DiPietro, 2010). As a normal response to this, inflammation is triggered to remove the invading bacteria (Diegelmann and Evans, 2004). However, if the bacterial cells continue to replicate, it can cause an infection. Consequently, this can lead to prolonged inflammation, which could result in increased levels of proteases, enzymes that can degrade the extracellular matrices (Guo and DiPietro, 2010). Additionally, the colonizing bacteria can also form biofilms (aggregation of bacterial cells covered with extracellular polymeric substance), and thus acquiring resistance towards antimicrobials (Davis et al., 2008). This could complicate the wound healing process as it can be difficult to treat the infection (Fux et al., 2005; Guo and DiPietro, 2010).

Apart from bacterial infection, oxidative stress is another factor that can impair wound healing. As previously mentioned, low ROS levels are essential for normal cellular functions. However, high ROS levels can damage cellular macromolecules, hence disrupt the wound repair process (Schäfer and Werner, 2008). Though ROS defense mechanisms exist in cells to maintain their balance, other factors such as age and disease, can adversely influence the efficacy of these mechanisms (Schäfer and Werner, 2008). Therefore, it is imperative that a wound healing agent also possesses antioxidant properties.

1.6 Significance and objectives of this study

Etlingera ginger plants have good medicinal potential as suggested through several ethnobotany reports and scientific studies. As *E. coccinea*, *E. pubescens*, and *E. sessilanthera* are understudied, this research will provide insights into the potential of these ginger plants for drug discovery. It is important to note that the conservation status of *E. pubescens* and *E. sessilanthera* are 'Near Threatened', hence, there is a great need to prove the medicinal potential of these ginger plants to support their conservation. The following lists the primary objectives of this study:

1. To obtain specimens of *E. coccinea*, *E. pubescens*, and *E. sessilanthera*, as well as their crude extracts.
2. To screen the crude extracts for antimicrobial, antioxidant, and wound healing properties.
3. To isolate and identify the compounds responsible for the observed activities.
4. To characterize the isolated compounds for their bioactivities

1.7 Outline of chapters presented in this thesis

Chapter 1 introduces the three ginger plants of interest, as well as justifies the need to study them to search for bioactive compounds that can be used to treat bacterial infections, diseases related to oxidative stress, and wounds.

Chapter 2 outlines the work carried out to achieve the objectives, and details the methodologies employed.

Chapter 3 describes the bioactivity of *E. sessilanthera*. The antioxidant properties of *E. sessilanthera*'s leaves, stems and rhizomes were evaluated via three different assays, using three

different percentages of methanol (50, 70, and 100%). Apart from this, antimicrobial activity testing was also performed on its various plant parts, in which the crude extracts were obtained through sequential solvent extraction. From its active extract, an antimicrobial compound was isolated, and characterized for its bacterial inhibition activities.

Chapter 4 describes the bioactivity of *E. coccinea*. Similar to the work done on *E. sessilanthera*, the antioxidant and antimicrobial properties of *E. coccinea* were assessed using the same methodologies. From its most active extract, an antimicrobial compound was identified, and tested for its bacterial inhibition activities. Moreover, the wound healing property of *E. coccinea* was investigated. Its water extract showed positive enhancements of cell proliferation and migration, thus the major constituents of the extract was analyzed. Further characterization on the extracts' antimicrobial and antioxidant activities were also performed to support the wound healing ability of *E. coccinea*.

Chapter 5 describes the bioactivity of *E. pubescens*. Its different plant parts were screened for antioxidant and antimicrobial properties. Subsequently, a compound which exhibits both antioxidant and antimicrobial activities was isolated. The compound's antioxidant activities were characterized by employing various assays, including two which involve the use of cell line models. Next, characterization of its antimicrobial activities was also conducted. This was achieved through antimicrobial activity testing, as well as mechanistic study (since its antimicrobial property has never been reported). Moreover, the compound's cytotoxicity was assessed to determine its potential use as an antioxidant drug to treat oxidative stress-related diseases.

Chapter 6 provides an overall discussion on the antioxidant and antimicrobial properties of *E. coccinea*, *E. pubescens*, and *E. sessilanthera*. Results for other ginger plants from literature were also included in the comparison to answer the question on how good the antioxidant and antimicrobial activities of the three ginger species studied are. Lastly, this chapter presents the conclusions of this study and several recommendations for future work.

CHAPTER 2

MATERIALS AND METHODS

Chapter 2 – Materials and Methods

2.1 Overview of research methodologies

The following figure depicts the research methodologies used in this study.

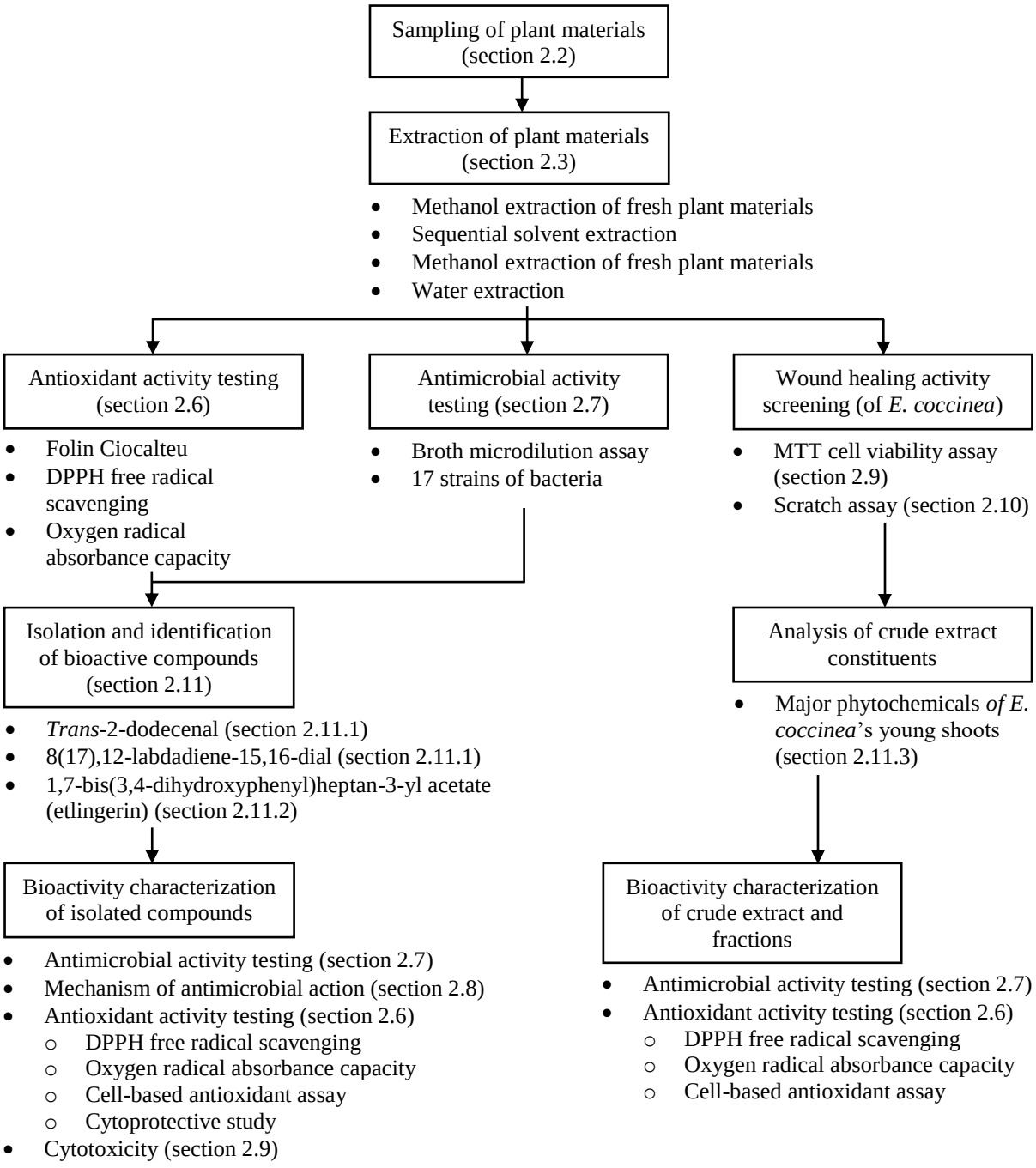

Figure 2: The overview of research methodologies employed in this study.

2.2 Sampling of plant materials

Specimens of *E. coccinea*, *E. pubescens*, and *E. sessilanthera* were sampled from Danum Valley, Sabah (GPS coordinates of 0*4°58'11.4"N, 117°48'41.5"E,* 0*4°57'59.3"N, 117°47'29.8"E,* and 0*4°57'57.1"N, 117°48'3"E,* respectively), with permission from Sabah Biodiversity Centre (SaBC) and Conservation and Environmental Management Division, Yayasan Sabah (CEMD) (see Appendix 1, 2, and 3 for the access, export, and approval licenses). Plant identification was done by Mr. Bernadus Bala Ola from Danum Valley Conservation Area (DVCA) and John Sugau from Forest Research Centre (FRC) Sabah. The young shoots, leaves, stems, and rhizomes were sampled haphazardly from plants with heights approximately between 1 to 2 meters tall. Voucher specimens were deposited at the Forest Department Herbarium Sandakan, Sepilok, 90000 Sandakan, Sabah, Malaysia, with the reference numbers 157373 (*E. coccinea*), 157375 (*E. pubescens*) and 157378 (*E. sessilanthera*).

2.3 Extraction of plant materials

Extraction processes were performed to obtain crude extracts of *E. coccinea*, *E. pubescens*, and *E. sessilanthera* for further analyses on their bioactive properties. This was achieved via several different methods, as outlined in the following sub-sections.

2.3.1 Methanol extraction of fresh plant materials

Methanol extraction of fresh plant materials was carried out to obtain plant crude extracts for antioxidant activity testing. Approximately 1 g of fresh plant materials were added with 50 mL of various concentrations of methanol (50 %, 70 %, and 100 %). After subjecting to sonication (35

kHz frequency, Elmasonic S130H, Elma, United States) for 15 min, the solutions were filtered and kept at –20 °C until further use.

2.3.2 Sequential solvent extraction (SSE)

SSE was done to obtain crude extracts that contain phytochemicals with different polarities. This was achieved via the sequential use of hexane, dichloromethane, ethyl acetate, methanol, and water, which have increasing polarities. Plant specimens were washed and dried using a freeze-drier (Freezone 4.5 Plus; Labconco, Kansas City, Missouri). With aid of liquid nitrogen, the specimens were crushed into smaller parts, and transferred into glass jars. Then, hexane was added until it reaches about 1 cm above the crushed specimens, and the glass jars were subjected to sonication (35 kHz frequency; Elmasonic S130H; Elma, Fremont, California) for 15 min, followed by filtration of the solution. These steps were conducted three times. The filtered solutions were pooled and concentrated under reduced pressure using a rotary evaporator (N-1110; Eyela, Tokyo, Japan). With the same plant material, extraction was conducted again using dichloromethane, ethyl acetate, methanol, and distilled water, sequentially. Finally, the extracts were dried using a freeze drier (Freezone 4.5 Plus; Labconco).

2.3.3 Methanol extraction of dried plant materials

Methanol extraction of dried plant materials was carried out to obtain plant crude extracts for antimicrobial activity testing. Plant specimens were washed and dried using a freeze-drier (Freezone 4.5 Plus; Labconco, Kansas City, Missouri). With aid of liquid nitrogen, the specimens were crushed into smaller parts, and transferred into glass jars. Methanol was added until it reaches about 1 cm above the crushed specimens, and the glass jars were subjected to sonication (35 kHz

frequency; Elmasonic S130H; Elma, Fremont, California) for 15 min, followed by filtration of the solution. These steps were conducted three times. The filtered solutions were pooled and concentrated under reduced pressure using a rotary evaporator (N-1110; Eyela, Tokyo, Japan).

2.3.4 Water extraction

Water extraction was performed to obtain extracts for wound healing study. The young shoots of *E. coccinea* were washed to remove any remaining dirt, blotted with paper towels and air dried for 30 min at room temperature. Two types of extraction procedures were employed to obtain extracts for use in subsequent assays: fresh water extract (FE) and dried water extract (DE). The former was done to imitate the plant's traditional use. The intention of preparing DE was to preserve the bioactive compounds for further identification as drying process can preserve phytochemicals. Also, accurate preparation of required concentrations for further analyses could be done on DE.

2.3.4.1 Preparation of fresh water extract (FE)

The air-dried samples (375 g) were pounded using mortar and pestle with aid of liquid nitrogen and placed into glass jars. Ultrapure water (1.85 L) was added in just enough to cover the samples. The jars were subjected to sonication for 15 min using an ultrasonic bath (35 kHz frequency, Elmasonic S130H, Elma, Singen, Germany). The solutions were filtered using Whatman Grade 1 filter papers (Whatman, Buckinghamshire, United Kingdom) and aliquoted in 1.5 mL microcentrifuge tubes. All tubes containing the extracts were stored in −80 ºC until further use.

2.3.4.2 Preparation of dried water extract (DE)

Similar procedure was repeated as described in section 2.3.4.1. The addition of ultrapure water, sonication and filtration were performed three times. The solutions were then pooled together, concentrated under reduced pressure using a rotary evaporator (EYELA OSB-2100, Japan), and dried using a freeze-drier (FreeZone 4.5 Plus, Labconco, United States). All dried extracts were kept at −80 °C until further use.

2.4 Bacterial cell culture

The following bacterial strains were purchased from American Type Culture Collection (ATCC): *Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC10145, *Salmonella enterica* serovar Typhimurium ATCC14028, *Shigella flexneri* ATCC12022, *Enterococcus faecalis* ATCC700802, *Enterococcus faecalis* ATCC29212, *Staphylococcus aureus* ATCC43300, *S. aureus* ATCC700699, *S. aureus* ATCC6538P, *S. aureus* ATCC29213, *Bacillus cereus* ATCC14579, *Streptococcus pneumonia*e ATCC49136, *Staphylococcus epidermidis* ATCC12228, *Bacillus subtilis* ATCC8188, *Proteus vulgaris* IMR, *Proteus mirabilis* ATCC12453, and *Salmonella enterica* serovar Enteritidis ATCC13076. All bacterial strains were maintained on tryptic soy agar (TSA) (Merck, Darmstadt, Germany) and Muller Hinton agar (MHA) (Oxoid, Hampshire, United Kingdom) at 37 °C for 24 hours and in 25% v/v glycerol at -80 °C for long term preservation.

2.5 Mammalian cell culture

HaCaT (human keratinocyte), SH-SY5Y (human neuroblastoma), HEK-293T (human embryonic kidney), and M10 (normal mammary epithelial) cell lines were cultured in high glucose

Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2 mM L-glutamine, 1 % (w/v) sodium pyruvate, 10 % of fetal bovine serum (FBS) and 1 % penicillin-streptomycin (10,000 U/mL). HT-29 (human colon carcinoma), SW-480 (human colon adenocarcinoma) and CCD-841 (human colon normal epithelial) cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 2mM L-glutamine, 10 % FBS and 1% penicillin-streptomycin (10,000 U/mL). All cell lines were cultured in T75 (75 cm^2) tissue culture flasks at 37°C with 5 % CO_2 supply in a biological incubator.

2.6 Antioxidant activity testing

To determine the antioxidant activities of the plants crude extracts and pure compounds, several assays were employed. The Folin Ciocalteu assay measures the amount of phenolic compounds in a crude extract. The assay also estimates the extracts' reducing ability (Tan and Lim, 2015). Conversely, the DPPH FRS (which is represented by AEAC) and ORAC measure antioxidant activities via different mechanisms: single electron transfer and hydrogen atom transfer, respectively (Tan and Lim, 2015). The cell-based antioxidant assay is a biologically-relevant method to determine the antioxidant properties of plant crude extracts and pure compounds, as the assay employs mammalian cells. Lastly, the cytoprotective study was conducted to assess the ability of pure compounds to protect cells from hydrogen peroxide challenge.

2.6.1 Folin Ciocalteu assay

Total phenolic content (TPC) was determined using the Folin Ciocalteu assay as described by Zhang et al. (2006), with modifications. In 96-well microtiter plates (SPL 30196, BioLab, Singapore) 30 µL of plant extract was mixed with 150 µL of 10 % Folin's reagent and 120 µL of

7.5 % sodium carbonate. After 10 min of incubation in the dark, absorbance was measured at 765 nm using a microtiter plate reader (Tecan Infinite M200, Mannedorf, Switzerland). Gallic acid was used to construct a standard curve and TPC was reported as mg GAE/ 100 g fresh sample.

2.6.2 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging (FRS)

The DPPH FRS assay was performed as described by Cheng et al. (2006), with modifications. In 96-well microtiter plates (SPL 30196, BioLab, Singapore), plant extracts/ pure compounds were serially diluted (with final volumes of 100 µL) and 200 µL of DPPH solution (6.0 mg/ 100 mL methanol) was added to each well. After 30 min of incubation, absorbance was measured at 517 nm using a microtiter plate reader (Tecan Infinite M200, Mannedorf, Switzerland). Ascorbic acid solution was used as a reference and the AEAC was reported as mg ascorbic acid equivalent (AAE)/ 100 g fresh sample. For comparison between pure compounds, the DPPH FRS IC_{50} (concentration that scavenges 50 % free radicals) values are reported in µM.

2.6.3 Oxygen radical absorbance capacity (ORAC)

ORAC assay was performed as described by Asensio et al. (2015). In 96-well black microtiter plates (SPL 30296, BioLab, Singapore), 25 µL of serially diluted plant extracts were added to 150 µL of 84 nM fluorescein, followed by 15 min incubation at 37 °C. Then, 25 µL of 153 mM of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) was added into each well. Immediately after this, the fluorescence values were measured for 90 min with 2 min intervals. The antioxidant, Trolox, was used to plot the standard curve, and the ORAC values were determined as µmol Trolox equivalent (TE)/ g fresh weight. For comparison between fractionated extracts and pure

compounds, the ORAC EC_{50} (concentration that provides 50 % protection) values were calculated and reported in µM.

2.6.4 Cell-based antioxidant assay

The cell-based antioxidant assay was performed according to Bender and Graziano (2015), with modifications. Approximately 50,000 HaCaT cells were loaded into wells of 96-well black microtiter plates (SPL 30296, BioLab, Singapore). After 24 hours incubation, the cells were pre-treated with various concentrations of the compounds, as well as 25 µM 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) (Merck, Darmstadt, Germany). Following one-hour incubation, the wells were washed and added with 600 µM AAPH. Fluorescence kinetics was measured every 5 min for 1 hr using a microtiter plate reader (Tecan Infinite M200, Mannedorf, Switzerland) with excitation and emission wavelengths of 485 nm and 540 nm, respectively. Quercetin was used as standard the cellular antioxidant activity (CAA) values were determined as quercetin equivalent (QE). Where appropriate, a graph of percentage protection (%) against concentration of compound (µg/mL) was plotted to enable comparison of antioxidant activity across various concentrations, in which the following formula was used: percentage protection = 100 % × (net area under curve for untreated – net area under curve for treatment) ÷ net area under curve for untreated.

2.6.5 Cytoprotective study

Determination of cellular protective effect on HaCaT cell line was performed according to Chen et al. (2011), with modifications. Approximately 5,000 cells of HaCaT cells in 100 µL culture media were loaded into the wells of a 96-well microtiter plate (SPL 30196, BioLab, Singapore).

After 24 hours incubation, the cells were treated with various concentrations of compounds (0 to 200 µg/mL) for one-hour. Then, the wells were washed to remove extracellular compounds, followed by addition of 500 µM hydrogen peroxide. After an additional 24 hours incubation, MTT assay was performed to determine cell viability. Determination of cell viability through trypan blue exclusion was done with the following modifications: Approximately 30,000 cells in 600 µL culture media were seeded in wells of a 24-well microtiter plate (NunC 150687, (Thermo Fisher Scientific, Massachusetts, United States). After treatment, the cells were washed with PBS, detached using 0.1 % trypsin-EDTA, mixed with equal volume of FBS, and enumerated on a hemocytometer. Visual assessment of cellular morphology was achieved using Nikon TS-100 (Nikon, Tokyo, Japan) inverted phase-contrast microscope. Following 24 hours treatment in 24-well microtiter plates (NunC 150687, (Thermo Fisher Scientific, Massachusetts, United States), images of three independent replicates were obtained.

2.7 Antimicrobial activity testing

Broth microdilution assay was conducted to determine the antimicrobial activities of the plant crude extracts and pure compounds. The assay was done by using 96-well microtiter plates (SPL 30196, BioLab, Singapore), as described by Daniel-Jambun et al. (2017), with modifications. Plant extracts were prepared in 5 % Tween 80 + 95 % acetonitrile (for hexane, dichloromethane, ethyl acetate extracts), methanol (for methanol extracts), or distilled water (for water extracts), while pure compounds were prepared in dimethyl sulfoxide (DMSO). Further dilutions were made using either tryptic soy broth (TSB) or Muller-Hinton broth (MHB) (Oxoid, Hampshire, United Kingdom). The respective solvents at 0.5% v/v final concentration were used as negative controls while 0.2 mg/mL chloramphenicol (Calbiochem, Merck, Darmstadt, Germany) was used as a

positive control. MIC values were determined from clear wells with the lowest concentration of extracts were taken as MIC values. The MBC values were determined by plating the clear wells on antibiotic-free TSA or MHA. The concentration with no bacterial growth was taken as MBC.

2.8 Assessment of antimicrobial mechanism

The antimicrobial mechanism was determined through several assays. The time-kill assay evaluates the killing kinetics and dose dependencies of an antimicrobial compound. SYTO-9/ propidium iodide (PI) uptake and fluorescence microscopy analysis were conducted to assess the effect of treatment with antimicrobial compound on the bacterial cell membrane. To further support this, the bacterial cellular leakage assay was performed, which measures the leakage of intracellular materials.

2.8.1 Time-kill assay

The time-kill assay was performed as described by CLSI (2012), with modifications. Various concentrations of etlingerin (in 2.5 mL) corresponding to 1× and 2× MIC were prepared in universal bottles and mixed with 2.5 mL of adjusted bacterial suspension (containing approximately 1×10^6 cfu/mL). At time = 0, 15, 60, 120 and 240 min, the contents of the universal bottles were diluted appropriately, spread plated on MHA and incubated at 37 °C aerobically. The number of viable colonies was enumerated and reported as log cfu/mL.

2.8.2 SYTO-9/ propidium iodide (PI) uptake assay

SYTO-9/ propidium iodide (PI) uptake assay was carried out using Invitrogen LIVE/DEAD BacLight Bacterial Viability Kit, as per manufacturer's instructions (Thermo Fisher Scientific,

Massachusetts, United States). Various concentrations of etlingerin (corresponding to 1× and 2× MIC) were prepared in 500 µL of 0.85% (w/v) saline in 1.5 mL microcentrifuge tubes. Then, equal volumes of adjusted bacterial suspension (containing approximately 1×10^8 cfu/mL) were added to the same tubes and incubated at 37 °C aerobically for 30 min. One hundred µL of the contents were mixed with 100 µL of SYTO-9/ PI dye mixture in black microtiter plates (SPL 30296, BioLab, Singapore) and further incubated for 30 min. The green and red fluorescence measurements were made using a microtiter plate reader (Tecan Infinite M200, Mannedorf, Switzerland) with excitation/emission wavelength of 485/530 nm and 485/630 nm, respectively. A positive control (70% isopropanol) was used and the dye uptake ratio was determined by the green/red fluorescence ratio.

2.8.3 Fluorescence microscopy analysis of SYTO-9/ propidium iodide (PI) uptake

The bacteria suspensions were prepared and treated as stated in previous section (SYTO-9/ PI uptake assay). Twenty µL of the bacteria-dye mixtures were pipetted onto glass slides and covered with glass cover slips. The slides were viewed under an Olympus BX60 fluorescence microscope (Olympus, Tokyo, Japan) using appropriate filters.

2.8.4 Bacterial cellular leakage assay

The bacterial cell suspensions were prepared and treated as stated in previous section (SYTO-9/ PI uptake assay). After 4 hours of treatment, the absorbance values of the solutions were measured using BioDrop DUO (BioDrop, Cambridge, United Kingdom) at 260 nm and 280 nm. The background absorbance values of 0.85% saline with bacteria as well as etlingerin at appropriate concentrations (62.5 and 125 µg/mL against *B. subtilis* ATCC 8188, and 125 and 250 µg/mL

against *S. aureus* ATCC 43300) were measured to determine the net absorbance values corresponding to leakage of intracellular materials.

2.9 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay

MTT cell viability assay was conducted to determine the viability of cells following treatment with plant crude extracts and pure compounds. The assay was employed as described by Sylvester (2011), with modifications. Approximately 5,000 cells in 100 µL culture media were loaded into the wells of a 96 well microtiter plate (SPL 30196, BioLab, Singapore) and incubated for 24 hours. After that, the cells were treated with various concentrations of compounds for an additional 24 hours. Then, the cells were exposed to 5 mg/mL MTT solution (Nacalai Tesque, Kyoto, Japan) for about 2-3 hours (depending on the cell line used), and the purple formazans formed were eluted with DMSO. Absorbance values were measured at 590 nm using a microtiter plate reader (Tecan Infinite M200, Mannedorf, Switzerland) and dose response curves were constructed. Cytotoxicity was determined as the concentration of compounds that inhibited 50 % cell growth (IC_{50}).

2.10 Scratch assay

Scratch assay was conducted to assess the effect of plant crude extracts on migration of cells (wound closure). The assay was performed as described by Liang et al. (2007), with modifications. Approximately 20,000 cells (in 500 µL) were loaded into each well of a 24-well microtitre plate (Thermo Fisher Scientific, Massachusetts, United States), followed by incubation at 37°C with 5% CO_2 supply. After a confluent cell monolayer was formed, a p200 pipette tip (200 µL Universal Fit Bevel Point Pipette Tip, Labcon, California, United States) was used to create a straight scratch in the middle of the cell monolayers. The wells were then rinsed with phosphate buffered saline

(PBS) twice to remove unbound cells, followed by addition of fresh media containing *E. coccinea* crude extracts at appropriate concentrations. Ten ng/mL epidermal growth factor (EGF) (Thermo Fisher Scientific, Massachusetts, United States) and 1 % ultrapure water were used as positive and negative controls, respectively. Images of the scratch in each well were captured at t = 0, 12, and 24 hours for BEAS-2B and HaCaT cells, and t = 0, 6, and 12 hours for NIH/3T3 cell, using Nikon TS-100 (Nikon, Tokyo, Japan) inverted phase-contrast microscope. The area of each scratch was measured by using NIS Element Basic Research software (Nikon Instruments, New York, United States) and the area of reduction was calculated according to the formula: Reduction in wound area ($\mu metre^2$) = wound area at 0 hour – wound area after 6, 12 or 24 hours.

2.11 Isolation and identification of bioactive compounds

Following antimicrobial and antioxidant activity screenings, three bioactive compounds, *trans*-2-dodecenal, 1,7-bis(3,4-dihydroxyphenyl)heptan-3-yl acetate (etlingerin), and 8(17),12-labdadiene-15,16-dial, were purified from *E. coccinea*, *E. pubescens*, and *E. sessilanthera*, respectively. These compounds were isolated through different chromatographic methods and identified via liquid chromatography mass spectrometry (LCMS) and nuclear magnetic resonance (NMR) analyses. The wound healing property of *E. coccinea* was also conducted, and the major phytochemicals present in the extract were assessed using LCMS and tandem MS (MS^2) methods. The following sub-sections describe the details of the methodologies used.

2.11.1 *Trans*-2-dodecenal and 8(17),12-labdadiene-15,16-dial

2.11.1.1 Thin layer chromatography (TLC)

TLC was performed to separate the components of the extracts with positive antimicrobial activities (leaf hexane extract of *E. coccinea* and rhizome hexane extract of *E. sessilanthera*). Ten mg/mL of extracts were prepared and spotted on a silica gel plate. The plate was then developed using HEX-EA (85:15) and visualized by heating the plate on a hot plate for 30 seconds after spraying with dilute sulphuric acid (0.1N). The visible spots were grouped into separate regions (see Figure 3). Partial purification of the antimicrobial compounds was carried out through preparative TLC method. Five hundred µL of extracts with concentration of 50 mg/mL were prepared and introduced on a preparative silica gel plate (175 - 225 µm layer thickness). The plates were developed using the same mobile phase as stated previously. Each region (as determined using analytical TLC) was scrapped off from the plate and extracted using MeOH. The solutions obtained were subjected to sonication (35 kHz frequency, Elmasonic S130H, Elma, United States), filtered, concentrated under reduced pressure using a rotary evaporator (N-1110, Eyela, Japan) and finally dried using freeze-drier (Freezone 4.5 Plus, Labconco, United States).

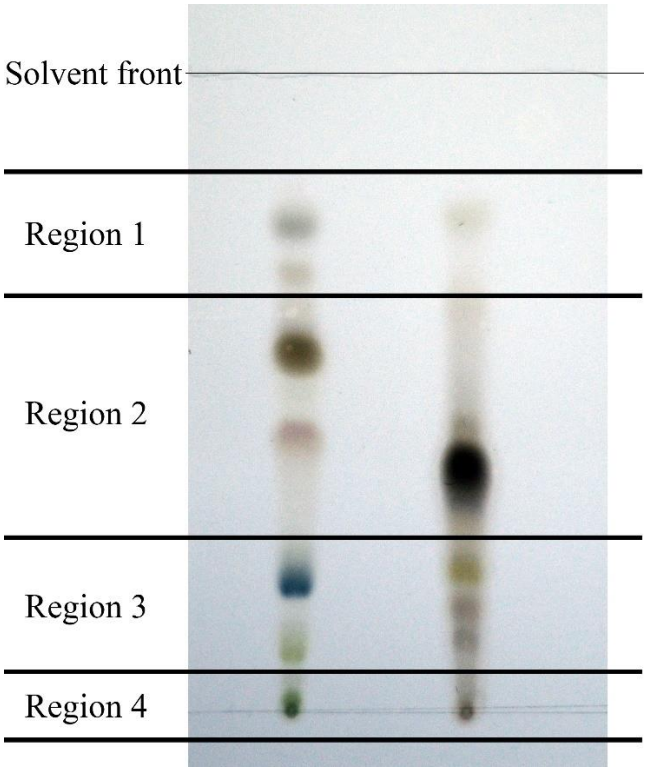

Figure 3: Images of developed thin layer chromatography (TLC) plates (after sulphuric acid treatment) showing the components in (left) *Etlingera sessilanthera* rhizome hexane extract and (right) *Etlingera coccinea* leaf hexane extract.

2.11.1.2 C18 preparative high-performance liquid chromatography (HPLC)

Only region 2 extracts of *E. coccinea* and *E sessilanthera* showed positive antimicrobial activity against two selected bacterial strains (*S. aureus* ATCC 43300 and *B. cereus* ATCC 14579) at 100 µg/mL. Therefore, preparative HPLC was employed to purify the antimicrobial compounds. The partially purified extracts from preparative TLC were dissolved in acetonitrile and filtered through 0.22 µm polytetrafluoroethylene (PTFE) membrane. Pure *trans*-2-dodecenal from *E. coccinea* was obtained by preparative HPLC with the following conditions: Cosmosil C18 preparative column (20 × 250 mm, 5-micron particle); solvent system: A-water, B-methanol; 90% B for 13 min; flow rate: 20 mL/min; injection volume: 100 µL; concentration: 100 mg/mL. The compound was

observed at t_R = 7.0 min with detection wavelength of 222 nm. Pure 8(17),12-labdadiene-15,16-dial from *E. sessilanthera* was obtained by preparative HPLC with the following conditions: Cosmosil C18 preparative column (20 × 250 mm, 5-micron particle), solvent system: A-water, B-acetonitrile; 80% B for 6 min, 100% B for 4 min; flow rate: 30 mL/min; injection volume: 100 µL; concentration: 100 mg/mL. The compound was observed at t_R = 7.5 min with detection wavelength of 235 nm.

2.11.1.3 Liquid chromatography mass spectrometry (LCMS)

The accurate masses of *trans*-2-dodecenal and 8(17),12-labdadiene-15,16-dial were determined using positive ion mode through electrospray ionization – mass spectrometry (ESI-MS). The following conditions were used to identify the mass of *trans*-2-dodecenal: Agilent 1290 Infinity LC instrument coupled to Agilent 6520 Accurate-Mass Q-TOF (Agilent, United StatesAgilent Zorbax SB-C18 (2.1 × 150 mm, 3.5-micron particle); solvent system: A-0.1 % formic acid, B-acetonitrile with 0.1 % formic acid; 90 % B for 15 min; flow rate: 0.5 mL/min; injection volume: 1 µL; concentration: 1 mg/mL. The compound was observed at t_R = 1.58 min. The following conditions were used to identify the mass of 8(17),12-labdadiene-15,16-dial: Acquity UPLC instrument (Waters, United States), Acquity UPLC BEH C18 column (2.1 × 50 mm, 1.7-micron particle); solvent system: A-0.1% formic acid, B-acetonitrile with 0.1 % formic acid; 40-100 % B over 2 min, 100 % B for 0.5 min; flow rate: 0.5 mL/min; injection volume: 5 µL; concentration: 1 mg/mL. The compound was observed at t_R = 2.18 min.

2.11.1.4 Nuclear magnetic resonance (NMR) spectroscopy

NMR analyses were performed using Varian Unity Plus instrument (Bruker, Germany) to aid structure elucidation. ^{1}H NMR (300 MHz) experiment was done for the identification of *trans*-2-dodecenal while ^{1}H NMR (700 MHz) and ^{13}C (176 MHz) experiments were done for the identification of 8(17),12-labdadiene-15,16-dial (see Appendix 4, 5, and 6 for the ^{1}H and ^{13}C NMR assignments).

2.11.2 1,7-bis(3,4-dihydroxyphenyl)heptan-3-yl acetate (etlingerin)

2.11.2.1 Solvent fractionation

SSE was carried out as outlined in section 2.3.2, on the dried methanol extract of *E. pubescens* leaves to separate out the non-polar compounds. Hexane, dichloromethane, and ethyl acetate (Merck, Darmstadt, Germany) were used as the solvents. The ethyl acetate fraction was kept for further purification work.

2.11.2.2 C18 gravity column chromatography

C18 gravity column chromatography was performed on the ethyl acetate fraction to further remove non-polar compounds. The following parameters were used: dimension of packed beads: 3 cm diameter × 20 cm height; stationary phase: LiChroprep RP-18 (40-63 µm) (Merck, Darmstadt, Germany); mobile phase: 90 % acetonitrile. One hundred mg/mL of the crude extract were introduced into the column and eluted with three column volumes of 90 % acetonitrile. The eluent was collected, filtered, concentrated under reduced pressure using a rotary evaporator (N-1110, Eyela, Tokyo, Japan) and finally dried using freeze-drier (Freezone 4.5 Plus, Labconco, Missouri, United States).

2.11.2.3 C18 preparative high-performance liquid chromatography (HPLC)

Preparative HPLC (Agilent 1200 Infinity, Agilent Technologies, California, United States) was employed to purify the bioactive compound. Pure etlingerin was obtained by using the following conditions: Cosmosil C18 preparative column, 20 × 250 mm, 5-micron particle (Nacalai Tesque, Kyoto, Japan); solvent system: A-water, B-acetonitrile; 30 to 50 % B in 20 min, 95 % B for 5 min; flow rate: 20 mL min/mL; injection volume: 100 µL; concentration: 100 mg/mL. The compound was observed at t_R = 17.0 min with detection wavelength of 210 nm.

2.11.2.4 Liquid chromatography mass spectrometry (LCMS)

The accurate mass of etlingerin was determined using negative ion mode through electrospray ionization – mass spectrometry (ESI-MS). The following conditions were used: Agilent 1290 Infinity LC instrument coupled to Agilent 6520 Accurate-Mass Q-TOF (Agilent, California, United States), Agilent Poroshell EC-C18 column, 2.1 × 100 mm, 2.7-micron particle (Agilent, California, United States); solvent system: A-0.1 % formic acid, B-methanol with 0.1 % formic acid; 40 to 100 % B in 13 min, 100 % B for 2 min; flow rate: 0.5 mL/min; injection volume: 1 µL; concentration: 1 mg/mL. The compound was observed at t_R = 4.96 min.

2.11.2.5 Nuclear magnetic resonance (NMR) spectroscopy

^{1}H NMR (700 MHz) and ^{13}C NMR (176 MHz) analyses were performed using Bruker Ascend instrument (Bruker, Karlsruhe, Germany) to aid structure elucidation (see Appendix 7 and 8 for the ^{1}H and ^{13}C NMR assignments).

2.11.3 Major phytochemicals of *E. coccinea*'s young shoots

2.11.3.1 High-performance liquid chromatography (HPLC) analysis

HPLC analysis was first performed to analyze the peaks present in the water extract. The following parameters were used to obtain the chromatogram: Agilent 1200 Infinity HPLC instrument (Agilent Technologies, California, United States), Cosmosil C18 preparative column (2.1 × 250 mm, 5-micron particle) (Nacalai Tesque, Kyoto, Japan); solvent system: A-3% formic acid, B-methanol with 3% formic acid; 10 to 60% B in 40 min, 60 to 95% B in 5 min, 95% B for 5 min; flow rate: 1 mL/min; injection volume: 50 µL; concentration: 2 mg/mL; detection wavelength: 280 nm. The major peaks were observed at t_R = 2 to 5 min (group A), t_R = 8 to 18 min (group B), and t_R = 24.09 min, 25.13 min, 25.60 min, 26.50 min, and 27.79 min (group C).

2.11.3.2 Liquid chromatography mass spectrometry (LCMS) and tandem mass spectrometry (MS^2)

The accurate masses of compounds found in the major peaks observed via HPLC analysis were determined using negative ion mode electrospray ionization – mass spectrometry (ESI-MS). The following conditions were used: Agilent 1290 Infinity LC instrument coupled to Agilent 6520 Accurate-Mass Q-TOF (Agilent, California, United States), Agilent Zorbax 300SB-C18 column, 2.1 × 150 mm, 5-micron particle (Agilent, California, United States); solvent system: A-0.1% formic acid, B-methanol with 0.1% formic acid; 10 to 60% B in 50 min, 60 to 95% B for 10 min, 95 to 100% B in 5 min; flow rate: 0.5 mL/min; injection volume: 2 µL; concentration: 1 mg/mL. The major peaks were observed at t_R = 0.5 to 1.5 min (group A), t_R = 2 to 10 min (group B), and t_R = 13.85 min, 14.30 min, 16.01 min, 18.86 min, 19.18 min, and 19.97 min (group C). MS^2 analysis was achieved using similar parameters as described for LCMS with the following

modifications: injection volume: 5 µL; collision energies: 10, 20, and 40V. Identification of major phytochemicals was achieved by comparing the fragmentation patterns observed with known compounds from the following mass spectrometry databases: METLIN (http://metlin.scripps.edu), MassBank (http://www.massbank.jp) and ReSpect (http://spectra.psc.riken.jp/).

2.11.3.3 Fractionation of the crude water extract of *E. coccinea*

Preparative HPLC was conducted to separate the crude extract into polar (F1) and less-polar (F2) fractions. The following parameters were utilized: Cosmosil C18 preparative column, 20 × 250 mm, 5-micron particle (Nacalai Tesque, Kyoto, Japan); solvent system: A-water, B-methanol; 10% B for 2 min, 95% B for 4 min, 10% B for 7 min; flow rate: 15 mL/min; injection volume: 500 µL; detection wavelength: 280 nm; concentration: 100 mg/mL. Fraction F1 was observed at t_R = between 2.8 to 5.3 min while fraction F2 was observed at t_R = 5.6 to 8.6 min. Both fractions were collected, concentrated under reduced pressure using a rotary evaporator (N-1110, Eyela, Tokyo, Japan), and dried using a freeze drier (Freezone 4.5 Plus, Labconco, Missouri, United States).

Sephadex LH-20 chromatography was employed, as described by Strumeyer and Malin (1975), to obtain non-tannin (F2A) and tannin-rich (F2B) fractions. The following parameters were used: dimension of packed beads: 2 cm diameter × 10 cm height; stationary phase: Sephadex LH-20 (25-100 µm) (Sigma-Aldrich, Missouri, United States); mobile phase: 100% ethanol. One hundred mg/mL of the fraction F2 was introduced into the column and eluted with three column volumes of 100 % ethanol. The eluent was collected as designated as F2A. Then, three column volumes of 70 % acetone was used to elute fraction F2B. All eluents were filtered, concentrated under reduced

pressure using a rotary evaporator (N-1110, Eyela, Tokyo, Japan) and finally dried using freeze-drier (Freezone 4.5 Plus, Labconco, Missouri, United States).

Analytical HPLC was performed to compare the chromatograms for F2, F2A, and F2B fractions. The following parameters were utilized: Cosmosil C18 column, 2.1 × 250 mm, 5-micron particle (Nacalai Tesque, Kyoto, Japan); solvent system: A-water, B-methanol; 10 to 60 % B for 25 min, 60 % B for 5 min; flow rate: 1 mL/min; injection volume: 20 µL; detection wavelength: 280 nm; concentration: 2 mg/mL.

2.11.3.4 Radial diffusion assay

Quantification of tannins was achieved through the radial diffusion assay, as described by Hagerman (1987), with modifications. Briefly, 1 % (w/v) agarose containing 0.1 % (w/v) bovine serum albumin was prepared in petri dishes. Then, wells of about 4 mm were made and various concentrations of extracts were added into the wells. After 96 hours of incubation at 30 °C, the diameters of hollow zones were measured. The tannin content was reported as mg tannic acid equivalent (TAE)/ g extract.

2.12 Determination of log P values

The log P values were calculated to estimate the lipophilicity of pure compounds. This was conducted using computational methods, as described by Daina et al. (2017). According to the authors, a common practice to obtain accurate log P values is by utilizing multiple predictors. Thus, five predictive models were used: XLOGP3, WLOGP, MLOGP, SILICOS-IT, and iLOGP,

as suggested by the authors. The consensus log P represents the mean of the values determined by the five methods.

2.13 Quantification of reactive oxygen species (ROS) in cells

Quantification of ROS was performed to determine whether treatment with a pure compound leads to intracellular ROS generation. The assay was carried out as described by Wang and Joseph (1999), with modifications. Briefly, HT-29 and SW-480 cells were seeded with a density of about 20,000 cells/well in 96-well black microtiter plates. After 24 hours incubation, the cells were pre-treated with various concentrations of etlingerin and curcumin (0, 1, 10, 50, and 100 µg/mL), for one hour. Then, the wells were washed once with HBSS, and 50 µM of DCFH-DA was added into the wells. After 5 min, the fluorescence values were measured using 490 nm excitation wavelength and 540 nm emission wavelength.

2.14 Statistical analysis

Statistical analysis was conducted using IBM SPSS Statistics Version 21.0. The significance of results for different assays were performed using either independent sample *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's Honest Significant Difference (Tukey's HSD) test at the level of significance, $\alpha = 0.05$. The differences with probability value $p \leq 0.05$ were considered statistically significant.

CHAPTER 3

ANTIOXIDANT AND ANTIMICROBIAL PROPERTIES

OF *ETLINGERA SESSILANTHERA*

Chapter 3 – Antioxidant and Antimicrobial Properties of *Etlingera sessilanthera*

3.1 Antioxidant activity of *E. sessilanthera*

Specimens of *E. sessilanthera* were obtained from Danum Valley (see section 2.2), and its methanol extracts were prepared as described in section 2.3.1. Then, the antioxidant activities of these extracts were evaluated through three different assays. The calibration equation determined for the quantification of TPC is as follow: $y = 0.008x \pm 0.0086$ ($R^2 = 0.9985$). As for the DPPH FRS, the IC_{50} value obtained for ascorbic acid was 3.91 ± 0.17 µg/mL, while the standard equation calculated for Trolox in the ORAC assay was $y = 0.3694\times + 1.3094$ ($R^2 = 0.9906$). A summary of the antioxidant activities of *E. sessilanthera* is presented in Table 1.

Table 1: The total phenolic content (TPC), ascorbic acid equivalent antioxidant capacity (AEAC), and oxygen radical absorbance capacity (ORAC) values determined for the methanolic extracts *Etlingera sessilanthera*.

Part of plant	Percentage methanol (%)	TPC (mg GAE/ 100 g fresh weight)	AEAC (mg AAE/ 100 g fresh weight)	ORAC (μmol TE/ g fresh weight)
Leaves	50	341.1 ± 3.2c	204.5 ±1.1c	ND
	70	473.5 ± 14.9b	230.9 ± 6.2b	ND
	100	658.7 ± 11.8aA	257.6 ± 12.8aA	145.4 ± 12.9A
Stems	50	52.53 ± 1.29c	64.66 ± 1.60c	ND
	70	54. 01 ± 0.24b	82.00 ± 0.21aB	4.567 ± 0.114B
	100	68.04 ± 1.59aB	74.73 ± 0.63b	ND
Rhizomes	50	55.13 ± 2.18c	63.52 ± 1.13c	ND
	70	59.77 ± 0.95b	78.54 ± 1.37aC	6.089 ± 0.106C
	100	67.01 ± 2.13aB	71.72 ± 0.15b	ND

Assays were performed with three replicates and values are reported as mean ± standard error. ND indicates that the experiment was not determined as ORAC values were only determined for the extracts with highest TPC and AEAC values. The significance of the results was assessed using ANOVA at $\alpha = 0.05$ with Tukey's post-hoc test. Small letters compare statistical differences between means of the three different percentages of methanol (50 %, 70 %, and 100 %) for the same part of the plant, while capital letters compare highest mean values between the three plant parts (leaves, stems, and rhizomes). GAE = gallic acid equivalent; AAE = ascorbic acid equivalent; TE = Trolox equivalent; ND = not determined.

The present work focuses on the phenolic group of phytochemicals due to their high antioxidant potential. The extraction of these compounds depends on two main factors: 1) their chemical nature (which affects their solubility in a given solvent), and 2) the solvent used for extraction itself (Garcia-Salas et al., 2010). Since the former is an intrinsic property of the compounds, the latter became an important factor to consider. Methanol was chosen to extract the phenolic compounds as past studies have proven its efficiency in extracting these compounds (Garcia-Salas et al., 2010; Yao et al., 2004). Additionally, methanol had also been shown to effectively extract antioxidant compounds from other *Etlingera* species (Chan et al., 2007). Apart from being able to extract the compounds, methanol is also known to inhibit polyphenol oxidase, which are enzymes that can degrade phenolic compounds (Lim and Murtijaya, 2007). This is an important property of methanol since the degradation could result in significant loss of antioxidant activity. Due to the basis that phenolic compounds have varying degree of solubility in different solvent polarities, three percentages of methanol (50, 70, and 100 %) were employed in the extraction process. This was to ensure that the phenolic compounds with different polarities could be extracted.

Referring to Table 1, when compared between the various percentages of solvent used, 100 % methanol was associated with highest TPC values for the different plant parts. However, while 100 % methanol resulted in highest AEAC value for its leaves, 70 % methanol showed higher AEAC values for its stems and rhizomes. These observations suggest that the antioxidant compounds present in all extracts, except for the leaf extract of *E sessilanthera*, were relatively more polar in nature as compared to the latter.

In general, the TPC, AEAC, and ORAC values reported in Table 1 were found to be significantly higher in the leaves than the stems and rhizomes, hence implying that *E. sessilanthera*'s leaves possess good antioxidant activity. This was not surprising as the leaves of other *Etlingera*, as well as other ginger plant species, also exhibited high antioxidant activities. (Chan et al., 2007; Chan et al., 2008). For example, the leaves of *E. elatior* displayed TPC and AEAC values of 2390 ± 329 mg GAE/ 100 g and 2280 ± 778 mg AAE/ 100 g, respectively. Conversely, the TPC and AEAC values determined for its rhizomes were 326 ± 76 g GAE/ 100 g and 295 ± 96 mg AAE/ 100 g, respectively.

3.2 Antimicrobial activity of *E. sessilanthera*

Apart from antioxidant, *E. sessilanthera* was also evaluated for its antimicrobial activity. The crude extracts were obtained via sequential solvent extraction, as described in section 2.3.2. Then, these extracts were tested for their antimicrobial properties using the broth microdilution method (see section 2.7), against 17 bacterial strains (see section 2.4). Table 2 lists the MIC values determined.

Table 2: Minimum inhibitory concentration (MIC) values of three different parts of *E. sessilanthera* against 17 strains of bacteria.

Part of plant	Solvent	Minimum inhibitory concentrations (mg/mL)																
		S. aureus ATCC 700699	*S. aureus* ATCC 43300	*S. aureus* ATCC 6538P	*S. aureus* ATCC 29213	*S. epidermidis* ATCC 12228	*B. subtilis* ATCC 8188	*B. cereus* ATCC 14579	*E. faecalis* ATCC 29212	*E. faecalis* ATCC 700802	*S. pneumoniae* ATCC 49136	*S. flexneri* ATCC 12022	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 10145	*P. vulgaris* IMR	*P. mirabilis* ATCC 12453	*S. enterica* ser. Enteritidis ATCC 13076	*S. enterica* ser. Typhimurium ATCC 14028
Leaf	HEX	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	DCM	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	EA	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	MeOH	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	H_2O	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
Stem	HEX	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	DCM	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	EA	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	MeOH	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	H_2O	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
Rhizome	HEX	1	1	>1	>1	1	0.5	0.125	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	DCM	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	EA	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	MeOH	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	H_2O	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1

MIC values were determined with 3 biological replicates. HEX = hexane, DCM = dichloromethane, EA = ethyl acetate, MeOH = methanol, H_2O = water.

Based on Table 2, only the hexane extract of *E. sessilanthera*'s rhizomes exhibited bacterial inhibition property. This extract was active against the gram-positive bacteria (see Table 2). The ineffectiveness towards gram-negative bacteria could be due to the presence of lipopolysaccharide (LPS) layer in their membrane which can act as a barrier that limits permeability of antimicrobials (Blair et al., 2015). Other species of *Etlingera* were also found to exhibit antimicrobial activity against gram-positive bacteria only (Chan et al., 2007; Chandarana et al., 2005; Wong et al., 2006a, b).

3.3 Isolation and identification of antimicrobial compound from *E. sessilanthera*

The hexane extract of *E. sessilanthera*'s rhizomes was subjected to compound isolation and identification work (see section 2.11.1). Briefly, the components in the crude extract were first separated and isolated through TLC, and following antimicrobial activity testing, only "region 2" extract showed inhibition activities. From this extract, 8(17),12-labdadiene-15,16-dial was purified via preparative HPLC, and identified through LCMS and NMR analyses. All spectroscopic data were consistent with previous literature (Forbes et al., 2014; González et al., 2010). The following lists the ^{1}H and ^{13}C NMR shifts determined for 8(17),12-labdadiene-15,16-dial, while Figure 4 illustrates the chemical structure of 8(17),12-labdadiene-15,16-dial.

8(17),12-labdadiene-15,16-dial: $[\alpha]_D^{25}$ = +44° (c = 0.04, ethanol). ^{1}H NMR (700 MHz, $CDCl_3$) δ 9.65 (t, *J* = 1.4 Hz, 1H), 9.42 (s, 1H), 6.78 (t, *J* = 6.6 Hz, 1H), 4.88 (s, 1H), 4.39 (s, 1H), 3.41 (d, *J* = 16.80 Hz, 1H), 3.48 (d, *J* = 16.64 Hz, 1H), 2.52 (ddd, *J* = 16.8, 6.3, 2.9 Hz, 1H), 2.44 (ddd, *J* = 12.9, 4.2, 2.4 Hz, 1H), 2.39 – 2.32 (m, 1H), 2.08 – 2.03 (m, 1H), 1.92 (d, *J* = 10.9 Hz, 1H), 1.78 (ddt, *J* = 12.9, 5.0, 2.4 Hz, 1H), 1.71 (d, *J* = 12.4 Hz, 1H), 1.63 – 1.51 (m, 2H), 1.44 (dd, *J* = 13.2, 1.2 Hz, 1H), 1.37 (qd, *J* = 13.0, 4.3 Hz, 1H), 1.21 (td, *J* = 13.4, 4.0 Hz, 1H), 1.16 (dd, *J* = 12.6, 2.7 Hz, 1H), 1.09 (td, *J* = 12.8, 3.9 Hz, 1H), 0.91 (s, 3H), 0.84 (s, 3H), 0.75 (s, 3H). ^{13}C NMR (176 MHz, $CDCl_3$) δ 197.34, 193.59, 159.98, 148.06, 134.86, 107.87, 56.47, 55.38, 41.97, 39.62, 39.37, 39.25, 37.86, 33.59, 24.68, 24.11, 21.73, 19.28, 14.41. LCMS (ESI) m/z 302.22.

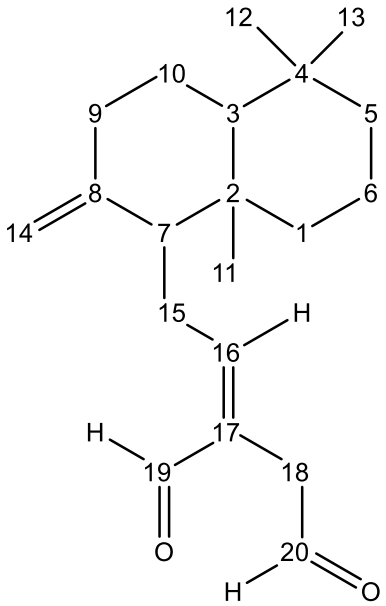

Figure 4: The chemical structure of 8(17),12-labdadiene-15,16-dial.

3.4 Antimicrobial activity of 8(17),12-labdadiene-15,16-dial

The antimicrobial activity of 8(17),12-labdadiene-15,16-dial was evaluated by employing the broth microdilution assay (as described in section 2.7) against several gram-positive and gram-negative bacterial strains. Table 3 lists the MIC values obtained.

Table 3: Minimum inhibitory concentration (MIC) values of 8(17),12-labdadiene-15,16-dial against several gram-positive and gram-negative bacteria.

Minimum inhibitory concentrations (µg/mL)							
S. aureus ATCC 43300	*S. aureus* ATCC 6538P	*B. cereus* ATCC 14579	*B. subtilis* ATCC 8188	*P. aeruginosa* ATCC 10145	*E. coli* ATCC 25922	*S. enterica* ser. Typhimurium ATCC 14028	*P. mirabilis* ATCC 12453
8	4	4	4	>1000	>1000	>1000	>1000

MIC values were determined with 3 biological replicates.

Classified as a labdane diterpene, 8(17),12-labdadiene-15,16-dial can be found in other ginger plants such as *Alpinia nigra*, *Alpinia zerumbet*, *Zingiber ottensii* and *Siphonochilus aethiopicus* (Akiyama et al., 2006; Chompoo et al., 2011; Ghosh et al., 2013; Igoli et al., 2012). To date, no other studies have discovered the presence of labdane diterpenes in the *Etlingera* genus except for 6-hydroxylabda-8(17),11,13-trien-15,16-olide which had been isolated from *Etlingera elatior* (Mohamad et al., 2005). 8(17),12-labdadiene-15,16-dial contains α,β-unsaturated aldehyde structures, thus can potentially act as an alkylating agent which could then interact with proteins and nucleic acids, hence affecting bacterial growth (Maillard, 2002; Witz, 1989). Several studies have evaluated the antimicrobial activity of 8(17),12-labdadiene-15,16-dial. One interesting finding was reported by Ghosh et al. (2013) in which 8(17),12-labdadiene-15,16-dial isolated from

Alpinia nigra seeds was found to be active against both gram-positive and gram-negative bacteria with MIC values ranging from 12.5 to 25 µg/mL. In this study, no activity was found against the gram-negative bacteria (see Table 3). This might be because 8(17),12-labdadiene-15,16-dial can exist as (+) and (−) stereoisomers and that different drug conformation can affect the efficacy of the said drug (González et al., 2010; Itokawa et al., 1980; McConathy and Owens, 2003). Due to the site-specific properties of many drug receptors, different stereoisomers can exhibit different pharmacokinetics and pharmacological properties. For example, the (–) isomer of ofloxacin was found to be much more active in inhibiting a wide range of gram-positive and gram-negative bacterial strains than its (+) isomer (Tanaka et al., 1990). In another study, (+)-*α*-pinene was found to be active against *S. aureus*, *E. coli*, *Candida albicans* and *Micrococcus luteus* while (−)-*α*-pinene failed to inhibit any of the aforementioned pathogens. The optical rotation of 8(17),12-labdadiene-15,16-dial determined in this study was (+44) while in the work by Ghosh et al. (2013), the optical rotation was not mentioned. Therefore, further work should investigate the structure-activity relationship of 8(17),12-labdadiene-15,16-dial stereoisomers.

In terms of cytotoxicity, 8(17),12-labdadiene-15,16-dial was found to exhibit IC_{50} values (concentration which inhibit 50 % of cells) of 13.1 ± 3.9 µg/mL and 19.5 ± 1.9 µg/mL against Vero and HeLa cell lines, respectively (González et al., 2010). The IC_{50} values of 8(17),12-labdadiene-15,16-dial are higher than the MIC values determined against gram-positive bacteria tested in this study, hence suggesting its potential to be used as an antimicrobial agent (i.e. the compounds can inhibit bacterial growth while not being toxic to normal cells at the required concentration).

3.5 Summary of Chapter 3

The leaves of *E. sessilanthera* showed good antioxidant properties, thus highlighting its potential to be mined for antioxidant compounds. In terms of antimicrobial activity, only its rhizomes were found to be active. The isolated compound, 8(17),12-labdadiene-15,16-dial, inhibited several clinically-relevant bacterial strains with low MIC values (4-8 µg/mL). Although it was suggested to be safe for use, further cytotoxicity studies using animal models should be performed. There is also an opportunity for future work to modify 8(17),12-labdadiene-15,16-dial to enhance its antimicrobial activity while further reducing toxicity towards mammalian cells. The work done on the antimicrobial property of *E. sessilanthera* was published in Journal of Applied Microbiology (see Appendix 9).

CHAPTER 4

ANTIOXIDANT, ANTIMICROBIAL, AND WOUND HEALING PROPERTIES OF *ETLINGERA COCCINEA*

Chapter 4 – Antioxidant, Antimicrobial, and Wound Healing Properties of *Etlingera coccinea*

4.1 Antioxidant activity of *E. coccinea*

Consumption of food that are rich in antioxidants is known to be beneficial for health. Since *E. coccinea* is traditionally eaten as pickled vegetable, it is only relevant to study its antioxidant properties. The leaves, stems, and rhizomes of *E. coccinea* were sampled from Danum Valley (see section 2.2), and its methanol extracts were prepared as outlined in section 2.3.1. Then, quantification TPC, AEAC, and ORAC (see section 2.6.1, 2.6.2, and 2.6.3) were conducted on the extracts to determine the antioxidant activities. The calibration equation determined for the quantification of TPC is as follow: $y = 0.008x \pm 0.0086$ ($R^2 = 0.9985$). As for the DPPH FRS, the IC_{50} value obtained for ascorbic acid was 3.91 ± 0.17 µg/mL, while the standard equation calculated for Trolox in the ORAC assay was $y = 0.3694\times + 1.3094$ ($R^2 = 0.9906$). Table 4 summarizes the results obtained.

Table 4: The total phenolic content (TPC), ascorbic acid equivalent antioxidant capacity (AEAC), and oxygen radical absorbance capacity (ORAC) values determined for the methanolic extracts of *Etlingera coccinea*.

Part of plant	Percentage methanol (%)	TPC (mg GAE/ 100 g fresh weight)	AEAC (mg AAE/ 100 g fresh weight)	ORAC (μmol TE/ g fresh weight)
Leaves	50	1126 ± 35b	2573 ± 94b	ND
	70	1246 ± 28aA	2702 ± 89aA	423.9 ± 18.2A
	100	1015 ± 45c	1508 ± 141c	ND
Stems	50	87.60 ± 6.78b	126.1 ± 5.6c	ND
	70	136.4 ± 1.9aB	203.5 ± 5.4aB	50.36 ± 2.80B
	100	131.5 ± 7.0a	182.5 ± 5.6b	ND
Rhizomes	50	97.97 ± 11.60b	154.0 ± 17.4c	ND
	70	141.2 ± 2.8aC	220.6 ± 6.4aC	43.79 ± 1.34C
	100	138.6 ± 4.3a	207.1 ± 7.8b	ND

Assays were performed with three replicates and values are reported as mean ± standard error. ND indicates that the experiment was not determined as ORAC values were only determined for the extracts with highest TPC and AEAC values. The significance of the results was assessed using ANOVA at $\alpha = 0.05$ with Tukey's post-hoc test. Small letters compare statistical differences between means of the three different percentages of methanol (50 %, 70 %, and 100 %) for the same part of the plant, while capital letters compare highest mean values between the three plant parts (leaves, stems, and rhizomes). GAE = gallic acid equivalent; AAE = ascorbic acid equivalent; TE = Trolox equivalent; ND = not determined.

In general, 70 % methanol resulted in highest TPC and AEAC values (see Table 4). This suggests that the antioxidant compounds present in *E. coccinea* are relatively more polar, as they could be extracted with aqueous methanol. When compared between the different plant parts, the leaves exhibited best antioxidant activity, similar to the results shown by *E. sessilanthera*. As previously discussed in Chapter 3, other ginger plants also displayed high antioxidant activities in their leaves.

The fact that *E. coccinea* possessed high antioxidant activity could be linked to its traditional uses in treating various illnesses. For example, it was mentioned in Chapter 1 that *E. coccinea* can be used to treat stomach ache and other gastric problems. These complications have been associated with ROS, which were thought to cause direct cytotoxicity, alterations in gut electrolyte transport and motility, mutagenicity, as well as gastric mucosal damage (Hahm et al., 1997). Therefore, the antioxidants present in *E. coccinea* could play a role in neutralizing the free radicals in the gut, hence diminishing their detrimental effects. Apart from this, *E. coccinea* has also been traditionally used to treat wounds. During wound healing, ROS are produced by cells such as macrophages to combat invading bacteria (Auf Dem Keller et al., 2006). High ROS levels can impair the wound healing process, however, the antioxidant compounds present in *E. coccinea* might be able to prevent the ROS from reaching extreme levels. While this is an interesting theory, further work is needed to investigate the role of *E. coccinea* in wound repair. Preliminary work on the wound healing ability of *E. coccinea* and the contribution of its antioxidant properties have been carried out and the findings are presented in section 4.5.

4.2 Antimicrobial activity of *E. coccinea*

The use of *E. coccinea* in treatment of wound and stomach ache drove the interest to study its antimicrobial property as these are often related to bacterial infections. Through literature search, only one published study had reported positive antimicrobial activity of *E. coccinea*. Although positive activity was found, Vairappan et al. (2012) only studied the essential oil extract from the plant's rhizomes. It is noteworthy that the indigenous people use the young shoots and stems for medicinal use (Poulsen, 2006). In this research, the leaves, stems and rhizomes of *E. coccinea* were investigated while the crude extracts were obtained via ultrasonic-assisted extraction using various solvents of different polarities. This provides a broader overview of the plant's antimicrobial property as different parts of plant as well as different polarities of phytochemicals were studied. The crude extracts of *E. coccinea* were prepared as described in section 2.3.2. Then, antimicrobial activity testing was conducted by using the broth microdilution method (see section 2.7) on 17 bacterial strains (as listed in section 2.4). Table 5 lists the MIC values obtained.

Table 5: Minimum inhibitory concentration (MIC) values of three different parts of *E. coccinea* against 17 strains of bacteria.

Part of plant	Solvent	Minimum inhibitory concentrations (mg/mL)															
		S. aureus ATCC 700699	*S. aureus* ATCC 43300	*S. aureus* ATCC 6538P	*S. aureus* ATCC 29213	*S. epidermidis* ATCC 12228	*B. subtilis* ATCC 8188	*B. cereus* ATCC 14579	*E. faecalis* ATCC 29212	*E. faecalis* ATCC 700802	*S. pneumoniae* ATCC 49136	*S. flexneri* ATCC 12022	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 10145	*P. vulgaris* IMR	*P. mirabilis* ATCC 12453	*S. enterica* ser. Enteritidis ATCC 13076	*S. enterica* ser. Typhimurium ATCC 14028
Leaf	HEX	0.031	0.031	0.031	0.031	0.016	0.016	0.016	0.063	0.063	0.016	>1	>1	>1	>1	>1	>1	>1
	DCM	0.063	0.063	0.063	0.063	0.031	0.031	0.031	0.031	0.031	0.031	>1	>1	>1	>1	>1	>1	>1
	EA	0.063	0.063	0.063	0.063	0.031	0.031	0.031	0.125	0.125	0.031	>1	>1	>1	>1	>1	>1	>1
	MeOH	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	H_2O	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
Stem	HEX	0.125	0.125	0.125	0.125	0.063	0.063	0.063	0.125	0.125	0.063	>1	>1	>1	>1	>1	>1	>1
	DCM	0.125	0.125	0.125	0.125	0.063	0.063	0.063	0.125	0.125	0.063	>1	>1	>1	>1	>1	>1	>1
	EA	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	MeOH	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	H_2O	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
Rhizome	HEX	0.5	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	DCM	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	EA	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	MeOH	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	H_2O	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1

MIC values were determined with 3 biological replicates. Concentration listed was based on final working concentration. HEX = hexane, DCM = dichloromethane, EA = ethyl acetate, MeOH = methanol, H_2O = water.

Based on Table 5, the crude extracts of *E. coccinea* were found to be active against the gram-positive bacteria only. As previously discussed in Chapter 3, the LPS layer might limit permeability of antimicrobials, hence making them ineffective against the gram-negative bacteria. However, Vairappan et al. (2012) reported that the essential oil extract from the rhizomes of *E. coccinea* was able to inhibit gram-negative bacteria. Even so, the differences between extraction protocols employed (extraction method affects type/amount of phytochemicals isolated), strains of bacteria used (different strains of the same bacteria species may possess different susceptibility towards the same antimicrobial), and location of plant specimens obtained (plants of the same species grown in different geographical area may contain different type/amount of phytochemical) could account for the differing observations (Crisóstomo et al., 2001; Silva et al., 1998; Wallaart et al., 2000). Not only that, since antimicrobial activity testing was done on crude extracts, there was a possibility of antagonism occurring between the extracts' constituents. For example, the combinations of carvacrol/thymol, carvacrol/eugenol and thymol/menthol were found to be ineffective against *S. aureus* and *E. coli* as compared to their individual counterparts (Gallucci et al., 2009). Regardless, the crude extracts could inhibit growth of wound-associated bacteria such as *S. aureus* and *S. epidermidis*, thus supporting their use in treatment of wounds by the indigenous community.

4.3 Isolation and identification of antimicrobial compound from *E. coccinea*

The leaves of *E. coccinea* were found to be better at inhibiting the bacterial strains, as compared to its stems and rhizomes. More specifically, its hexane extract was associated with lowest MIC values (ranging from 0.016 to 0.063 mg/mL). Therefore, it was of interest to isolate and identify the antimicrobial compound. The protocol used to achieve this is outlined in 2.10.1. Briefly, the

leaf hexane extract of *E. coccinea* was first subjected to TLC, and it was found that "region 2" was positive for antimicrobial activity. Then, preparative HPLC was performed on this extract, yielding *trans*-2-dococenal as the major antimicrobial compound. All spectroscopic data were consistent with those reported in previous literature (Itokawa et al., 1980). The following lists the ^{1}H and ^{13}C NMR shifts for *trans*-2-dodecenal, while Figure 5 shows the chemical structure of *trans*-2-dodecenal.

***Trans*-2-dodecenal**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.51 (d, *J* = 7.9 Hz, 1H), 6.85 (dt, *J* = 15.6, 6.8 Hz, 1H), 6.12 (ddt, *J* = 15.6, 7.9, 1.5 Hz, 1H), 2.33 (ddd, *J* = 14.6, 7.0, 1.5 Hz, 2H), 1.51 (dt, *J* = 14.3, 7.3 Hz, 2H), 1.27 (s, 12H), 0.88 (t, *J* = 6.7 Hz, 3H). LCMS (ESI) m/z 182.17. ^{13}C NMR (Forbes et al., 2014) δ 194.1 (CH), δ 159.0 (CH), δ 132.9 (CH), δ 32.7 (CH_2), δ 31.8 (CH_2), δ 29.4 (CH_2), δ 29.3 (CH_2), δ 29.2 (CH_2), δ 29.16 (CH_2), δ 27.8 (CH_2), δ 22.6 (CH_2), δ 14.0 (CH_3).

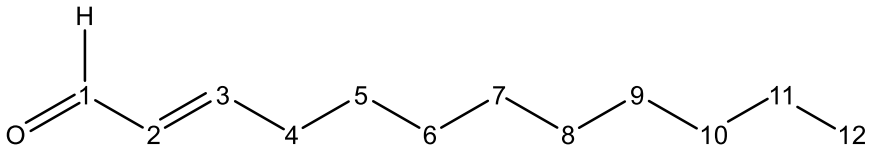

Figure 5: The chemical structure of *trans*-2-dodecenal.

4.4 Antimicrobial activity of *trans*-2-dodecenal

The antimicrobial activity of *trans*-2-dodecenal was assessed by using the broth microdilution assay (see section 2.7) against several bacterial strains. Table 6 lists the MIC values obtained.

Table 6: Minimum inhibitory concentration (MIC) values of *trans*-2-dodecenal against several gram-positive and gram-negative bacteria.

Minimum inhibitory concentrations (µg/mL)							
S. aureus ATCC 43300	*S. aureus* ATCC 6538P	*B. cereus* ATCC 14579	*B. subtilis* ATCC 8188	*P. aeruginosa* ATCC 10145	*E. coli* ATCC 25922	*S. enterica* ser. Typhimurium ATCC 14028	*P. mirabilis* ATCC 12453
8	4	4	4	>1000	>1000	>1000	>1000

MIC values were determined with 3 biological replicates.

Trans-2-dodecenal, or (E)-2-dodecenal, can be found in various sources such as culantro (*Eryngium foetidum*), coriander (*Coriandrum sativum*), in several bacteria species, and even in the millipede, *Rhinocricus insulates* (Blom et al., 2011; Eyres et al., 2005; Wheeler et al., 1964). This study is the first to report the presence of this compound in the genus *Etlingera*. In terms of its antimicrobial activity, this compound was hypothesized to exert its killing effect through membrane disruption, as reported by Kubo et al. (2004). In addition, work by Trombetta et al. (2002) on several 2E-alkenals suggested that apart from alterations in membrane permeability, these compounds could affect other membrane functions, as well as penetrate the bacterial cells and interact with other cell constituents. As *trans*-2-dodecenal possesses α,β-unsaturated aldehyde structure, it can potentially act as an alkylating agent which could then interact with proteins and nucleic acids, thus affect bacterial growth (Maillard, 2002; Witz, 1989).

Based on the results obtained, *trans*-2-dodecenal was found to be inactive against the gram-negative bacteria (see Table 6). One plausible reason for this is could be because gram-positive bacteria utilizes the redox buffer bacillithiol (BSH), while the tripeptide glutathione (GSH) is predominant in gram-negative bacteria (Loi et al., 2015). Glutathiones have been associated with

resistance to alkylating agents and therefore might explain the ineffectiveness of *trans*-2-dodecenal towards gram-negative bacteria (Colvin et al., 1993). Contrariwise, Kubo et al. (2004) reported that the same compound was active against *Salmonella* Choleraesuis, a gram-negative bacterium (Kubo et al., 2004). This could be due to the differences in experimental procedures such as bacterial strains and culture media used (different strains might have different susceptibility while culture media can affect a compound's antimicrobial activity) (Ando et al., 2010; Crisóstomo et al., 2001). Nonetheless, its remarkably low MIC value against clinically relevant pathogens such as *S. aureus* warrants further work into its possible uses. One potential application of *trans*-2-dodecenal would be in wound dressings. As this compound is volatile (due to its strong smell), it might be able to exhibit its antimicrobial effect while in its vapour phase. This effect can be seen in cinnamon and clove essential oils (Goni et al., 2009).

In terms of its cytotoxicity, the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) concluded that there are no safety concerns on the use of *trans*-2-dodecenal (FAO, 2005). The basis that this compound is not toxic to humans while able to inhibit bacterial strains with low MIC values highly suggest its potential to be used as an antimicrobial agent. However, further testing on its antimicrobial activity should be performed using animal models.

4.5 Wound healing property of *E. coccinea*

As previously mentioned, *E. coccinea* is traditionally used by the indigenous community in Sabah to treat wounds. Hence, its wound healing property was evaluated in this study. Specimens of *E. coccinea*'s young shoots were obtained from Danum Valley. Then, water extraction was

performed (see section 2.3.4) to prepare the crude extracts. Water was used as the extraction solvent to replicate the traditional use of *E. coccinea*.

4.5.1 Effects of *E. coccinea* water extracts on cell proliferation and migration

To determine the wound healing property of *E. coccinea*, its water extracts were first tested for their effects on proliferation of BEAS-2B, HaCaT, and NIH/3T3 cell lines. This was achieved by employing the MTT assay (see section 2.9), and Figure 6 depicts the cell viability values determined.

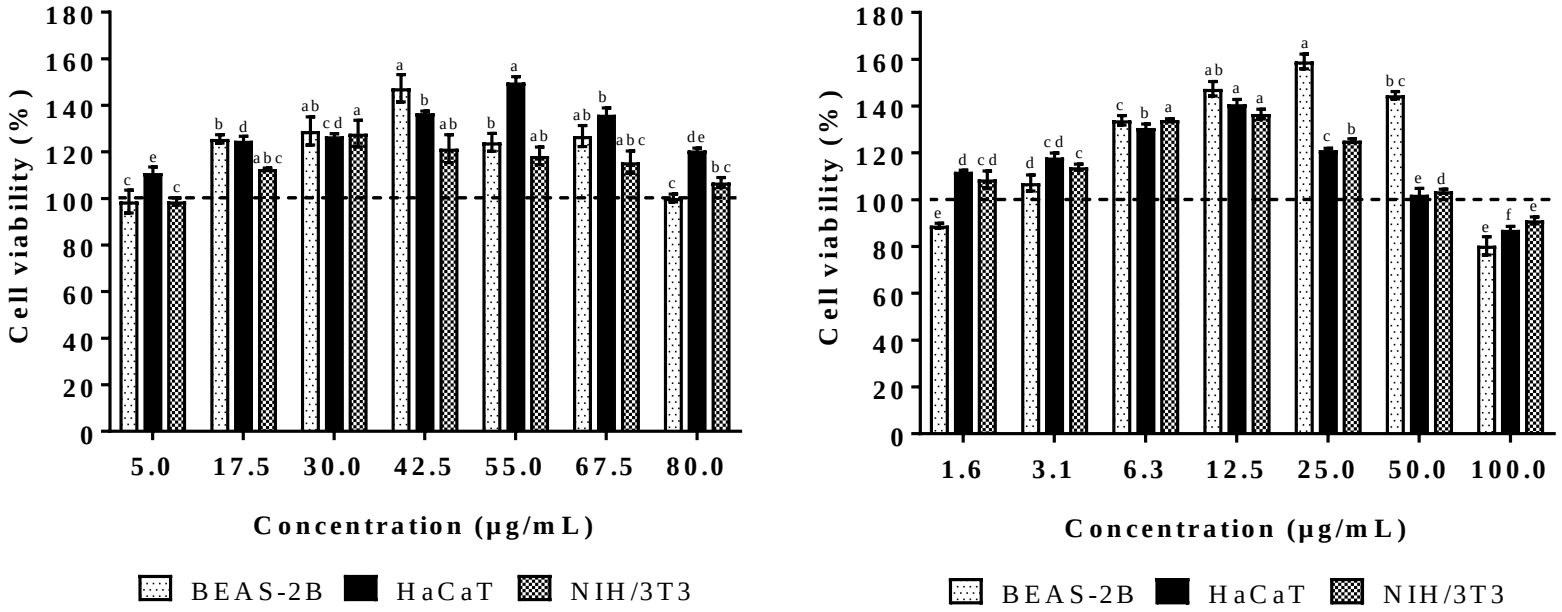

Figure 6: Cell viabilities of BEAS-2B, HaCaT, and NIH/3T3 cells following treatment with various concentrations of *E. coccinea* young shoots fresh water extract (FE) (left) and dried water extract (DE) (right). Assays were performed with three independent replicates and bar heights are reported as mean ± standard error. Different alphabets indicate significant differences between the mean cell viabilities across difference concentrations, for the same cell line, at $\alpha = 0.05$ (as determined using ANOVA, with Tukey's post-hoc test).

Dose-dependent effects were observed when the cells were treated with various concentrations of FE and DE (see Figure 6). Comparatively, highest cell viability values for each cell line for each treatment is as follows: treatment with 42.5, 55, and 30 µg/mL FE resulted in cell viability values of 147.3 ± 5.8, 149.7 ± 2.4, and 127.6 ± 5.9 % on BEAS-2B, HaCaT, and NIH/3T3 cell lines, respectively. Also, treatment with 25, 12.5, and 12.5 µg/mL DE resulted in cell viability values of 158.7 ± 3.3, 140.8 ± 2.0, and 136.5 ± 2.1 % on BEAS-2B, HaCaT, and NIH/3T3 cell lines, respectively. At higher concentrations, the cell viability values were observed to decrease in a dose-dependent manner. This could be due to the presence of compounds that are harmful at higher concentrations, hence causing cytotoxicity. Conversely, at lower concentrations, the bioactive compounds might not be sufficient to effectively enhance cell proliferation. Similar to this study, a study by Annan and Houghton (2008) showed that the extracts of *Gossypium arboretum* and *Ficus asperifolia* enhanced proliferation of 142BR fibroblast cells in a dose-dependent manner, with 50 µg/mL being the optimum concentration determined for both extracts, after which cytotoxic effects were observed (at higher concentrations). It was also noted that the cell viability of BEAS-2B cells treated with 1.6 µg/mL DE was lower than 100 % (see Figure 6), and this might be due to the presence of compounds that can lead to decreased cell proliferation. For example, ROS are known to play a role in cell proliferation, and that antioxidants can reduce ROS levels (Huo et al., 2009; Pan et al., 2011). The decrease in ROS might prevent cell proliferation from proceeding at its optimum state.

Apart from cell proliferation, FE and DE were also evaluated for their effects on cell migration. The scratch assay (see section 2.10) was conducted, and the reduction in wound areas at different time points were determined and compared. The optimum concentrations that resulted in highest

viabilities were used: 42.5, 55, and 30 µg/mL FE, as well as 25, 12.5, and 12.5 µg/mL DE, on BEAS-2B, HaCaT, and NIH/3T3 cell lines, respectively. An example of the wound area assessment conducted is shown in Figure 7.

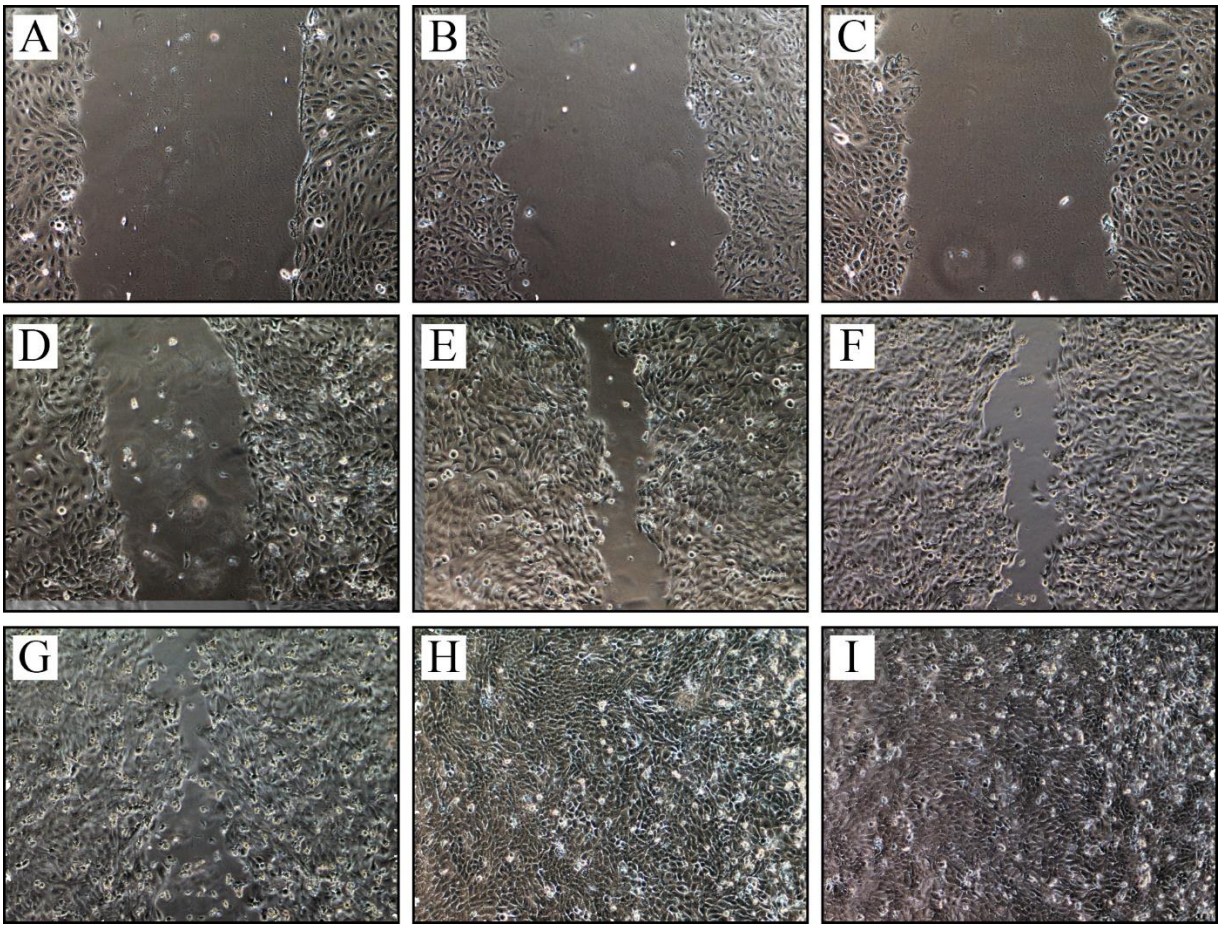

Figure 7: Scratch assay images of HaCaT cells with wound areas at t = 0 hour (A, B, and C), 12 hours (D, E, and F), and 24 hours (G, H, and I), following treatment with 1 % ultrapure water (negative control) (A, D, and G), 10 ng/mL epidermal growth factor (EGF) (positive control) (B, E, and H), and 55 µg/mL *E. coccinea* young shoots fresh water extract (FE) (C, F, and I).

As shown in Figure 7, when compared with HaCaT cells treated with 1 % ultrapure water (negative control), treatment with 10 ng/mL EGF resulted in better wound closure at t = 12 hours, while complete closure was observed at t = 24 hours. This supports the use of EGF as a positive control. Similar observations were made for the cells treated with 55 µg/mL FE. Images of three independent replicates were obtained for each treatment on the three cell lines, and the reductions in wound area were calculated and statistically analyzed. A summary of the results is presented in Figure 8.

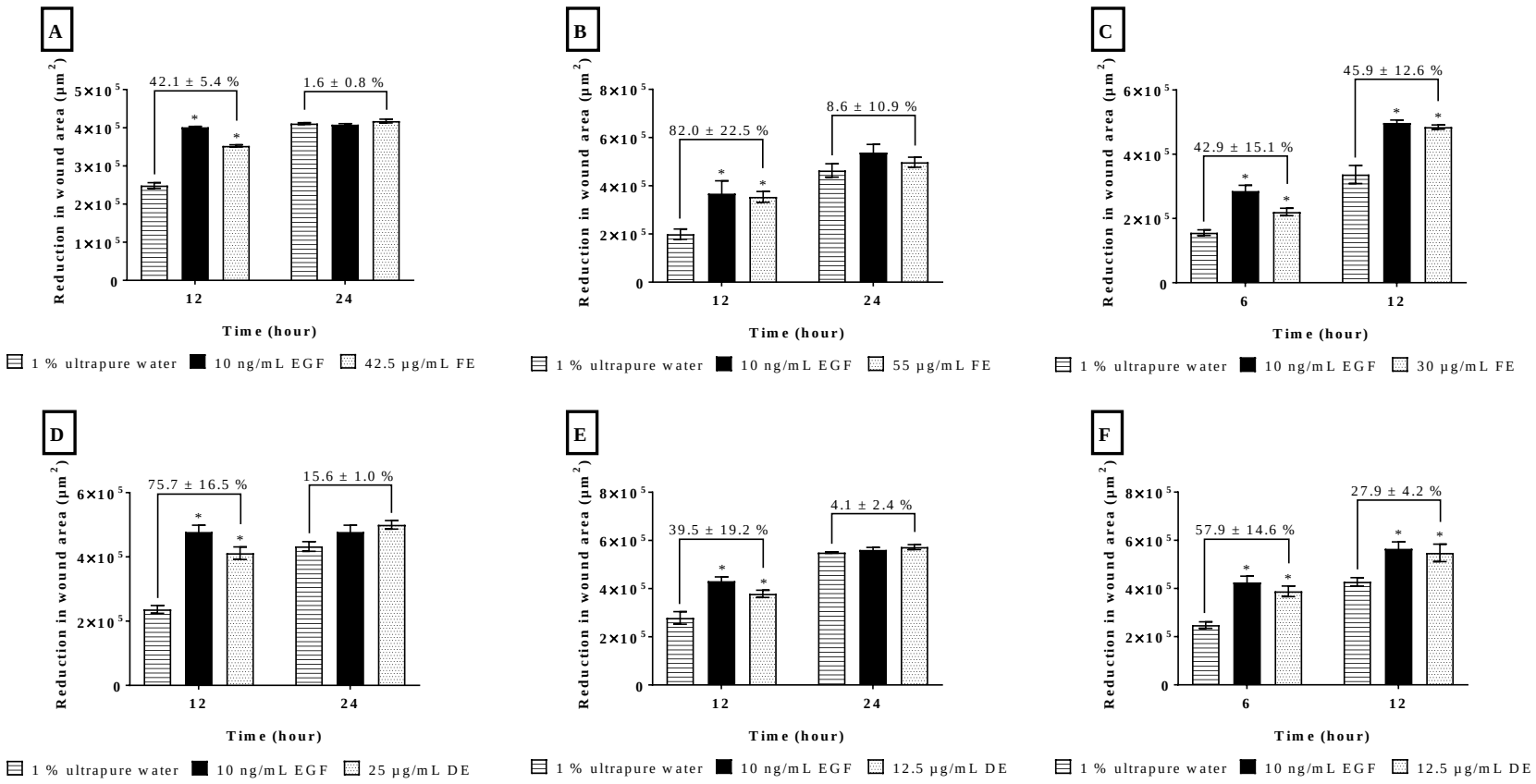

Figure 8: Reduction in wound areas measured at different time points for BEAS-2B (A and D), HaCaT (B and E), and NIH/3T3 (C and F) cell lines, following treatments with *E. coccinea* young shoots fresh water extract (FE) (A, B, and C), and dried water extract (DE) (D, E, and F). One % ultrapure water and epidermal growth factor (EGF) were used as negative and positive controls, respectively. Assays were performed with three independent replicates and error bars are reported as mean ± standard error. Asterisks indicate significant difference between treatment and negative control at $\alpha = 0.05$ (using independent sample *t*-test). Values above bars represent the percentage improvement of reduction in wound areas associated with *E. coccinea* extracts as compared to their respective negative controls.

In Figure 8, treatment with 10 ng/mL EGF significantly enhanced wound closure at t = 6 hours for NIH/3T3 cell, and at t = 12 hours for BEAS-2B and HaCaT cells (see graphs A to F). This proved the effectiveness of EGF as a promoter of cell migration. EGF is known to stimulate cell migration by binding to the EGF receptor (EGFR), which subsequently triggers several mechanisms linked to cell motility such as PLCγ-dependent pathway, mitogen-activated protein (MAP) kinases, and calcium-dependent proteases (calpains) (Jorissen et al., 2003; Peplow and Chatterjee, 2013; Wells, 1999). Treatment with FE and DE on BEAS-2B, HaCaT, and NIH/3T3 cells also resulted in increased reduction in wound areas as compared to their respective negative controls, with percentage improvements determined ranging from 1.6 ± 0.8 to 82.0 ± 22.5 %.

It is noteworthy that the water extracts could enhance proliferation and migration of the three cell lines used. This is because different types of cells play different roles in the wound healing process. The rapid migration and proliferation of fibroblasts during the early phase of wound healing are essential as these cells deposit new extracellular matrices needed to repair the tissue injury (Diegelmann and Evans, 2004). On the other hand, epithelial cells are known to migrate from the wound edges within a few hours of wounding, and subsequently proliferate over the provisional matrix below them, hence closing the wound (Velnar et al., 2009). The migration and proliferation of keratinocytes are also important as they are responsible for stimulating fibroblasts to synthesize growth factors, as well as cause differentiation of fibroblasts into myofibroblasts (Werner et al., 2007). Myofibroblasts are cells that contract to bring the margins of open wounds together during the later stages in wound healing (Grinnell, 1994). The positive effects shown by *E. coccinea*'s water extracts on different cell types suggest that the overall wound healing process could be hastened.

Apart from providing evidence for its traditional use, it was also of interest to compare the wound healing ability of *E. coccinea* with those of other plants (see Table 7). Only DE was used for comparison due to the similarity in extraction methods: the extracts obtained from other studies were also dried, hence their concentrations were expressed in µg extract/mL (as opposed to FE in which its concentrations were expressed in µg plant material/mL).

Table 7: The effects of *E. coccinea* young shoot dried water extract (DE) on the cell proliferation and migration of BEAS-2B, HaCaT, and NIH/3T3 cell lines in comparison with the reported wound healing effects of several other plant species.

Plant species	Effective concentration (µg/mL)	Cell line used	Reported wound healing effect	Reference
Etlingera coccinea	25	BEAS-2B epithelial	158.7 ± 3.3 % (cell proliferation) 75.7 ± 16.5 % (cell migration)	This study
	12.5	HaCaT keratinocyte	140.8 ± 2.0 % (cell proliferation) 39.5 ± 19.2 % (cell migration)	
	12.5	NIH/3T3 fibroblast	136.5 ± 2.1 % (cell proliferation) 57.9 ± 14.6 % (increased cell migration)	
Aloe vera	25	L929 fibroblast	137.15 % (cell proliferation) 19.7 ± 2.63 % (increased cell migration)	(Manoj et al., 2009)
Bridelia ferruginea	5	FS5 fibroblast	133.3 ± 9 % (cell proliferation)	(Adetutu et al., 2011)
Calendula officinalis	10	Swiss 3T3 fibroblast	70.53 ± 2.64 % (cell migration)	(Fronza et al., 2009)
Parkia biglobosa	30	FS5 fibroblast	131.3 ± 12 % (cell proliferation)	(Adetutu et al., 2011)

The enhancement of cell proliferation shown by *E. coccinea* was comparable to other plant species that are known for their wound healing effects, as displayed in Table 7. For example, the cell viabilities of fibroblast cells treated with *A. vera*, *Bridelia ferruginea* and *Parkia biglobosa* extracts

range from 131 to 137 %, while *E. coccinea*'s extract improved NIH/3T3 fibroblast proliferation with cell viability determined at 136.5 ± 2.1 %. In terms of cell migration, *E. coccinea* also showed comparable activity with *C. officinalis* and *A. vera* (57.9 ± 14.6 % against 70.53 ± 2.64 and 19.7 ± 2.63 %, respectively). The fact that *E. coccinea* showed comparable enhancement of cell proliferation and migration activities further suggests its effectiveness as a wound healing treatment. In vivo wound healing studies should be conducted to provide more evidence on its efficacy.

4.5.2 Major phytochemicals present in *E. coccinea* young shoot water extract

The water extracts of *E. coccinea*'s young shoots were shown to enhance cell proliferation and migration, thus suggesting that they possess wound healing ability. Thereafter, it was of interest to determine the major compounds present. Identification work was done on DE due it being in powder form, hence enabled accurate preparation of the required concentrations for HPLC and LCMS analyses. Also, the absence of water could reduce the risks of the bioactive compounds being degraded over time during storage. The contents of DE were first analyzed via analytical HPLC (see section 2.11.4.1) and the chromatogram obtained is presented in Figure 9.

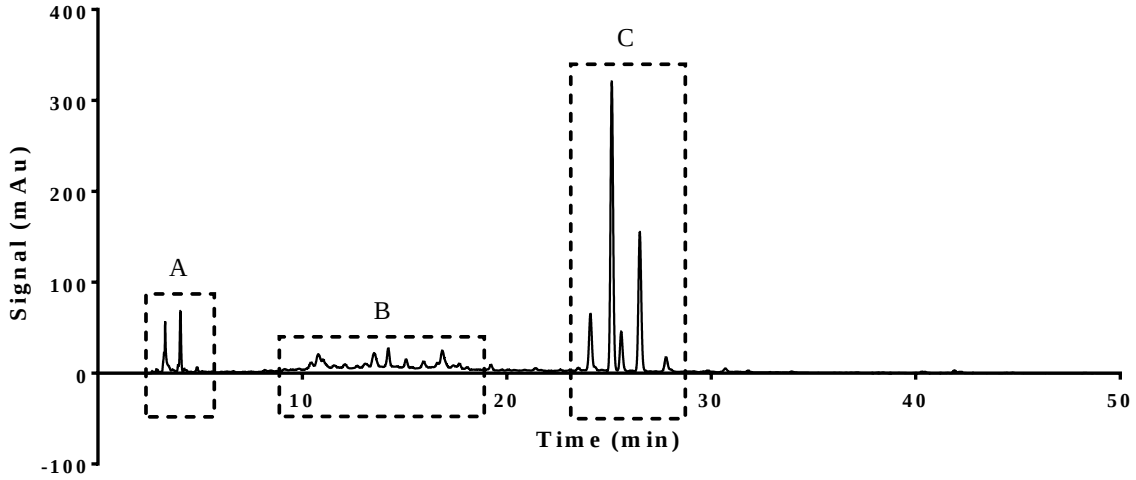

Figure 9: High performance liquid chromatography (HPLC) chromatogram of *E. coccinea* young shoots dried water extract (DE) showing peaks detected at 280 nm. Three major regions of compounds were observed as indicated by boxes A, B, and C.

As displayed in Figure 9, the major peaks found in DE can be grouped into three regions (A, B, and C). Although only two peaks were observed in region A, this region could contain many highly polar compounds that do not have strong interactions with the hydrophobic C18 column, thus elute simultaneously. In region B, the peaks appear unresolved at t_R = between 8 to 18 min. As for region C, five major peaks were observed at t_R = 24.09, 25.13, 25.60, 26.50, and 27.79 min. The peaks in these three regions were subjected to LCMS and MS^2 analyses (see section 2.11.4.2) to determine the major compounds' accurate masses as well as their fragmentation patterns. Table 8 lists the compounds' accurate masses, ion fragments, and their identities based on database search.

Table 8: Identities of the major compounds present in *E. coccinea* young shoots water extract as determined via liquid chromatography mass spectrometry (LCMS) and tandem mass spectrometry (MS^2).

Group	t_R (min)	Accurate mass of [M] (m/z)	MS^2 ion fragment mass (m/z)	Compound identification
A	2-5	612.1532	128.0359, 160.0098, 254.0819, 272.0911, 304.0596, 306.0779, 307.0845, 338.0499, 611.1459, 612.1513	Glutathione (oxidized) [M] 612.1520 m/z
		196.0597	57.0346, 59.0146, 61.9867, 68.9968, 71.0154, 75.0086, 83.0108, 87.0068, 99.0090, 129.0173	Gluconic acid [M] 196.0583 m/z
		134.0227	115.0046, 116.0081, 133.0155, 134.0184, 135.0285	Malic acid [M] 134.0215 m/z
		192.0284	101.0266, 111.0108, 111.1028, 112.0143, 129.021, 131.0014, 154.9993, 173.0111, 191.0220, 192.0253	Citric acid [M] 192.0270 m/z
		174.0164	59.0134, 60.9963, 67.0191, 68.9993, 85.0307, 88.9884, 107.3141, 111.0091, 130.0839	Aconitic acid [M] 174.0164 m/z
B	8-18	578.1439	125.0243, 161.0260, 287.0572, 289.0733, 290.0768, 407.0791, 408.0820, 425.0891, 451.1053, 577.1384	Procyanidin B2 [M] 578.1425 m/z
		866.2064	287.0565, 425.0877, 575.1201, 577.1352, 578.1380, 695.1409, 713.1502, 865.2000, 866.2030, 867.2085	Procyanidin C1 [M] 866.2058 m/z
C	24.09	310.0704	115.0033, 117.0341, 134.0374, 135.0415, 149.0604, 178.0267, 193.0512, 194.0543, 195.0554	Unidentified
	25.13	620.1402	115.0034, 133.0143, 193.0509, 194.0537, 287.0540, 309.0607, 331.0444, 332.0482, 409.0910, 525.1041	Unidentified
	25.60	623.1281/ 624.1352	121.0268, 151.0014, 178.9981, 300.0283, 301.0354, 302.0390, 303.0440, 321.0830, 623.1258, 624.1293, 625.1323	Unidentified
	26.50	478.0688	113.0234, 151.0035, 178.9984, 301.0353, 301.1829, 302.0375, 303.0391, 477.0654, 478.0728	Quercetin-3-glucuronide [M] 478.0750 m/z
		608.1400	113.0239, 163.0031, 229.0507, 285.0413, 286.0445, 287.0460, 321.0835, 322.0863, 607.1316, 608.1346, 609.1379	Unidentified
	27.79	610.1531	Undetected	Unidentified

Identification of major phytochemicals was achieved by comparing the fragmentation patterns observed with known compounds from the following mass spectrometry databases: METLIN (http://metlin.scripps.edu), MassBank (http://www.massbank.jp) and ReSpect (http://spectra.psc.riken.jp/).

The major phytochemicals that can be found in region A are organic acids: gluconic, malic, citric, and aconitic acids. These compounds are commonly found in plants and they play important roles in metabolism (Bennet-Clark, 1933; Vickery and Pucher, 1940). Apart from organic acids, glutathione was also suggested to be present, though in oxidized form, which could be due to oxidation by air molecules. Glutathione is an essential component to many organisms including bacteria, plants, and animals, as it functions in oxidative stress response mechanisms (Frova, 2003; Lushchak, 2011).

Although region B appeared unresolved, only two major compounds with the masses 578.1439 and 866.2064 m/z were found throughout the region. These compounds were then identified as procyanidin B2 and procyanidin C1, respectively, as suggested by their matching fragmentation patterns with those reported in databases. In region C, each of the five peaks corresponded to one parent compound, except for the peak at t_R = 26.50 min, which contains two parent compounds. Their corresponding accurate masses and MS^2 ion fragments detected are listed in Table 8. In this region, quercetin-3-glucuronide was suggested to be present along with four other compounds with masses 310.0704, 620.1402, 624.1352, and 608.1400 m/z. The identities of the latter four were unable to be determined as no matching fragmentation patterns were found on the databases. However, the compounds with masses 310.0704, 624.1352, and 608.1400 m/z might consist of ferulic acid ([M] 194.0579 m/z), quercetin ([M] 302.0427 m/z), and fisetin ([M] 286.0477 m/z) moieties, respectively, due to the matching fragmentation patterns of these moieties with those reported in databases. Analysis via NMR experiments is needed to determine the identities of the compounds. The MS^2 ion fragments for the compound with mass 610.1531 m/z were unidentified

and this could be due to its low abundance, or its inability to be fragmentized. The chemical structures of the major compounds and possible moieties are displayed in Figure 10.

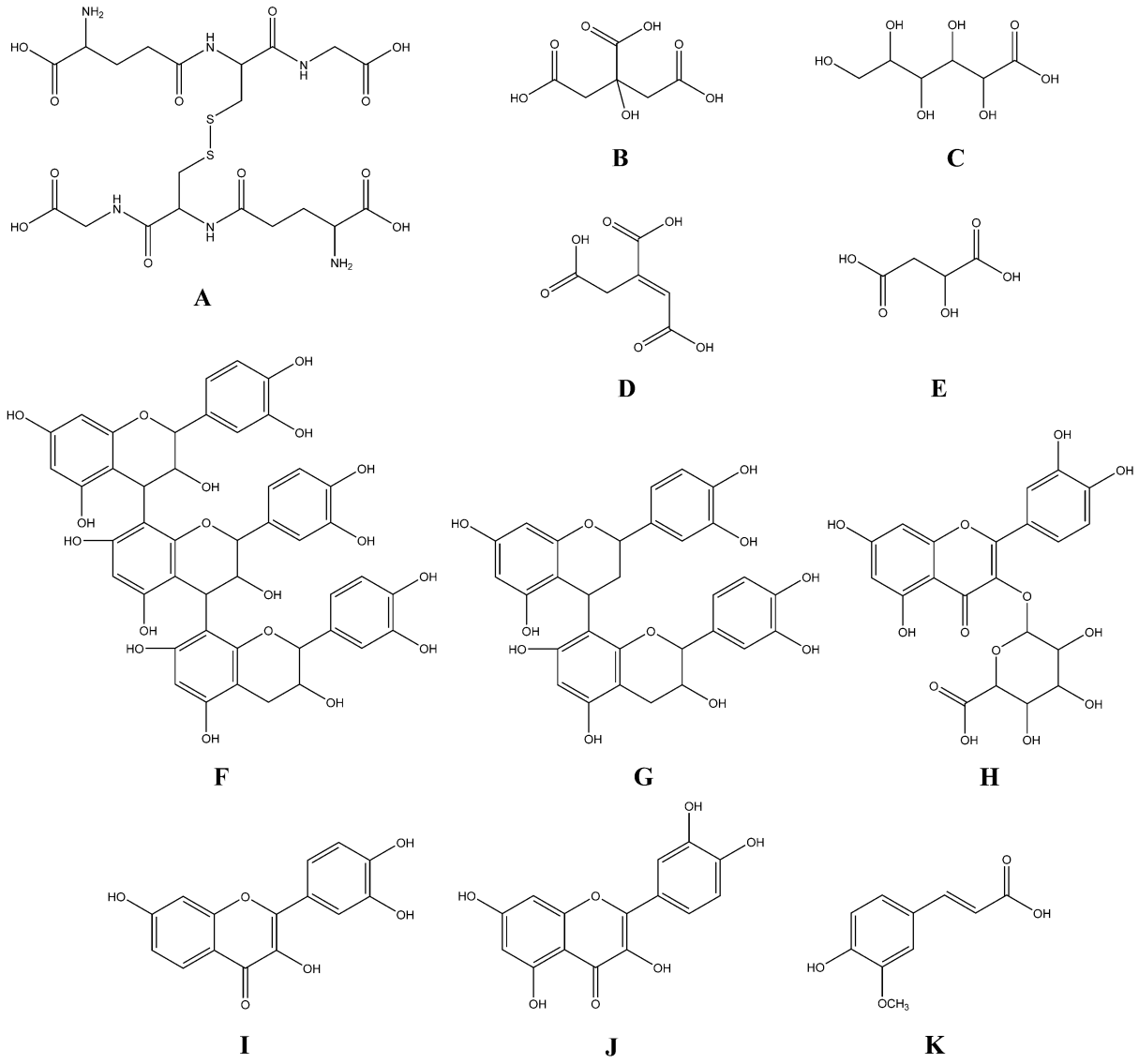

Figure 10: The chemical structures of compounds identified from *E. coccinea* young shoots water extract as suggested by mass spectrometry analyses: glutathione (oxidized) (A), citric acid (B), gluconic acid (C), aconitic acid (D), malic acid (E), procyanidin C1 (F), procyanidin B2 (G), and quercetin-3-glucuronide (H). Several other unidentified compounds were also present which possibly possess the following moieties: fisetin (I), quercetin (J), and ferulic acid (K).

Procyanidin B2 and procyanidin C1 (see Figure 10 for their chemical structures) are classified as tannins, which are a diverse group of high molecular weight phenolic compounds (ranging from 500 to more than 3000 Da) (Hassanpour et al., 2011; Khanbabaee and van Ree, 2001). Tannins play a role in a plant's defense mechanism against mammalian and insect herbivores because: 1) they have a bitter taste, thus acting as feeding deterrents, and 2) they possess protein and metal ion-binding abilities which can reduce their nutritive values when ingested (Robbins et al., 1987; War et al., 2012). Although tannins can be harmful when consumed, past research had shown that they also have beneficial effects such as anticarcinogen, antimutagenic, antibacterial, and antiviral activities (Chung et al., 1998; Yang et al., 2013). Additionally, wound healing has also been attributed to tannins and tannin-rich plant extracts (Agyare et al., 2009; Bueno et al., 2014; Deters et al., 2001; Hupkens et al., 1995; Li et al., 2011; Lopes et al., 2005; Su et al., 2017).

The wound healing mechanisms of tannins are still not well understood, however, several theories have been proposed. Firstly, tannins can promote capillary vasoconstriction, causing a local anti-inflammatory effect. This reduces the build-up of inflammatory exudates, and thus prevent growth of microorganisms at the wound site (Edwin et al., 2008; Lopes et al., 2005). This anti-inflammatory effect is also important as prolonged inflammation can lead to increased levels of proteases, which are enzymes that can degrade ECM and growth factors (Edwards and Harding, 2004; Guo and DiPietro, 2010; Menke et al., 2007). Some studies suggest that tannins might improve wound healing by preventing the build-up of excessive edema that can retard cicatrization (scar tissue formation process) (Lopes et al., 2005; Sanchez Neto et al., 1993). Procyanidins in particular, have been associated with upregulation of vascular endothelial growth factor (VEGF) in keratinocytes (Khanna et al., 2001). VEGF is known for its role in angiogenesis (generation of

new blood vessels) (Bernatchez et al., 2002). This process is important as the new vasculature delivers essential nutrients as well as remove wastes from the developing tissues (Pettet et al., 1996).

Another mechanism is related to the good antioxidant activities possessed by tannins and other phenolic compounds, such as those suggested to be present in this study (quercetin-3-glucuronide as well as other compounds with ferulic acid, quercetin, and fisetin moieties). As previously mentioned, ROS are secreted by cells such as macrophages during the wound healing process, and high amounts of these free radicals can damage cellular macromolecules (it is important to note that the ROS being referred to are extracellular). When this occurs, tissues that are otherwise healthy could be damaged, consequently resulting in impaired wound healing. Tannins and other phenolic compounds might prevent this from occurring by acting as antioxidants to lower ROS levels at wound site, hence preventing cellular damage (Süntar et al., 2012).

As opposed to being antioxidants, tannins and other phenolic compounds can also exhibit pro-oxidant activities (generation of ROS molecules) at particular concentrations (Fukumoto and Mazza, 2000; Khan et al., 2000; Shao et al., 2003). Past studies have linked optimum intracellular ROS levels with improvement in wound healing due to their ability to promote cell adhesion, proliferation, and migration (Huo et al., 2009; Pan et al., 2011). The tannins and other phenolic compounds present in the water extracts of *E. coccinea* might have caused a mild increase in intracellular ROS, thereby enhancing cell proliferation and migration. Although several theories have been discussed, further studies are required to explore the wound healing mechanism by *E. coccinea*.

The basis that tannins and other phenolic compounds possess pro-oxidant properties could also explain the dose-dependent effects observed in Figure 6, whereby cells treated with the extracts showed decreasing viabilities at higher concentrations. As previously discussed, high levels of ROS can be detrimental to cells. Thus, high concentrations of the extracts might have resulted in high ROS levels, which led to decreased cell viability. Curcumin is a well-known example of a phenolic compound that can kill cells via generation of ROS, at high concentrations (Lee et al., 2016).

4.5.3 Antimicrobial and antioxidant activities of *E. coccinea* water extracts

Apart from having the ability to enhance cell proliferation and migration, it is also important for a wound healing agent to possess antimicrobial and antioxidant properties. This is because bacterial infection and oxidative stress can negatively affect the wound healing mechanism. Thus, the bacterial inhibition and free radical scavenging activities of the water extract of *E. coccinea*'s young shoots were investigated. The tannin-rich and non-tannin phenolic fractions were also obtained and evaluated for the aforementioned properties, since these were the major phytochemicals found in the crude extract (see section 4.5.2).

4.5.3.1 Fractionation of the crude water extract of *E. coccinea*

The crude water extract of *E. coccinea* (DE) was first separated into polar (F1) and less-polar (F2) fractions via C18 preparative HPLC, as outlined in section 2.11.4.3. The latter was expected to contain tannins and other phenolic compounds, and thus, its TPC value was determined and compared to the crude extract and fraction F1. A summary of the TPC values obtained is presented in Table 9.

Table 9: Total phenolic content (TPC) determined for *E. coccinea* crude extract, as well as fractions F1 and F2.

Extract	TPC (mg GAE/ g extract)
Crude water extract	157 ± 1b
Fraction F1	25.4 ± 0.8c
Fraction F2	693 ± 19a

The assay was performed with three replicates, and the values are presented as mean ± standard error. Different letters indicate significant difference at $\alpha = 0.05$ (using ANOVA with Tukey's post-hoc test). GAE = gallic acid equivalent.

As shown in Table 9, fraction F2 exhibited higher TPC value as compared to fraction F1 (693 ± 19 and 25.4 ± 0.8 mg GAE/ g dried extract, respectively), hence suggesting that most of the phenolic compounds were retained in fraction F2. On the other hand, fraction F1 should possess mainly organic acids, as previously postulated (see section 4.5.2). The phenolic-rich fraction (F2) was then subjected to Sephadex LH-20 chromatography (see section 2.11.4.3), which yielded non-tannin phenolics (F2A) and tannin-rich (F2B) fractions. HPLC analysis was also conducted to compare the contents of these fractions, and the chromatograms obtained are shown in Figure 11.

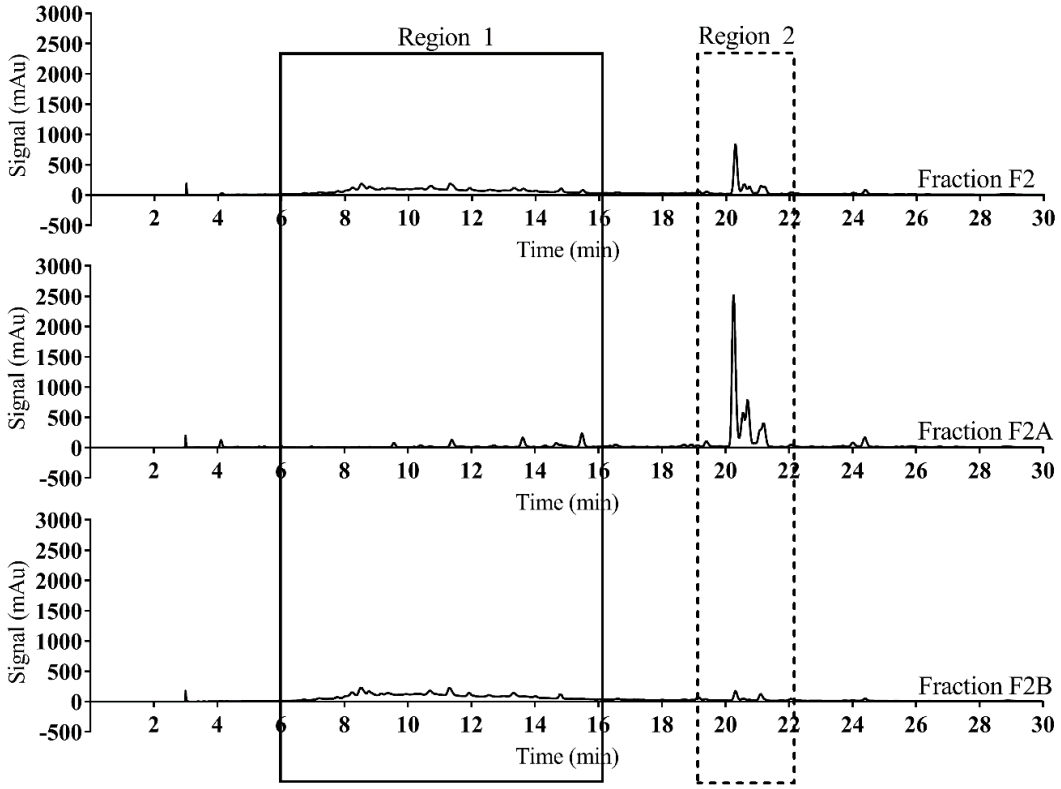

Figure 11: The high-performance liquid chromatography (HPLC) chromatograms obtained for fractions F2 (top), F2A (middle), and F2B (bottom). Region 1 and region 2 indicate the ranges which contain the tannins (procyanidin B2 and C1) and other phenolic compounds, respectively, as suggested in section 4.5.2.

HPLC analysis on fraction F2 (see Figure 11) produced a similar chromatogram as shown in Figure 8 (see section 4.5.2). Therefore, region 1 should contain the tannins while the non-tannin phenolics should be present in region 2. Following Sephadex LH-20 chromatography, it can be observed that the components from region 1 and region 2 were mainly separated into fractions F2B and F2A, respectively. This means that fractions F2A and F2B primarily contained the non-tannin phenolics and tannins, respectively. To further support this, quantification of tannins was carried out via radial diffusion assay. This assay relies on the principle that tannins can interact with proteins,

hence becoming a reliable method to detect their presence (Hagerman, 1987). Table 10 lists the tannin contents of fractions F2, F2A, and F2B.

Table 10: Tannin content of *E. coccinea* fractions, as determined through the radial diffusion assay.

Extract	Tannin content (mg TAE/ g extract)
Crude extract	88.2 ± 25.5a
Fraction F1	0a
Fraction F2	341 ± 39b
Fraction F2A	0a
Fraction F2B	641 ± 48c

Tannin content was determined with three replicates and the error was estimated based on the error in radius measurement. TAE = tannic acid equivalent.

The results in Table 10 confirmed that the tannins were present in fraction F2. Furthermore, when compared between fractions F2A and F2B, only the latter was found to possess tannins, while the former did not contain any. In summary, current results suggest that fractions F1, F2, F2A and F2B, mainly consist of organic acids, phenolics, non-tannin phenolics, and tannins, respectively. These fractions, as well as the crude extract, were assessed for their antimicrobial and antioxidant activities.

4.5.3.2 Antimicrobial activity of *E. coccinea* water extracts

The antimicrobial activities of *E. coccinea*'s crude extract, as well as fractions F1, F2, F2A, and F2B, were determined through broth microdilution assay (as described in section 2.7) against six bacterial strains. Table 12 lists the MIC values determined.

Table 11: Minimum inhibitory concentration (MIC) values of *Etlingera coccinea* extracts against several gram-positive and gram-negative bacteria strains.

Extract	MIC (mg/mL)					
	S. aureus ATCC 43300	*S. aureus* ATCC 6538P	*B. cereus* ATCC 14579	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 10145	*K. pneumoniae* ATCC 10031
Crude water extract	1	1	1	>1	>1	>1
Fraction F1	>1	>1	>1	>1	>1	>1
Fraction F2	0.25	0.25	0.125	>1	>1	>1
Fraction F2A	>1	>1	>1	>1	>1	>1
Fraction F2B	0.125	0.125	0.063	>1	>1	>1

The MIC values were determined with three biological replicates.

As shown in Table 11, the crude extract inhibited the gram-positive bacteria (*S. aureus* ATCC 43300, *S. aureus* ATCC 6538P, and *B. cereus* ATCC 14579), thus proving that it has antimicrobial property. When compared between the organic acid-rich (F1) and phenolic-rich (F2) fractions, only the latter displayed positive inhibition activity. In addition, strong activity was observed for the tannin-rich fraction (F2B), which exhibited MIC values determined as low as 0.063 mg/mL. Conversely, the non-tannin phenolics fraction (F2A) did not inhibit any of the bacterial strains used.

The trend shown in Table 11 implies that the tannins were mostly responsible for *E. coccinea*'s antimicrobial property. This group of phytochemicals are known to inhibit bacterial growth via several mechanisms. Firstly, tannins can inhibit microbial enzymes due to their astringent character (Scalbert, 1991). Particularly, procyanidins have been associated with high protein binding efficiency, and this might explain the antimicrobial activity of fraction F2B, which contains procyanidin B2 and C1 (Scalbert, 1991). Apart from this, tannins can cause a reduction in the availability of essential metal ions (such as iron and zinc), hence affect the activities of metalloenzymes (Daglia, 2012; Scalbert, 1991). This is due to their multi-catecholate nature, which contributes to their metal ion-binding abilities (Kennedy and Powell, 1985; Okuda et al., 1982). Moreover, destabilization and permeabilization of the bacterial membrane were also believed to play a role in tannins' antimicrobial mechanism (Daglia, 2012; Scalbert, 1991; Smith et al., 2005).

Although antimicrobial activity was observed against the gram-positive bacteria, the gram-negative bacteria were unaffected. Similar to the present work, a study by Jones et al. (1994) reported that the tannins from *Onobrychis viciifolia* could inhibit the growth of *Butyrivibrio fibriosolvens* A38 and *Streptococcus bovis* 45S1 (gram-positive bacteria), but had little effect on *Prevotella ruminicola* B14 and *Ruminobacter amylophilus* WP225 (gram-negative bacteria). The polyphenol structure is known to have a weak interaction with LPS (as opposed to strong interaction with peptidoglycan); hence LPS can act as a barrier to polyphenols, thus limiting their antimicrobial action (Yoda et al., 2004). Apart from this, some bacteria can overcome tannin inhibition through overproduction of enzymes, or, by producing tannin-resistant enzymes (Smith et al., 2005). As an example, Bae et al. (1993) reported that when *Fibrobacter succinogenes* (a

gram-negative bacteria) was treated with condensed tannins, an increase in cell-associated endoglucanase activity (enzyme that helps in breakdown of cellulose materials) was observed (Bae et al., 1993; Sun and Cheng, 2002).

Although no antimicrobial activity was found against the gram-negative bacteria, *E. coccinea*'s extracts were able to inhibit gram-positive bacterial strains associated with wounds (*S. aureus* and *B. cereus*). This highlights its potential antimicrobial contribution in wound healing.

4.5.3.3 Antioxidant activity of *E. coccinea* water extracts

Apart from having antimicrobial property, a good wound healing agent should also possess the ability to scavenge excessive ROS at the site of wound. Thus, the crude extract of *E. coccinea*, as well its fractions (F1, F2, F2A, and F2B), were evaluated for their antioxidant activities. Particularly, their TPC, DPPH FRS IC_{50}, and ORAC EC_{50} values were determined. The protocols employed for these assays are described in sections 2.6.1, 2.6.2, and 2.6.3. The calibration equation determined for the quantification of TPC is as follow: $y = 0.0067x \pm 0.0182$ ($R^2 = 0.9998$). Table 12 summarizes the results of these assays.

Table 12: Total phenolic content (TPC), DPPH free radical scavenging (FRS) IC_{50}, and oxygen radical absorbance capacity (ORAC) values determined for *Etlingera coccinea* extracts.

Extract / compound	TPC (mg GAE/ g extract)	DPPH FRS IC_{50} (µg/mL)	ORAC EC_{50} (µg/mL)
Crude water extract	1317 ± 10d	18.66 ± 0.27b	2.899 ± 0.068c
Fraction F1	97.36 ± 0.21e	>333.3d	8.025 ± 0.199e
Fraction F2	4939 ± 55b	4.819 ± 0.078a	0.1130 ± 0.0019a
Fraction F2A	1482 ± 9c	44.55 ± 0.22c	0.1938 ± 0.0298a
Fraction F2B	5528 ± 20a	4.171 ± 0.036a	0.1961 ± 0.0925a
Ascorbic acid	ND	4.499 ± 0.009a	3.322 ± 0.040d
Trolox	ND	ND	2.066 ± 0.027b

Assays performed included three replicates and values are reported as mean ± standard error. Different letters indicate significant difference between mean values within the same assay, at $\alpha = 0.05$ (using ANOVA with Tukey's post-hoc test). GAE = gallic acid equivalent; ND = not determined.

In Table 12, the crude extract shows higher DPPH FRS IC_{50} value than ascorbic acid (18.66 ± 0.27 against 4.499 ± 0.009 µg/mL, respectively). However, its ORAC EC_{50} value was comparable to ascorbic acid and Trolox (2.899 ± 0.068, 3.322 ± 0.040, and 2.066 ± 0.027 µg/mL, respectively). This suggests that although in crude form, *E. coccinea*'s extract possesses free radical scavenging activities that can rival pure compounds.

The TPC values determined for fractions F1 and F2 (see Table 12) supported the results presented in section 4.5.3.1, whereby fraction F2 contains most of the phenolic compounds. Additionally, when compared for their DPPH FRS and ORAC, fraction F2 showed much lower IC_{50} and EC_{50}

values (4.819 ± 0.078 and 0.1130 ± 0.0019 µg/mL, respectively) as compared to fraction F1 (>333.3 and 8.025 ± 0.199 µg/mL, respectively). Therefore, it can be inferred that the phenolic compounds were mainly responsible for *E. coccinea*'s antioxidant activity.

Comparison between fractions F2B and F2A revealed that the former displayed a higher TPC value as compared to the latter (5528 ± 20 and 1482 ± 9 mg GAE/ g sample, respectively). Moreover, fraction F2B also exhibited significantly lower DPPH FRS IC_{50} than fraction F2A (4.171 ± 0.036 and 44.55 ± 0.22 µg/mL, respectively). These results suggest that the tannins play a significant role in the antioxidant activity of *E. coccinea*'s water extract. However, there were no significant differences in the ORAC EC_{50} values determined for fractions F2B and F2A (0.1961 ± 0.0925 and 0.1938 ± 0.0298 µg/mL, respectively). The lack of correlation could be due to the following: 1) the DPPH FRS and ORAC assays evaluate antioxidant activities via different mechanisms, and 2) the intrinsic properties of a compound can affect its antioxidant mechanism (Tan and Lim, 2015). Nevertheless, though still in partially purified forms, the antioxidant activities of the tannins and non-tannin phenolics present in *E. coccinea* were comparable to ascorbic acid and Trolox (see Table 12), which are well-known pure antioxidant compounds.

Apart from in vitro chemical-based assays, the cell-based antioxidant assay was also performed. This assay is biologically-relevant as it evaluates antioxidant responses at a cellular level (López-Alarcón and Denicola, 2013; Wolfe and Liu, 2007). The HaCaT (skin keratinocyte) cell line was used due to its relevance to the traditional use of *E. coccinea* in treating wounds (by topical application). Also, since the cell proliferation and migration studies were conducted by utilizing the same cell line (see section 4.5.1), the results presented here could be implicated with the

aforementioned properties of *E. coccinea* extracts. The concentrations chosen for evaluation ranged from 6.25 to 50 µg/mL. This range includes the optimum concentrations that enhanced cell proliferation and migration, as revealed in section 4.5.1. Figure 12 shows the CAA of the extracts in comparison to quercetin, which acts as a control.

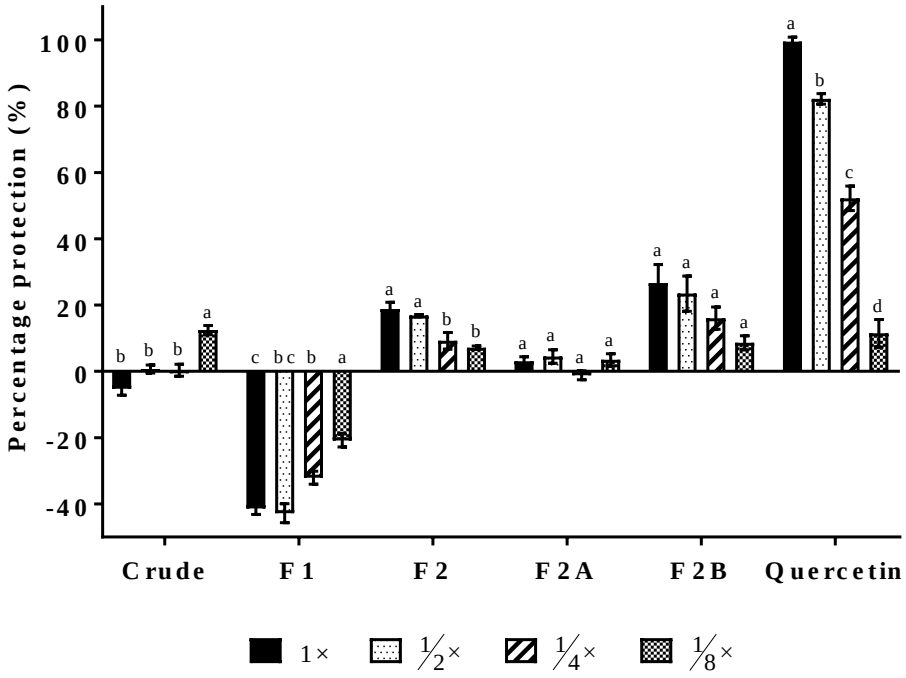

Figure 12: Cellular antioxidant activity (CAA) of *E. coccinea* crude extract and fractions, as indicated by the percentage protection (%) of DCFH. Quercetin was used as a control. Cell-based antioxidant assay was performed with three independent replicates, and error bars indicate mean ± standard error. The legend represents the concentration of the extracts/ compound: highest concentration (1×) tested for *E. coccinea*'s extracts (crude, F1, F2, F2A, and F2B) as well as quercetin were 50 and 20 µg/mL, respectively. Different alphabets shown above bars indicate significant differences between the mean percentage protection values for the same extract/ compound, at $\alpha = 0.05$ (using ANOVA with Tukey's post-hoc test).

As shown in Figure 12, the crude extract of *E. coccinea* protected the DCFH from free radical damage at a lower concentration (12.44 ± 1.37 % at 6.25 µg/mL). However, a slight negative percentage was observed at a high concentration (−5.221 ± 1.958 % at 50 µg/mL), indicating pro-oxidant activity. The compounds with this property could have arisen from fraction F1, which also exhibited negative percentage values. This fraction was suggested to contain organic acids, and previous studies have reported that organic acids such as ascorbic acid and citric acid can act as pro-oxidants in the presence of redox-active metal ions, such as Fe^{3+} and Cu^{2+} (Foti and Amorati, 2009; Mahoney and Graf, 1986). Cells require these transition metals for growth, and culture media such as DMEM usually contains added iron(III) nitrate (Halliwell, 2003; Wee et al., 2003). Halliwell (2003) also mentioned that unless specially prepared, all laboratory solutions and cell culture media are contaminated with transition metals. Therefore, the presence of these metal ions could catalyze free radical reactions due to the pro-oxidant property of the organic acids.

As for fraction F2, it was able to protect the DCFH from free radical damage with percentage protection determined as high as 15.69 ± 1.67 %. When compared between fractions F2A and F2B, the latter showed significantly higher percentage protection values (as high as 26.67 ± 5.56 % at 50 µg/mL), hence implying that the tannins largely contribute to *E. coccinea*'s CAA.

The CAA values exhibited by *E. coccinea*'s extracts were found to be much lower than quercetin, although the latter was tested at lower concentrations (20 µg/mL and below). However, *E. coccinea*'s crude extract and fractions (F2, F2A, and F2B) showed good free radical scavenging activities, as evidenced by their low DPPH FRS IC_{50} and ORAC EC_{50} values, while also being comparable to ascorbic acid and Trolox (see Table 13). The lack of agreement between the results

from these assays could be because the cell-based antioxidant assay accounts for essential aspects such as cellular uptake (or a compound's ability to penetrate the lipid bilayers), distribution, and metabolism of compounds (Saija et al., 1995; Wolfe and Liu, 2008). As an example, Wolfe and Liu (2008) reported that several polyphenols (such as rutin, apigenin, and catechin) possessed high ORAC values (13.7 ± 1.7, 10.7 ± 1.5, and 12.4 ± 4.0 µmol Trolox equivalent/ µmol compound, respectively), but showed weak CAAs (no activities were found). The authors postulated that lipophilicity might have a crucial effect on a compound's CAA: compounds with low lipophilicity might weakly interact with the cell membrane, and are thus removed during the washing step. Lipophilicity can be influenced by the degree of hydroxylation of the aromatic rings, whereby high degree of hydroxylation is associated with low lipophilicity (Koleckar et al., 2008; Wolfe and Liu, 2008). Since tannins have a high degree of hydroxylation, their low lipophilicity might limit their ability to pass through the cell membrane.

Although the crude water extract of *E. coccinea* showed weak CAA, its exceptional free radical scavenging activity (as demonstrated by its low DPPH FRS IC_{50} and ORAC EC_{50}) still elicits its potential to aid the wound healing process. For example, during the 'inflammation' phase, ROS are secreted by cells such as macrophages to prevent bacterial growth (Auf Dem Keller et al., 2006). High ROS levels can impair wound healing, however, since these free radicals are secreted outside of the cells, the antioxidant compounds in *E. coccinea* could scavenge the excessive extracellular ROS. It might be of concern, should the ROS levels drop significantly, hence adversely affecting their role in combating the invading bacteria. If this occurs, the extract itself could aid in preventing bacterial growth at the site of wound, since it possesses antimicrobial activity (results are shown in section 4.5.3.2).

Although high ROS levels are detrimental, appropriate intracellular ROS levels are essential in cells during the wound healing process. Thus, it might be advantageous for *E. coccinea* to possess weak CAA. Its inability to effectively scavenge intracellular free radicals could enable the cells to benefit from the effects of ROS such as enhancement of cell proliferation and migration. Additionally, the pro-oxidant compounds present in the extract might help to generate small amounts of intracellular ROS, thus contributing to the beneficial effects as well. Though these mechanisms are only probable, further investigations are needed. The ROS levels could be monitored prior to, and after treatment with the extract. From this, molecular studies can also be conducted to assess the correlation between the ROS levels and expression of genes associated with cell proliferation and migration.

4.6 Summary of Chapter 4

The methanolic extracts of *E. coccinea* were shown to have good antioxidant activities, with 70 % methanol being the best solvent to extract the antioxidant compounds. When compared between the different plant parts, the leaves showed highest TPC, AEAC, and ORAC values. Antimicrobial activity screening also revealed that *E. coccinea*'s leaves (particularly the hexane extract) displayed best bacterial inhibition activity. From the active extract, *trans*-2-dodecenal was purified and identified as the major antimicrobial compound. This compound inhibited the growth of several gram-positive bacteria, with MIC values determined ranging from 4-8 µg/mL. *Trans*-2-dodecenal was considered safe for use, hence has a potential to be developed as an antimicrobial agent. The work done on the antimicrobial property of *E. coccinea* was published in Journal of Applied Microbiology (see Appendix 9). The wound healing property of *E. coccinea* was also explored in the present work. Its water extracts enhanced proliferation and migration of BEAS-

2B, HaCaT, and NIH-3T3 cell lines. Assessment of its major constituents suggested the presence of procyanidin B2, procyanidin C1, as well as other phenolic compounds with ferulic acid, quercetin, and fisetin moieties. These compounds might be responsible for the observed effects. Apart from having the ability to enhance cell proliferation and migration, the extracts also possessed antimicrobial and antioxidant activities, which could be beneficial to the wound healing process. A manuscript which included the work done on the effects of *E. coccinea*'s water extracts on cell proliferation and migration, as well as identification of the major phytochemicals present, has been submitted to the Journal of Ethnopharmacology and is currently under review (see Appendix 10).

CHAPTER 5

ANTIOXIDANT AND ANTIMICROBIAL PROPERTIES

OF *ETLINGERA PUBESCENS*

Chapter 5 – Antioxidant and Antimicrobial Properties of *Etlingera pubescens*

5.1 Antioxidant activity of *E. pubescens*

Oxidative stress plays an important role in the development of degenerative diseases and metabolic syndromes. Plant phenolics are good candidates to be developed as treatment agents due to their superb antioxidant properties. Therefore, the present work focused on the phenolic antioxidants of *E. pubescens*. Its specimens were obtained from Danum Valley (see section 2.2), and the crude extracts were prepared as outlined in section 2.3.1. These extracts were then assessed for their TPC, AEAC, and ORAC, in which the protocols employed are described in sections 2.6.1, 2.6.2, and 2.6.3, respectively. The calibration equation determined for the quantification of TPC is as follow: $y = 0.008x \pm 0.0086$ ($R^2 = 0.9985$). As for the DPPH FRS, the IC_{50} value obtained for ascorbic acid was 3.91 ± 0.17 µg/mL, while the standard equation calculated for Trolox in the ORAC assay was $y = 0.3694\times + 1.3094$ ($R^2 = 0.9906$). Table 13 summarizes the antioxidant activities of *E. pubescens*.

Table 13: The total phenolic content (TPC), ascorbic acid equivalent antioxidant capacity (AEAC), and oxygen radical absorbance capacity (ORAC) values determined for *E. pubescens* samples extracted using various percentages of methanol.

Part of plant	Percentage methanol (%)	TPC (mg GAE/ 100 g fresh weight)	AEAC (mg AAE/ 100 g fresh weight)	ORAC (µmol TE/ g fresh weight)
Leaves	50	254.9 ± 12.6c	376.3 ± 22.1c	ND
	70	428.6 ± 7.8b	504.7 ± 11.1b	ND
	100	1019 ± 15aA	1129 ± 28aA	150.5 ± 26.0A
Stems	50	58.95 ± 0.55b	58.11 ± 1.22b	ND
	70	67.57 ± 1.54b	81.44± 1.66b	ND
	100	108.8 ± 3.8aB	137.3 ± 9.2aB	14.00 ± 2.12B
Rhizomes	50	49.62 ±0.67c	54.76 ± 1.22c	ND
	70	59.34 ± 1.64b	79.37 ±2.36b	ND
	100	91.96 ± 2.39aB	115.2 ±2.87aB	7.993 ± 0.178B

Assays were performed with three replicates and values are reported as mean ± standard error. ND indicates that the experiment was not determined as ORAC values were only determined for the extracts with highest TPC and AEAC values. The significance of the results was assessed using ANOVA at $\alpha = 0.05$ with Tukey's post-hoc test. Small letters compare statistical differences between means of the three different percentages of methanol (50 %, 70 %, and 100 %) for the same part of the plant, while capital letters compare highest mean values between the three plant parts (leaves, stems, and rhizomes). GAE = gallic acid equivalent; AAE = ascorbic acid equivalent; TE = Trolox equivalent; ND = not determined.

In general, the crude extracts obtained using 100 % methanol showed higher TPC and AEAC values, as compared to 70 % and 50 % methanol (see Table 13), hence suggesting that the antioxidant compounds present in *E. pubescens* are relatively non-polar in nature. This was desirable as compounds that are more soluble in organic solvents tend to have higher lipophilicity, thus could pass through the cell membrane and exert their effects from within the cells (Andrés et al., 2015; Wolfe and Liu, 2008). When contrasted between the different plant parts, the leaf extract of *E. pubescens* exhibited significantly better antioxidant activity than the stems and rhizomes, as suggested by the former's high TPC, AEAC, and ORAC values (1019 ± 15 mg GAE/ 100 g fresh weight, 1129 ± 28 mg AAE/ 100 g fresh weight, and 150.5 ± 26.0 µmol TE/ g fresh weight). The high antioxidant properties shown by *E. pubescens* warrant further work to isolate the compounds responsible for the observed effects.

5.2 Antimicrobial activity of *E. pubescens*

Apart from oxidative stress, antimicrobial resistance is also an alarming health issue that necessitates the search for more antimicrobial agents. Hence, *E. pubescens* was investigated for its antimicrobial activity. Its crude extracts were screened using the broth microdilution assay (described in section 2.7) against 17 bacterial strains (listed in section 2.4). Table 14 shows the MIC values determined.

Table 14: Antimicrobial activity of *E. pubescens*' leaf, stem, and rhizome methanol extracts.

Part of plant	Minimum inhibitory concentration (mg/mL)									
	S. aureus ATCC 700699	*S. aureus* ATCC 43300	*S. aureus* ATCC 6538P	*S. aureus* ATCC 29213	*S. epidermidis* ATCC 12228	*B. subtilis* ATCC 8188	*B. cereus* ATCC 14579	*E. faecalis* ATCC 29212	*E. faecalis* ATCC 700802	*S. pneumoniae* ATCC 49136
Leaf	0.125	0.125	0.125	0.125	0.25	0.125	0.125	0.5	0.5	0.25
Stem	0.25	0.25	0.25	0.25	1	0.25	0.25	0.125	0.125	1
Rhizome	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1

Minimum inhibitory concentration (MIC) values were determined with three biological replicates. No inhibition was observed against the gram-negative bacteria (MIC values >1 mg/mL).

When compared between the different plant parts, the leaves of *E. pubescens* exhibited best antimicrobial activity, with MIC values determined ranging from 0.125 to 1 mg/mL. The gram-negative bacteria were unaffected, and this might be due to the permeability-limiting property of their LPS layer, as previously discussed. Though only the gram-positive bacteria were affected, the extracts of *E. pubescens* inhibited several clinically-relevant bacterial strains such as methicillin-resistant *S. aureus* ATCC 43300, thus suggesting its potential to be mined for antimicrobial compounds.

5.3 Isolation and identification of bioactive compound from *E. pubescens*

The leaf methanol extract of *E. pubescens* showed good antioxidant and antimicrobial properties. Therefore, this extract was subjected to compound isolation work (see section 2.11.2). Briefly, the extract was fractionated via SSE to remove unwanted non-polar compounds such as chlorophylls and fatty acids. The resulting ethyl acetate fraction was subsequently introduced into a C18 gravity column and the eluent was collected and dried. This procedure was conducted to remove

compounds that strongly bind to the C18 material. Then, preparative HPLC was performed to purify etlingerin. The following lists the ^{1}H and ^{13}C NMR shifts determined for etlingerin.

1,7-bis(3,4-dihydroxyphenyl)heptan-3-yl acetate (etlingerin): syrup; ^{1}H NMR (CD_3OD, 700 MHz) δ 6.67 (d, *J* = 8.0 Hz, 1H, H-5a), 6.67 (d, *J* = 8.0 Hz, 1H, H-5b), 6.61 (s, broad, 2H, H-2a, H-2b), 6.48 (d, broad, *J* = 8.0 Hz, 2H, H-6a, H-6b), 4.86 (p, *J* = 6.3 Hz, 1H, H-3), 2.50 (m, 2H, H-1), 2.44 (m, 2H, H-7), 2.01 (s, 3H, NCOOC*H*$_3$), 1.78 (m, 2H, H-2), 1.59 (m, 2H, H-4), 1.53 (m, 2H, H-6), 1.31 (m, 2H, H-5); ^{13}C NMR (176 MHz, MeOD) δ 171.56 (N*C*OOCH$_3$), 144.75 (C, C-3a), 144.63 (C, C-3b), 142.93 (C, C-4a), 142.72 (C, C-4b), 133.9672 (C, C-1a), 133.1472 (C, C-1b), 119.2672 (CH, C-6a), 119.1872 (CH, C-6b), 115.1272 (CH, C-2a), 115.0572 (CH, C-2b), 114.9172 (CH, C-5a), 114.8072 (CH, C-5b), 73.8972 (CH, C-3), 35.7772 (CH_2, C-2), 34.5972 (CH_2, C-7), 33.5572 (CH_2, C-4), 31.0972 (CH_2, C-6), 30.7272 (CH_2, C-1), 24.2872 (CH_2, C-5), 19.7172 (CH_3, NCOO*C*H$_3$); LCESIMS m/z 374.1769 (calcd for $C_{21}H_{26}O_6$, 374.1730).

Etlingerin (see Figure 13 for its chemical structure) is a diarylheptanoid that was once isolated from *Amomum muricarpum* (Giang et al., 2012). The spectroscopic data reported by Giang et al. (2012) match with those reported in this study. Although previously isolated, this study is the first to describe its bioactivity. Diarylheptanoids are a small group of compounds containing two aromatic rings joined by a heptane chain. They can be found in various plants, including the ginger plant genera: *Zingiber*, *Curcuma*, *Alpinia*, *Betula*, *Alnus*, and *Etlingera* (Chan et al., 2013; Novaković et al., 2015). These compounds are known to possess good antioxidant properties (Kuroyanagi et al., 2005; Tung et al., 2010).

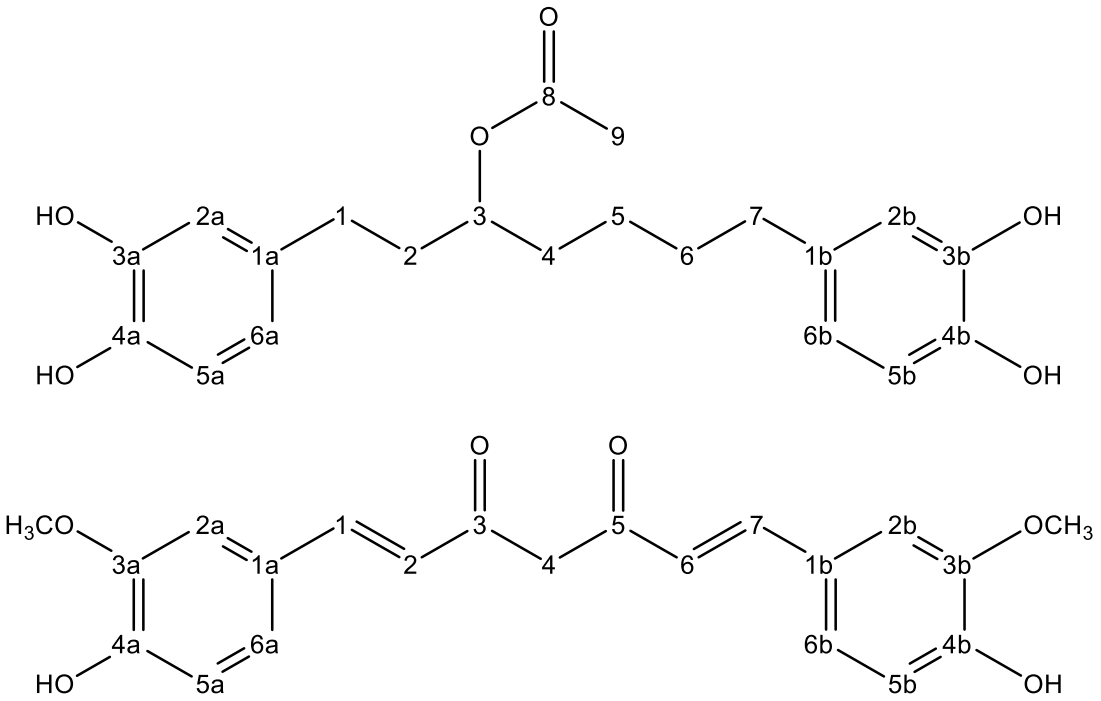

Figure 13: The chemical structures of 1,7-bis(3,4-dihydroxyphenyl)heptan-3-yl acetate (etlingerin, top) and (1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (curcumin, bottom).

Another diarylheptanoid which is well-known is curcumin (see Figure 12 for its chemical structure). It is one of the most extensively studied compound of plant origin due to its dual properties that enable it to exhibit a wide variety of biological effects. For example, it can protect normal cells from oxidative damage at low concentrations (by acting as an antioxidant), while killing cancer cells at high concentrations (by acting as a prooxidant) (Ahmida, 2012). Additionally, curcumin had also been shown to possess antimicrobial activity (Prasad et al., 2014). Therefore, to determine the antioxidant and antimicrobial potentials of etlingerin, all subsequent assays were carried out with curcumin as a reference.

5.4 Antioxidant, antimicrobial, and cytotoxic activities of etlingerin

The bioactivity of etlingerin was assessed in terms of its antioxidant, antimicrobial, as well as cytotoxicity. Curcumin was used as a reference compound to enable comparison of etligerin's potential to be developed as a drug to treat bacterial infections and oxidative stress-related diseases.

5.4.1 Antioxidant activity of etlingerin

The antioxidant activity of etlingerin was first tested via in vitro chemical-based assays (DPPH FRS and ORAC, as described in section 2.6.2 and 2.6.3) to evaluate its ability to scavenge free radicals. Apart from curcumin, ascorbic acid and Trolox were also used as reference compounds, and the results are shown in Table 15.

Table 15: The DPPH free radical scavenging (FRS) IC_{50} and oxygen radical absorbance capacity (ORAC) EC_{50} values of etlingerin in comparison with curcumin, ascorbic acid, and Trolox.

Compound	DPPH FRS IC_{50} (µM)	ORAC EC_{50} (µM)
Curcumin	26.1 ± 0.2bc	2.59 ± 0.03a
Etlingerin	9.91 ± 0.06a	2.73 ± 0.02a
Ascorbic acid	21.6 ± 0.2b	ND
Trolox	ND	14.3 ± 0.1b

Assays were performed with three replicates and values are reported as mean ± standard error. Different letters indicate significant difference between mean values within the same assay, at $\alpha = 0.05$ (using ANOVA with Tukey's post-hoc test). ND = not determined.

Etlingerin exhibited significantly lower DPPH FRS IC_{50} value as compared to curcumin (9.91 ± 0.06 and 26.1 ± 0.2 µM, respectively), therefore suggesting that the former is a better free radical

scavenger than the latter. This could be due to etlingerin possessing four phenolic groups while curcumin has only two phenolic groups (see Figure 13). A compound with more phenolic groups can transfer more hydrogen ions to the free radicals (Michalak, 2006; Priyadarsini et al., 2003). However, the differences between the ORAC EC_{50} values determined for etlingerin and curcumin were found to be insignificant (2.73 ± 0.02 and 2.59 ± 0.03 μM, respectively). The contradiction might be explained by several reasons. Firstly, as previously mentioned, the two assays evaluate antioxidant activities via different mechanisms. Secondly, in the ORAC assay, the solutions were buffered using phosphate buffer (pH 7.4), whereas the DPPH FRS assay utilized unbuffered methanol. The pH of a solution can affect a compound's antioxidant activity. For example, a study by Altunkaya et al. (2016) showed that pH increase is associated with stabilization of phenolic species, hence leading to polymerization reaction. This subsequently results in new oxidizable hydroxyl moieties being formed. Nonetheless, it can be inferred that etlingerin possesses comparable free radical scavenging activity with curcumin. When contrasted with ascorbic acid and Trolox, etlingerin showed significantly lower DPPH FRS IC_{50} and ORAC EC_{50} values (see Table 15), thus implying that etlingerin is a better free radical scavenger than ascorbic acid and Trolox.

Though it is important for an antioxidant to be able to effectively scavenge free radicals, it is also crucial that the compound can exert this effect from within cells. This warrants the use of the cell-based antioxidant assay. In the present work, the ability of etlingerin to protect intracellular DCFH was evaluated in comparison with curcumin as well as quercetin. The protocol employed was as outlined in section 2.6.4. Figure 14 shows the percentage protection values determined across various concentrations of these compounds.

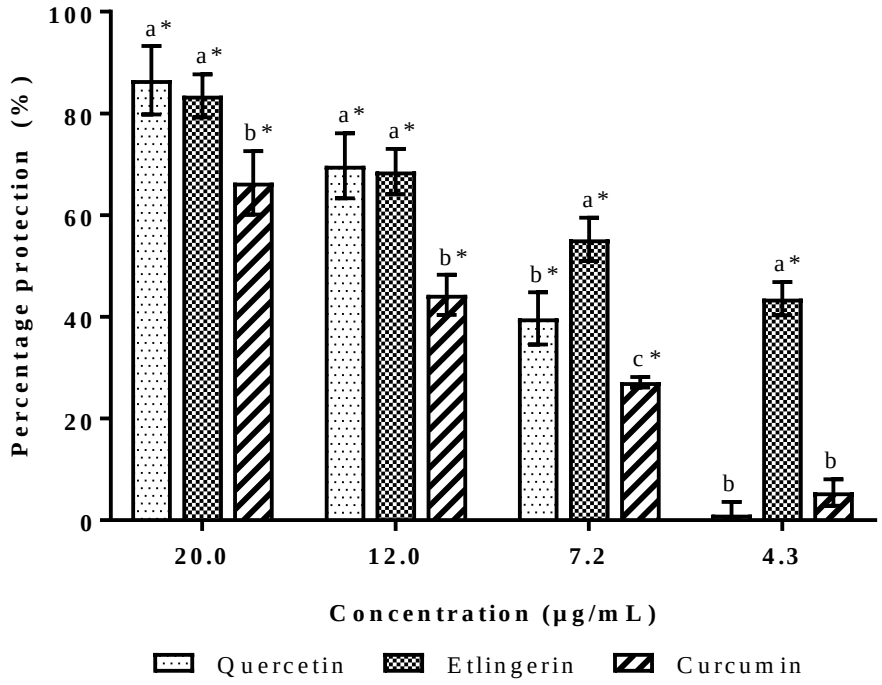

Figure 14: Cellular antioxidant activity (CAA) of various concentrations of quercetin, etlingerin, and curcumin, represented by percentage protection (%) of DCFH. Four independent replicates were performed, and error bars represent mean ± standard error. Different letters indicate significant differences between the mean percentage protection values of the different compounds within the same concentration, at $\alpha = 0.05$ (as determined using ANOVA with Tukey's post-hoc test). Asterisks indicate significant differences between pre-treatment for each compound and the untreated cells (that has mean percentage protection value of $-2.013\times10^{-15} \pm 5.648\times10^{-15}$ %, not shown on graph), at $\alpha = 0.05$ (as determined using independent samples *t*-test).

Based on Figure 14, quercetin and etlingerin showed highest percentage protection values (68.6 ± 2.9 and 67.6 ± 2.2 %, respectively) at the highest concentration tested (20 µg/mL). Curcumin also exhibited positive protective effect at the same concentration, though at a significantly lower percentage (55.9 ± 4.2 %). These suggest that all three compounds could cross the cell membrane

and protect the intracellular DCFH. A dose-dependent decrease in percentage protection values could also be observed at decreasing concentrations of the three compounds. However, the protective effect of etlingerin became more prominent than quercetin and curcumin, hence implying that etlingerin can still be an effective antioxidant, even at low concentrations. This is highly desirable as beneficial effects could still be achieved while posing low toxicity towards cells.

As previously mentioned, the cell-based antioxidant assay is biologically-relevant as it can measure a compound's ability to protect the intracellular DCFH. It also takes into account several important aspects such as cellular uptake. However, it does not provide any information on the consequences of the treatments to the viability of the cells. The prolonged exposure to free radicals is also not considered, as the assay only proceeds for an hour. Thereafter, to address these limitations, the cytoprotective study was carried out (as described in section 2.6.5). Hydrogen peroxide was chosen as the free radical generator as it is one of the most prominent ROS in mammalian cells (Lushchak, 2014). Other well-known antioxidants (curcumin, quercetin, Trolox, catechin, and caffeic acid) were also tested for comparison purpose, and the results are displayed in Figure 15.

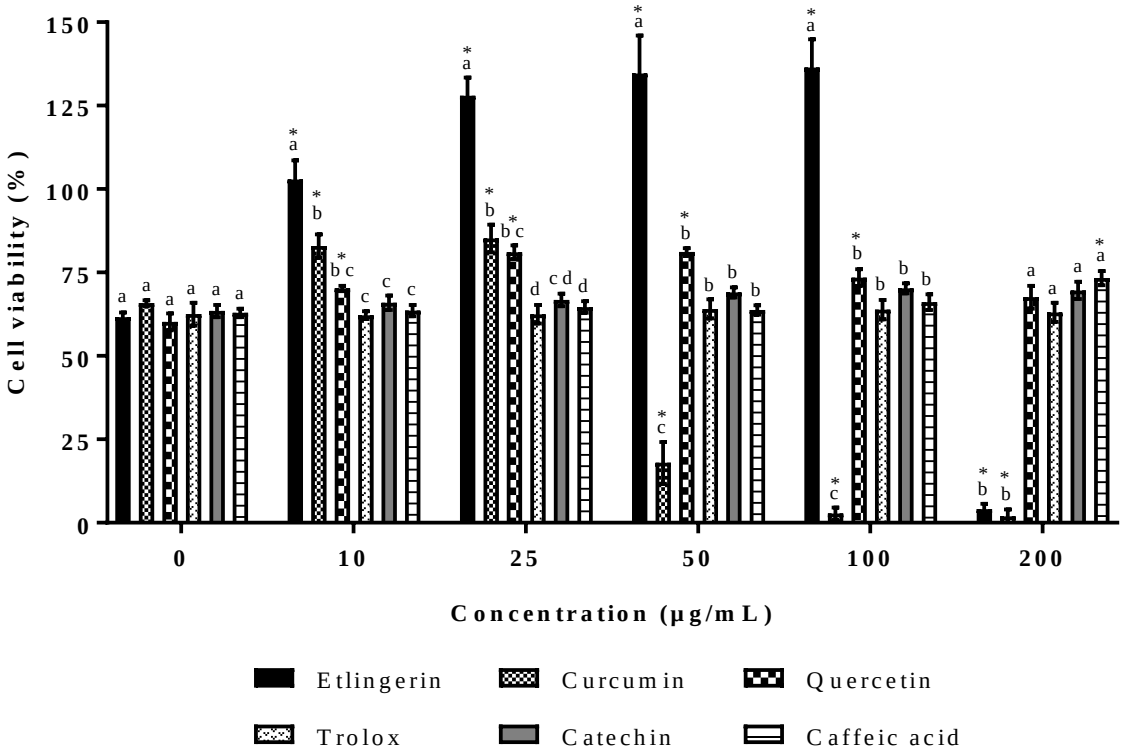

Figure 15: HaCaT cell viability (as determined through MTT cell viability assay) following treatment with hydrogen peroxide alone and in combination with either etlingerin, curcumin, quercetin, Trolox, catechin, or caffeic acid pre-treatments. Three independent replicates were performed, and values are reported as mean cell count ± standard error. Different letters indicate significant differences between pre-treatments within the same concentrations, at $\alpha = 0.05$ (using ANOVA with Tukey's post-hoc test). Asterisks indicate significant differences between pre-treatments (10, 25, 50, 100, and 200 µg/mL concentrations) with hydrogen peroxide treatment only (0 µg/mL concentration) for the same compound, at $\alpha = 0.05$ (using ANOVA with Tukey's post-hoc test).

As demonstrated in Figure 15, treatment with hydrogen peroxide caused an approximately 40 % drop in cell viability as compared to untreated cells (100 %), thereby validating the detrimental

effects of hydrogen peroxide. When the cells were pre-treated with the antioxidant compounds, varying degrees of changes in cell viability were observed. For example, at 50 µg/mL, etlingerin and quercetin pre-treatments resulted in significantly higher cell viability values (134.6 ± 11.3 and 81.19 ± 1.13 %, respectively) than hydrogen peroxide treatment only (61.63 ± 1.35 and 60.18 ± 2.52 %, respectively), while lower cell viability was associated with curcumin pre-treatment (17.99 ± 6.26 against 65.82 ± 0.83 %, respectively). Also, no significant differences were observed between the cell viability values linked to Trolox, catechin, and caffeic acid pre-treatments (64.08 ± 2.85, 69.06 ± 1.49, and 63.76 ± 1.40 %, respectively), with hydrogen peroxide treatment only (62.53 ± 3.45, 63.48 ± 1.84, and 62.88 ± 1.23 %, respectively). These findings might be associated with the lipophilicity of the antioxidant compounds tested. Therefore, to assess this hypothesis, the log P values were determined (as described in section 2.12) and summarized in Table 16.

Table 16: Lipophilicity of curcumin, etlingerin, quercetin, Trolox, catechin, and caffeic acid, as determined using computational methods.

Computational method	Curcumin	Etlingerin	Quercetin	Trolox	Catechin	Caffeic acid
iLOGP	3.27	2.58	1.63	2.11	1.47	0.97
XLOGP3	3.20	4.29	1.54	2.84	0.36	1.15
WLOGP	3.15	3.79	1.99	2.49	1.22	1.09
MLOGP	1.47	2.51	-0.56	1.81	0.24	0.70
SILICOS-IT	4.04	3.92	1.54	3.11	0.98	0.75
Consensus	3.03	3.42	1.23	2.47	0.85	0.93

iLOGP, XLOGP3, WLOGP, MLOGP, and SILICOS-IT are five different predictive models used to determine the log P values. The consensus log P values are the mean values of the five models.

Based on the predicted log P values listed in Table 16, quercetin, Trolox, catechin, and caffeic acid possess low lipophilicity, as compared to curcumin and etlingerin. Therefore, these compounds might not readily penetrate the cell membrane during the one-hour pre-treatment period, hence unable to effectively protect the cells from hydrogen peroxide-induced damage. Conversely, etlingerin possesses highest lipophilicity among the antioxidants tested, as suggested by its consensus log P value predicted (3.42). A lipophilic antioxidant is advantageous as it can modulate intracellular free radicals (Wolfe and Liu, 2008). This, together with the fact that etlingerin is a good free radical scavenger, could account for the results shown in Figure 15, whereby etlingerin exhibited highest percentage cell viability as compared to the other antioxidants, across various concentrations. Though etlingerin has excellent protective effect at low concentrations, it was noted that cytotoxicity was observed at a high concentration (200 µg/mL).

In general, etlingerin demonstrated better protective effects than the other antioxidants. However, at 12.5 to 100 µg/mL, its associated cell viability values exceeded 100 %. This could be due to the possible interaction between etlingerin and the MTT dye, as some antioxidant compounds can reduce MTT in the absence of cells (Bruggisser et al., 2002; Talorete et al., 2006). This means that the observed increase in cell viability might be caused by 1) its true antioxidant (protective) effect, and/ or 2) etlingerin-MTT interaction. Therefore, direct cell enumeration (via trypan blue exclusion, as outlined in section 2.6.5) was performed to validate the results displayed in Figure 15. The concentration 50 µg/mL was chosen as varying degrees of change in cell viability could be observed. Figure 16 summarizes the cell counts following pre-treatment with etlingerin, quercetin, as well as curcumin, and subsequent treatment with hydrogen peroxide.

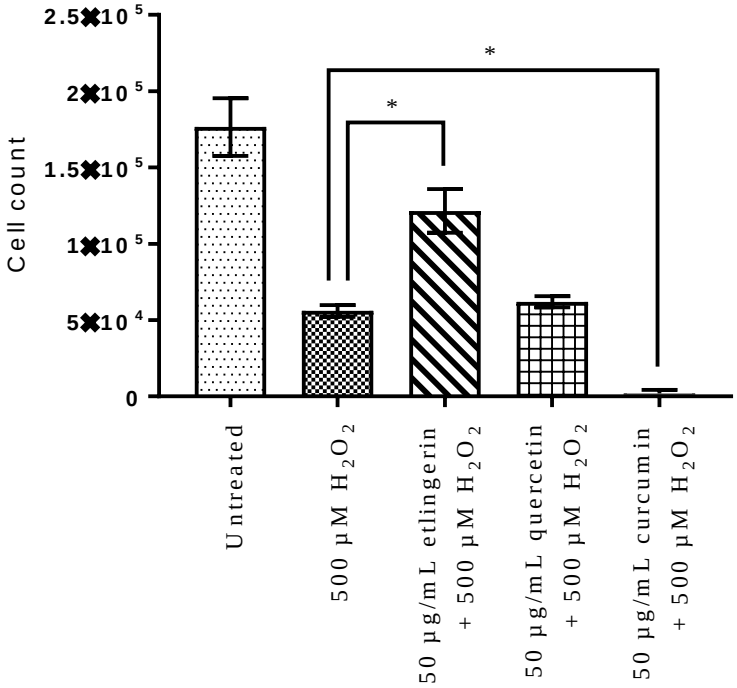

Figure 16: HaCaT cell viability (as determined via trypan blue exclusion) following treatment with hydrogen peroxide alone and in combination with either etlingerin, quercetin, or curcumin pre-treatments. Three independent replicates were performed, and values are reported as mean cell count ± standard error. Asterisks indicate significant difference at $\alpha = 0.05$ (using independent-samples *t*-test) compared to HaCaT cells treated with hydrogen peroxide alone.

In Figure 16, hydrogen peroxide treatment caused a significant reduction in cell viability compared to the untreated cells ($0.56\times10^5 \pm 0.04\times10^5$ cells against $1.77\times10^5 \pm 0.19\times10^5$ cells, respectively). However, when pre-treated with etlingerin, the number of viable cells ($1.22\times10^5 \pm 0.14\times10^5$ cells) were significantly higher than cells treated with hydrogen peroxide only. This supports etlingerin's cytoprotective effect. On the other hand, no protective effect was found for curcumin pre-treatment; instead, a significant reduction in cell viability was observed (as also seen in Figure 15). This was not surprising as previous studies reported that curcumin pre-treatment has no obvious

protective effect on hydrogen peroxide induced cell injury (Chen et al., 2011; Mahakunakorn et al., 2003). However, co-treatment was found to have a positive protective effect (Mahakunakorn et al., 2003).

Figure 16 also demonstrated that the quercetin pre-treatment did not significantly changed the number of viable cells as compared to hydrogen peroxide treated cells ($0.62\times10^5 \pm 0.04\times10^5$ cells and $0.56\times10^5 \pm 0.04\times10^5$ cells, respectively). This contradicts with the results from previous experiments in which 1) quercetin protected DCFH from degradation with percentage protection determined as high as 86.55 ± 6.73 % at 20 µg/mL (see Figure 14), and 2) pre-treatment with 50 µg/mL quercetin resulted in increased cell viability (81.19 %) following exposure to hydrogen peroxide (see Figure 15). The following reasons might explain these contradictions. The first reason can be linked to the basis that the cell-based antioxidant assay does not consider the consequences of the treatments to the viability of the cells. AAPH and hydrogen peroxide can generate free radicals that not only cause damage to DCFH, but also to cellular components, which in turn can lead to cell death (Minotti and Aust, 1987; Terao et al., 1994; Wijeratne et al., 2005). In addition, the assay only proceeded for 90 min whereas the treatments in the cytoprotective study were conducted for 24 hours. Hence, depletion of antioxidant compounds (in this case, quercetin) might affect their ability to protect cells in a longer period. Thirdly, several phenolic compounds, including quercetin, can reduce MTT in the absence of cells, leading to a false positive (Bruggisser et al., 2002; Talorete et al., 2006). To further validate the current findings, phase-contrast microscopy was conducted (as outlined in section 2.6.5) to enable visual assessments on changes in cellular morphology. The images obtained are presented in Figure 17.

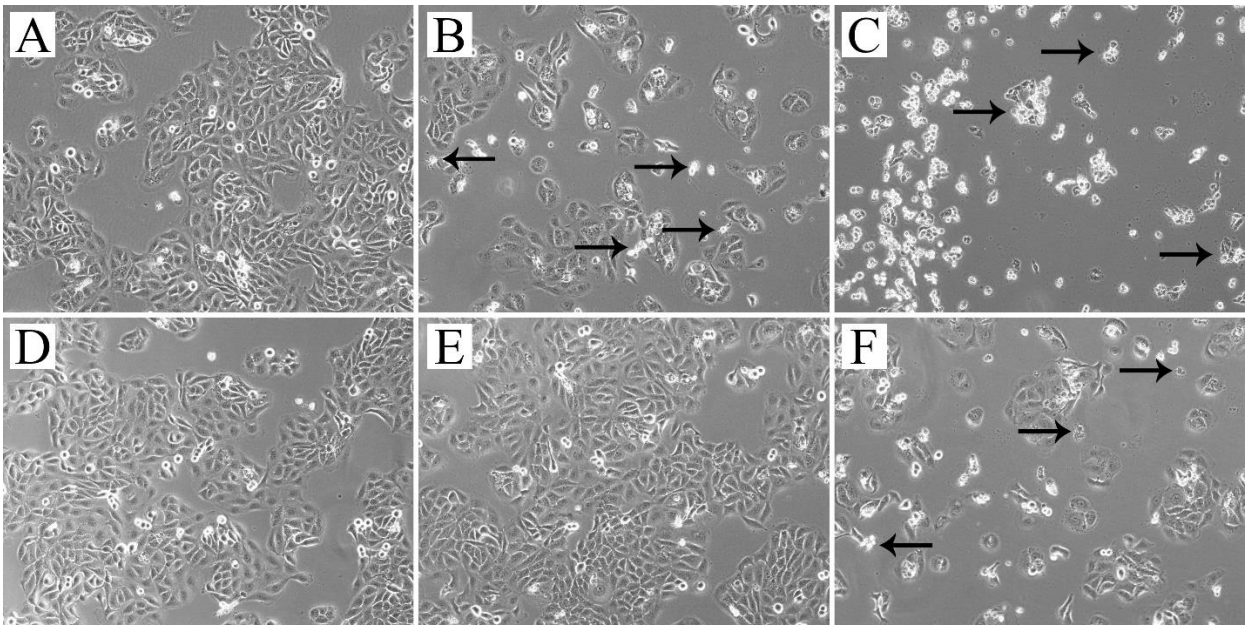

Figure 17: Phase-contrast microscopy images of HaCaT cells morphology after pre-treatment with different antioxidant compounds and subsequent treatment with hydrogen peroxide. (A) untreated cells, (B) hydrogen peroxide (500 µM) treated cells, (C) pre-treatment with 50 µg/mL curcumin, (D) untreated cells, (E) pre-treatment with 50 µg/mL etlingerin, and (F) pre-treatment with 50 µg/mL quercetin. Arrows indicate examples of apoptosis-like morphology and floating cells.

In Figure 17, the untreated cells (A and D) show typical endothelial cell morphology, with high cell density. Treatment with hydrogen peroxide (B) resulted in apoptosis-like morphology and increased number of floating cells, indicating cell death. On the other hand, pre-treatment with etlingerin (E) proved effective in protecting the cells from hydrogen peroxide damage, as low number of apoptosis-like and floating cells were observed. Also, there were no significant changes in the cellular morphology as compared to the untreated cells, and this suggests that etlingerin has good biocompatibility. Figure 17 also implied that the quercetin pre-treatment (F) did not effectively protect the cells, as large numbers of apoptosis-like morphology and floating cells could be seen. As for curcumin pre-treatment (C), significant changes in cellular morphology were

noted whereby most cells showed apoptosis-like appearance. This supports the results shown in Figure 15 and Figure 16, whereby curcumin did not protect the cells, but instead further promoted cell death (at concentration tested), similar to the outcomes reported in another study whereby curcumin was found to enhance hydrogen peroxide-induced cytotoxicity (Chen et al., 2011).

5.4.2 Antimicrobial activity of etlingerin

Apart from antioxidant, the antimicrobial activity of etlingerin was also evaluated in comparison with curcumin, using the broth microdilution assay (as described in section 2.7) against eight bacterial strains (*S. aureus* ATCC 43300, *S. aureus* ATCC 6538P, *B. cereus* ATCC 14579, *B. subtilis* ATCC 8188, *P. aeruginosa* ATCC 10145, *E. coli* ATCC 25922, *S. enterica* ser. Typhimurium ATCC 14028, and *P. mirabilis* ATCC 12453). The results are presented in Table 17.

Table 17: The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values determined for 1,7-bis(3,4-dihydroxyphenyl)heptan-3-yl acetate (etlingerin) and curcumin.

Compound	Bacterial strains							
	S. aureus ATCC 43300		*S. aureus* ATCC 6538P		*B. cereus* ATCC 14579		*B. subtilis* ATCC 8188	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Etlingerin	0.125	0.125	0.125	0.125	0.0625	0.0625	0.0625	0.0625
Curcumin	0.250	>1	0.250	>1	0.125	>1	0.125	>1

MIC and MBC values were determined with 3 biological replicates. All values are expressed in mg/mL. Etlingerin and curcumin did not inhibit the gram-negative bacteria tested (MIC values >1 mg/mL).

The MIC values of etlingerin against *S. aureus* ATCC 43300, *S. aureus* ATCC 6538P, *B. cereus* ATCC 14579, and *B. subtilis* ATCC 8188 were found to be two-fold lower than curcumin (see Table 17). Although both compounds were effective against the gram-positive bacterial strains, the gram-negative strains were unaffected up to the highest concentration used (1 mg/mL). This was expected for curcumin, as a similar finding was reported in another study (Tajbakhsh et al., 2008). Also, the presence of LPS layer in gram-negative bacteria might hindered the entry of these compounds. The present study also revealed that etlingerin was bactericidal (as the MIC values determined were equal to their respective MBC values), whereas curcumin was only bacteriostatic (MBC values were more than 1 mg/mL).

It is noteworthy that in vitro and in vivo studies have suggested the potential of curcumin as an antimicrobial agent, as reviewed by Zorofchian Moghadamtousi et al. (2014). Although so, curcumin suffers from major disadvantages which include low bioavailability and poor water solubility (Anand et al., 2007). Future studies should investigate the pharmacokinetics and pharmacodynamics of etlingerin. Nevertheless, current results suggest that etlingerin has superior antimicrobial property as compared to curcumin, hence highlighting the former's potential to be developed as an antimicrobial agent.

It is essential to determine etlingerin's antimicrobial mechanism to gain an understanding on its effects on bacterial cells. To achieve this, a time kill study was first conducted against *S. aureus* ATCC 43300 and *B. subtilis* ATCC 8188. The assay was conducted as outlined in section 2.8.1, and Figure 18 summarizes the outcomes of the experiment.

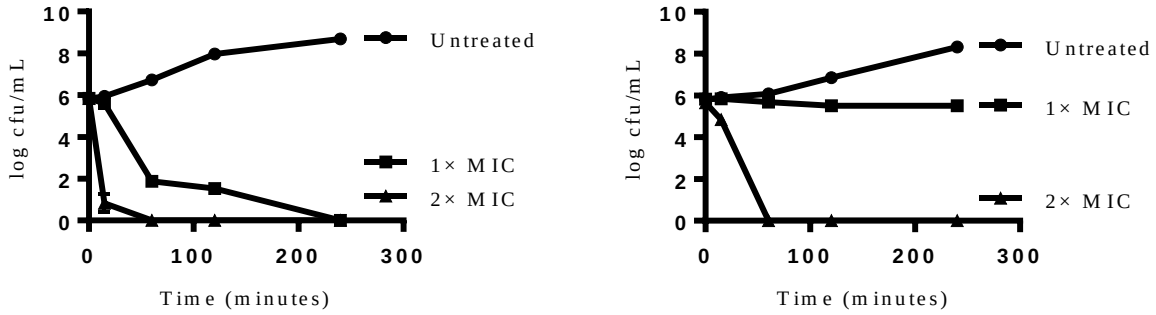

Figure 18: Time-kill curves showing the dose- and time-dependent effects of etlingerin at 1× and 2× MIC against *B. subtilis* ATCC 8188 (62.5 and 125 µg/mL, respectively) (left) and *S. aureus* ATCC 43300 (125 and 250 µg/mL, respectively) (right). At 24 hours, both concentrations of etlingerin resulted in complete killing of both bacterial strains (results not shown). Assay was performed with three replicates and values are reported as mean log cfu/mL ± standard error.

The killing action of etlingerin follows a dose-dependent manner as 2× MIC resulted in a larger reduction in bacterial cell count as compared to 1× MIC over the same duration against both bacterial strains (see Figure 18). In addition, a complete killing effect was achieved in as short as 60 min after treatment with etlingerin. One of the main target sites for antimicrobials with rapid action is the bacterial membrane (Hancock, 2005). Many phenolic compounds have been shown to affect the bacterial membrane, primarily by decreasing permeability/fluidity of the phospholipid bilayers (Borges et al., 2013; Cushnie and Lamb, 2005; Simoes et al., 2009). The rapid action observed prompted the need to investigate the effect of etlingerin treatment on the bacterial membrane. This was achieved by employing the SYTO-9/ PI uptake assay (as described in section 2.8.2). The results are displayed in Figure 19.

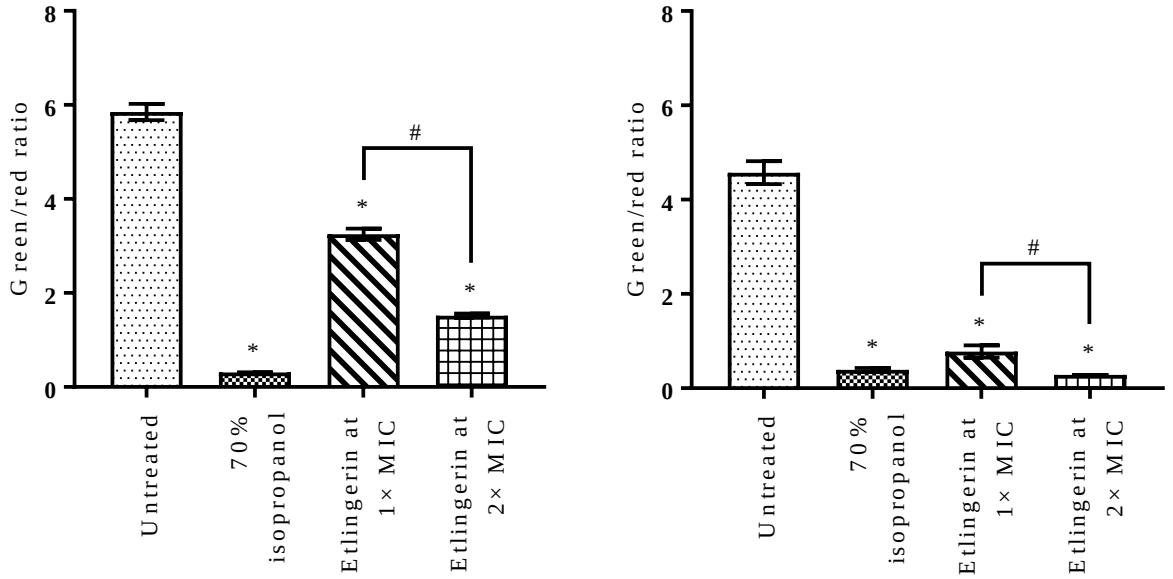

Figure 19: Graph bars representing the membrane damage effect of etlingerin at 1× and 2× MIC against *B. subtilis* ATCC 8188 (62.5 and 125 µg/mL, respectively) (left) and *S. aureus* ATCC 43300 (125 and 250 µg/mL, respectively) (right). Assay was performed with three replicates and the green/red ratios were reported as mean ± standard error. Asterisks (*) indicate significant differences between untreated bacteria and those treated with either isopropanol or etlingerin at α = 0.05 (using independent-samples *t*-test). Hashes (#) indicate significant differences between etlingerin at 1× MIC and etlingerin at 2× MIC.

The dye uptake assay incorporates two important nuclei stains, SYTO-9 and PI (Huang and Yousef, 2014). Untreated bacterial cells have intact membranes, and upon addition of SYTO-9 (a membrane permeable dye), its binding to DNA results in the emission of green fluorescence. On the other hand, when the membrane is compromised, PI (a membrane impermeable dye) can enter the cells, bind to DNA and emit red fluorescence. Aqueous isopropanol was used as a positive control since it is a known membrane permeabilizer, and a significant reduction in the green/red

ratio was observed, as shown in Figure 19. Treatment with etlingerin also significantly reduced the green/red ratio, with 2× MIC causing a larger reduction than 1× MIC against both bacterial strains. As lower green/red ratios indicate membrane damage, these results suggest that etlingerin caused an increase in bacterial membrane permeability. To rule out the possible interaction between etlingerin and the dyes, fluorescence microscopy analysis was performed (as outlined in section 2.8.3, and the results are presented in Figure 20.

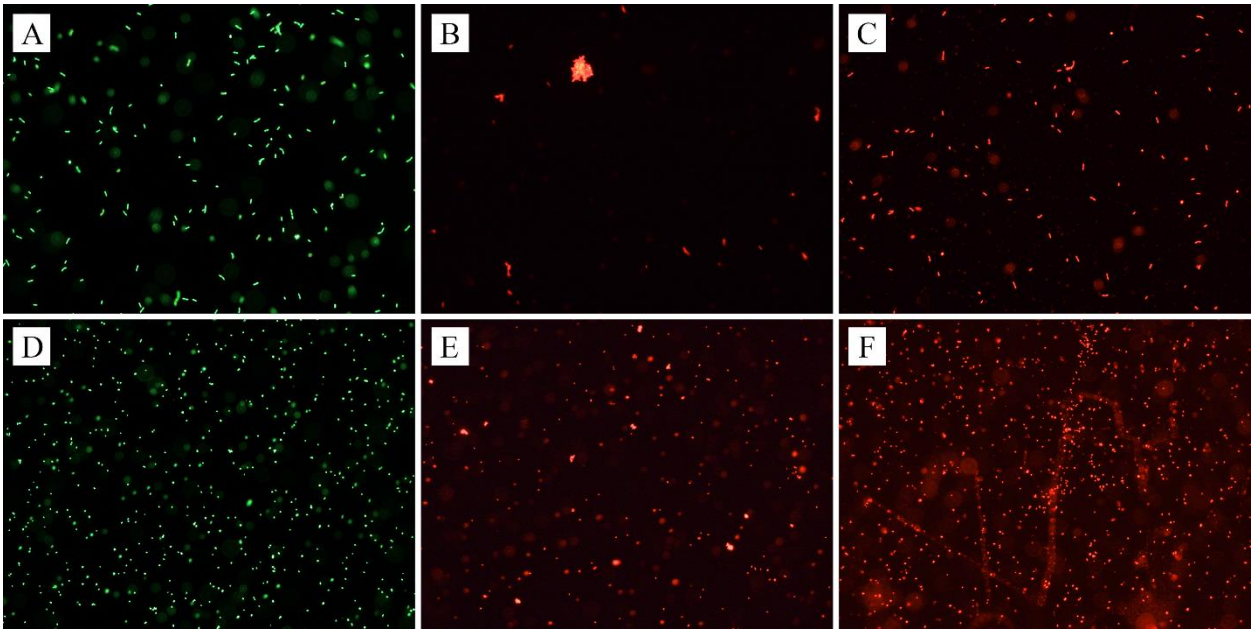

Figure 20: Fluorescence microscopy analysis of untreated *B. subtilis* ATCC 8188 (A), *B. subtilis* treated with 70 % isopropanol (B), *B. subtilis* treated with 2× MIC of etlingerin (125 µg/mL) (C), untreated *S. aureus* ATCC 43300 (D), *S. aureus* treated with 70% isopropanol (E), and *S. aureus* treated with 2× MIC of etlingerin (500 µg/mL) (F) stained with SYTO-9/propidium iodide (PI) dye mixture. Fluorescent green appearance indicates an intact membrane, while fluorescent red suggests an increase in membrane permeability.

Images in Figure 20 showed that only the bacterial cells appeared red following treatment with 70 % isopropanol and etlingerin, whereas the untreated bacterial cells appear green. These support the results presented in Figure 19, while also ruled out the possible interaction between etlingerin and the dyes. Apart from assessment via dye uptake, cellular leakage assay was also carried out (as outlined in section 2.8.4) to evaluate the effect of etlingerin treatment on the leakage of intracellular materials. A summary of the findings is shown in Figure 21.

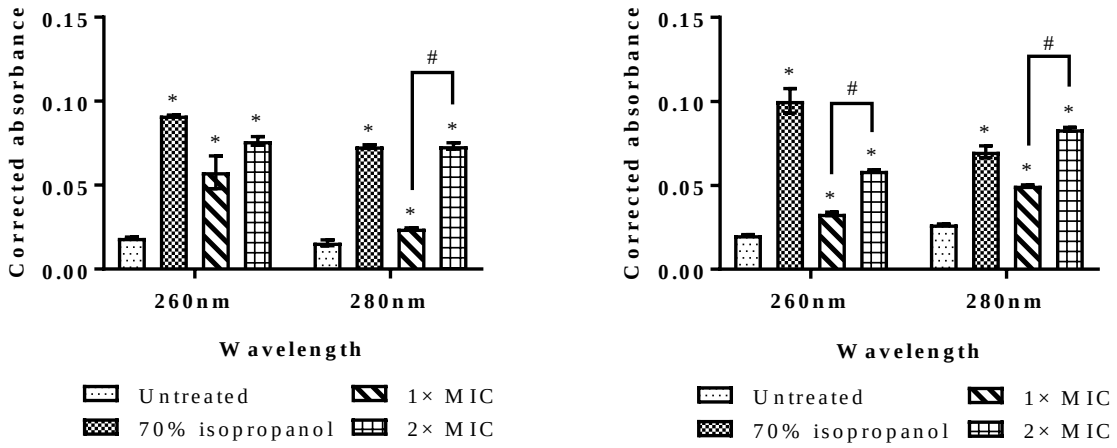

Figure 21: Leakage of cellular constituents following 1× and 2× MIC treatment with etlingerin on *B. subtilis* ATCC 8188 (62.5 and 125 µg/mL, respectively) (left) and *S. aureus* ATCC 43300 (125 and 250 µg/mL, respectively) (right) after 4 hours. The absorbance values were measured at 260 nm and 280 nm. The assay was performed with three replicates and error bars represent the mean corrected absorbance ± standard error. Asterisks (*) indicate significant differences between untreated bacteria and those treated with either isopropanol or etlingerin at $\alpha = 0.05$ (using independent-samples *t*-test). Hashes (#) indicate significant differences between etlingerin at 1× MIC and etlingerin at 2× MIC.

In the cellular leakage assay, 260 and 280 nm wavelengths were chosen because intracellular materials such as nucleic acids and proteins absorb at these wavelengths, respectively (Wong and Kitts, 2006). If the membrane is compromised, these intracellular components could leak out from the bacterial cells. Figure 21 revealed that the bacterial cells treated with etlingerin resulted in increased absorbance at 260 and 280 nm (the background absorbance from etlingerin itself was accounted for), as compared to the untreated cells. In addition, a dose dependent effect was found as 2× MIC generally caused a larger increase in absorbance values compared to 1× MIC against both bacterial strains. Therefore, it can be inferred that leakage of intracellular materials had occurred. The present work suggests that etlingerin exhibits its antimicrobial mechanism by altering the bacterial membrane permeability. Further work is required to confirm the interaction between etlingerin and the bacterial membrane. Electron microscopy could be done to assess the changes in the membrane structure, while molecular docking studies could unveil the site where the compound binds to.

5.4.3 Cytotoxicity of etlingerin

Cytotoxicity study was performed to assess the effects of prolonged treatment with etlingerin on various cell lines. A combination of colon normal (CCD-841) and cancer (HT-29 and SW-480) cell lines were also employed to determine the cytotoxic selectivity of etlingerin, as well as whether its cytotoxic mechanism involves prooxidant activity (curcumin is known to kill cancer cells by being a prooxidant). MTT assay was used to determine the IC_{50} values (as described in section 2.9), and the results are summarized in Table 18.

Table 18: Cytotoxicity of curcumin and etlingerin on various cell lines.

Compound	IC_{50} (μg/mL)						
	HaCaT	M10	HEK-293T	SH-SY5Y	HT-29	SW-480	CCD-841
Curcumin	4.207 ± 0.927b	0.0130 ± 0.0014b	0.0106 ± 0.0003b	0.0055 ± 0.0002b	13.71 ± 2.01b	10.54 ± 0.50b	30.86 ± 1.60b
Etlingerin	48.34 ± 3.72a	0.1037 ± 0.0069a	0.1242 ± 0.0028a	0.0301 ± 0.0016a	59.56 ± 5.59a	45.52 ± 3.59a	143.8 ± 2.9a

Assays were performed with three independent replicates, and values are reported as mean ± standard error. Different letters indicate significant differences between curcumin and etlingerin for the same cell line, at $\alpha = 0.05$ (using independent-samples *t*-test).

In general, etlingerin was found to be less cytotoxic (about 4 to 12-fold) as compared to curcumin (see Table 18). It is noteworthy that curcumin itself is not considered toxic as clinical trials had proven that it was safe even when consumed at a daily dose of 12 g for 3 months (Gupta et al., 2013). Therefore, the results suggest that etlingerin could be safe when administered in high doses. The reason for curcumin's low toxicity in humans is mainly due to its low bioavailability. This is because curcumin is unstable at physiological pH, susceptible to autoxidation, has poor absorption through the gastrointestinal tract, is rapidly metabolized, and is rapidly eliminated, thus resulting in low bioavailability (Anand et al., 2007b; Metzler et al., 2013).

When compared for their cytotoxicity on the colon normal and cancer cell lines, etlingerin and curcumin showed higher IC_{50} values against CCD-841 (normal) as compared to HT-29 (cancer) and SW-480 (cancer) cell lines (see Table 18). This means that both compounds were relatively more cytotoxic towards the two colon cancer cell lines. To enable better comparison of their cytotoxic selectivity, the therapeutic index (TI) values were calculated (TI = IC_{50} of normal cell line ÷ IC_{50} of cancer cell line). Curcumin and etlingerin exhibited TI values of 2.59 ± 0.20 and

2.42 ± 0.27 against HT-29, respectively, as well as 3.33 ± 0.39 and 3.13 ± 0.19 against SW-480, respectively. The TI values determined for both compounds were not significantly different (at $\alpha = 0.05$, as determined using independent sample *t*-test), hence suggesting that both compounds possess similar cytotoxic selectivity. Subsequently, the possible cytotoxic mechanism of etlingerin was explored. Curcumin has been shown to kill cancer cells by acting as a prooxidant. At high concentrations, it was associated with an increase in intracellular ROS generation, hence causing DNA damage, and subsequently leading to induction of apoptosis (Lee et al., 2016; Yoshino et al., 2004). Therefore, quantification of ROS levels in cancer cells treated with etlingerin and curcumin were conducted (as outlined in section 2.13) to determine whether these compounds possess similar mechanism. Figure 22 summarizes the results obtained.

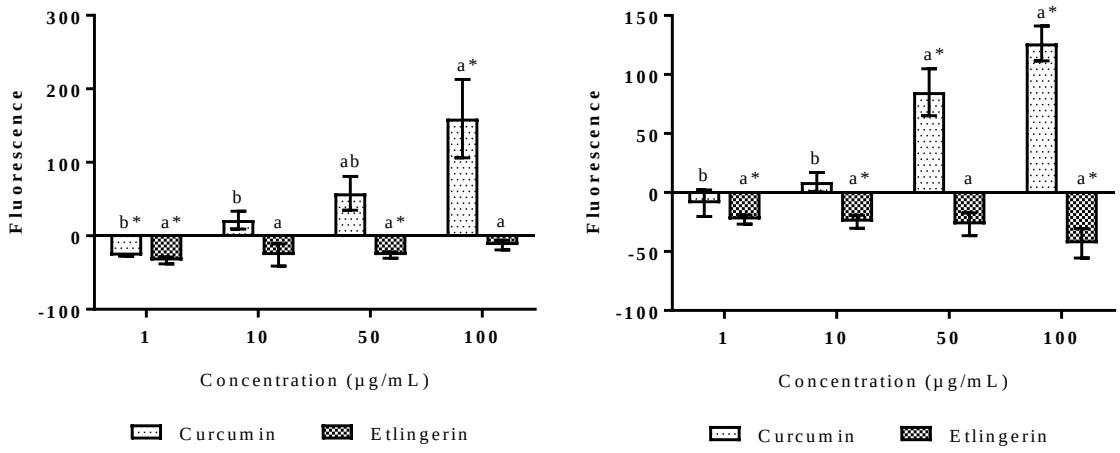

Figure 22: Fluorescence levels measured following treatment using various concentrations (100, 50, 10 and 1 µg/mL) of etlingerin and curcumin on HT-29 (left) and SW-480 (right) colon cancer cells. Three independent replicates were performed, and error bars are reported as mean ± standard error. Different letters indicate significant difference between mean values of the various concentrations for the same compound and within the same cell line (as determined using ANOVA with Tukey's post-hoc test). Asterisks indicate significant difference between mean values of treatment and negative control, at $\alpha = 0.05$ (using *t*-test).

In Figure 22, there is a dose-dependent increase in fluorescence measured for curcumin treated cancer cells, thus proving that curcumin promoted ROS generation. Conversely, the fluorescence levels determined for etlingerin remain unchanged with increasing concentrations. This means that the cytotoxic mechanism of etlingerin might not be associated with prooxidant activity. Therefore, further work is needed to elucidate its mechanism. This can be achieved via flow cytometry as well as gene/ protein expression studies to determine the cell death pathway.

5.5 Summary of Chapter 5

The crude extracts of *E. pubescens* were shown to possess good antioxidant and antimicrobial properties. In addition, its bioactive compound, etlingerin, exhibited free radical scavenging activities that are comparable to curcumin, and better than those of ascorbic acid and Trolox. It is also noteworthy that etlingerin protected cells that were challenged with hydrogen peroxide, while the other antioxidants did not. This highly supports the potential application of etlingerin in treating oxidative stress-related diseases. Apart from this, it was also revealed that etlingerin which inhibited the growth of clinically-relevant bacterial strains, can potentially be developed as an antimicrobial agent. Current data suggests that etlingerin exerts its bactericidal effect on gram-positive bacteria via altering membrane permeability, which in turn causes leakage of intracellular materials from the bacterial cells. However, further work is needed to investigate its interaction with the bacterial membrane. Lastly, etlingerin was found to be significantly less cytotoxic than curcumin, thus suggesting its safety. Although both compounds have similar chemical structures, etlingerin did not cause any increase in ROS generation. It might exert its cytotoxic effect through a different mechanism, hence warrant further investigation.

CHAPTER 6

OVERALL DISCUSSION, CONCLUSIONS, AND FUTURE WORK

Chapter 6 – Overall Discussion, Conclusions, and Future Work

6.1 Overall discussion on the antioxidant and antimicrobial activities of *E. coccinea*, *E. pubescens*, and *E. sessilanthera*

The antioxidant activities of the three *Etlingera* species were screened by using three different assays, and their TPC, AEAC, and ORAC values were determined, as outlined in sections 2.6.1, 2.6.2, and 2.6.3. The values obtained were presented in sections 3.1, 4.1, and 5.1. Here, comparisons are made between the three species, as well as with other ginger plant species reported in literature. The work by Chan et al. (2008) was used for comparison due to the similar methods employed in preparation and evaluation of the crude extracts.

In previous chapters, it was shown that the leaves of *E. coccinea*, *E. pubescens*, and *E. sessilanthera* exhibited highest antioxidant activities as compared to their stems and rhizomes. According to Chan et al. (2008), it is generally believed that the antioxidants produced by ginger plants are transported to and accumulated in their rhizomes. This means that the latter should have higher antioxidant activities than the former. However, based on the authors' results, as well as those reported in this study, most of the ginger species displayed significantly higher antioxidant activities in their leaves as compared to their rhizomes. Other vegetables such as celery, lettuce, and asparagus have also been reported to contain much greater concentrations of flavones and flavonols (which are antioxidant compounds) in the leaves than in their roots (Herrmann, 1988). This might be due to the need for these compounds in the leaves to prevent oxidative damage from exposure to sunlight (Masuda et al., 1999). Hence, comparison of the antioxidant activities was made on the leaves of the ginger plants. Table 19 lists the TPC, AEAC, and ORAC values of the leaf methanol extract of several ginger species.

Table 19: Comparison of the antioxidant activities of the leaf methanol extracts of several ginger plant species.

Plant species	TPC (mg GAE/ 100 g fresh weight)	AEAC (mg AAE/ 100 g fresh weight)	ORAC (μmol TE/ g fresh weight)	Reference
Etlingera coccinea	1246 ± 28	2702 ± 89	423.9 ± 18.2	This study
Etlingera pubescens	1019 ± 15	1129 ± 28	150.5 ± 26.0	This study
Etlingera sessilanthera	658.7 ± 11.8	257.6 ± 12.8	145.4 ± 12.9	This study
Etlingera elatior	2390 ± 329	2280 ± 778	ND	Chan *et al*., 2008
Etlingera rubriostrata	2250 ± 113	2290 ± 118	ND	Chan *et al*., 2008
Etlingera littoralis	2150 ± 94	1990 ± 87	ND	Chan *et al*., 2008
Etlingera fulgens	1280 ± 144	845 ± 158	ND	Chan *et al*., 2008
Etlingera maingayi	1110 ± 93	963 ± 169	ND	Chan *et al*., 2008
Alpinia galanga	392 ± 50	90 ± 36	ND	Chan *et al*., 2008
Zingiber officinale	291 ± 18	96 ± 7	ND	Chan *et al*., 2008
Curcuma longa	230 ± 19	113 ± 18	ND	Chan *et al*., 2008
Kaempferia galanga	146 ± 9	77 ± 7	ND	Chan *et al*., 2008

Different percentages of methanol (50, 70, and 100 %) were used to determine the antioxidant activities of *E. coccinea*, *E. pubescens*, and *E. sessilanthera*, and this table shows the highest values obtained. TPC = total phenolic content; AEAC = ascorbic acid equivalent antioxidant capacity; ORAC = oxygen radical absorbance capacity; GAE = gallic acid equivalent; AAE = ascorbic acid equivalent; TE = Trolox equivalent; ND = no data available.

As shown in Table 19, the antioxidant activities of *E. coccinea*, *E. pubescens*, and *E. sessilanthera* were found to be comparable to those of other *Etlingera* species. Between the three species, *E. coccinea* showed highest activity, followed by *E. pubescens*, and then *E. sessilanthera*. One possible explanation for this could be due to the specimens of *E. coccinea* being obtained from the forest open area, while the specimens of *E. pubescens* and *E. sessilanthera* were collected from the shaded areas. It is known that plants which receive more sunlight have efficient antioxidant properties to combat oxidative stress (Masuda et al., 1999). Next, when compared with other ginger genera (*Alpinia*, *Zingiber*, *Curcuma*, and *Kaempferia*), the *Etlingera* plants exhibit superior

antioxidant activities (see Table 21). According to Khaw (2001) and Larsen et al. (1999), the *Etlingera* are the largest among the ginger plants, while the other ginger plants (*Alpinia*, *Zingiber*, *Curcuma*, and *Kaempferia*) are small- to medium-sized herbs. Additionally, *Etlingera* plants are also commonly found growing in gaps of disturbed forests and are continually exposed to sunlight (Chan et al., 2008). A positive correlation was reported by Chan et al. (2008) in which larger ginger plants that grow in exposed sites have better antioxidant activities than smaller plants that grow in shaded area. These reasons might account for the superior antioxidant properties of *Etlingera* plants, thus making them great sources to mine for antioxidant compounds.

Apart from antioxidant, the antimicrobial activities of *E. coccinea*, *E. pubescens*, and *E. sessilanthera* have also been investigated (via broth microdilution method, as described in section 2.7), and the results were presented in previous chapters of this thesis (see sections 3.2, 4.2, and 5.2). To determine the effectiveness of the crude extracts in inhibiting bacteria, a comparison was made and shown in Table 20. The antimicrobial activities of several other ginger plants, based on those reported in literature, were also included.

Table 20: Comparison of the antimicrobial activities of several *Etlingera* species.

Species	Extract that exhibits best activity	Range of MIC values (mg/mL)		Reference
		Gram-positive bacteria	Gram-negative bacteria	
Etlingera coccinea	Leaf hexane extract	0.016 to 0.063	>1	This study
Etlingera pubescens	Leaf methanol extract	0.125 to 0.5	>1	This study
Etlingera sessilanthera	Rhizome hexane extract	0.125 to >1	>1	This study
Alpinia japonica	Rhizome methanol extract	0.05 to 0.08	0.05	Chen et al. (2008)
Curcuma longa	Rhizome methanol extract	0.05	0.05 to 1	Chen et al. (2008)
Etlingera brevilabrum	Stem acetone extract Leaf water extract	1.56 >50	>50 1.56	Mahdavi et al. (2012)
Etlingera elatior	Flower methanol extract	1.563 to 50	12.5 to 25	Lachumy et al. (2010)
Etlingera sayapensis	Leaf methanol extract	1.17	ND	Mahdavi et al. (2017)
Zingiber officinale	Rhizome essential oils	0.16 to 0.31	0.31 to 0.63	Sivasothy et al. (2011)

ND = not determined.

Between the three ginger species studied, *E. coccinea* displayed best antimicrobial activity, followed by *E. pubesens* and *E. sessilanthera* (see Table 20). The major antimicrobials isolated from these ginger plants were described in sections 3.2, 4.2, and 5.4.2. When compared for their bacterial inhibition properties, *trans*-2-dodecenal and 8(17),12-labdadiene-15,16-dial exhibited lower MIC values (4 to 8 µg/mL) than etlingerin (62.5 to 125 µg/mL), hence suggesting that the two former compounds are better antimicrobials than the later. Although so, all three compounds were effective in inhibiting the gram-positive bacteria, while potentially having low cytotoxicity.

Therefore, these compounds have the potential to be used as antimicrobials to treat infections by resistant bacteria, though further studies using animal models are required. Table 20 also compares the antimicrobial activities of *E. coccinea*, *E. pubescens*, and *E. sessilanthera* to other ginger species. In general, the three ginger plants have comparable activities to other *Etlingera* species. However, ginger plants from other genera (*Alpinia*, *Curcuma*, and *Zingiber*) seem to have better antimicrobial properties, as indicated by their lower MIC values. These ginger plants could also inhibit the gram-negative bacteria with low MIC values, as opposed to the *Etlingera*, which could only affect the gram-positive bacteria. Although *E. elatior* was reported to inhibit the gram-negative bacteria, its MIC values were relatively high (12.5 to 25 mg/mL). Nonetheless, this is not surprising as different plants can synthesize different compounds, therefore having different bioactivities.

6.2 Final conclusions

E. coccinea, *E. pubescens*, and *E. sessilanthera* were found to possess good free radical scavenging activities. Their TPC and AEAC values are comparable to other *Etlingera* species, while being better than ginger plants of other genera (*Alpinia*, *Zingiber*, *Curcuma*, and *Kaempferia*). From the active extract of *E. pubescens*, etlingerin was isolated, which not only showed good free radical scavenging activities, but it also protected cells that were challenged with hydrogen peroxide. In addition, when compared with curcumin, etlingerin was found to have superior antioxidant properties. These reasons highlight etlingerin's potential to be used as a drug to treat oxidative stress-related diseases.

Apart from having good antioxidant properties, the three ginger species studied also exhibited good antimicrobial activities, with *trans*-2-dodecenal, etlingerin, and 8(17),12-labdadiene-15,16-dial being identified as the major antimicrobials present in *E. coccinea*, *E. pubescens*, and *E. sessilanthera*, respectively. These compounds exhibited low MIC values (as low as 4 µg/mL) against clinically-relevant bacterial strains, thus could potentially be used as antimicrobial agents. Also, since this study is the first to investigate the antimicrobial activity of etlingerin, mechanistic work was also carried out, in which it was proposed that it exerts its bactericidal effect by altering the bacterial membrane permeability.

In the wound healing study, the water extracts of *E. coccinea*'s young shoots enhanced the proliferation and migration of BEAS-2B, HaCaT, and NIH/3T3 cell lines. Tannins (procyanidin B2 and C1) as well as other phenolic compounds were suggested to be present, which could contribute to the effects observed. In addition to having positive effects on cell proliferation and migration, the water extracts also inhibited the growth of bacterial strains that are relevant to wounds, such as *S. aureus* ATCC 43300, as well as displayed good free radical scavenging activities. Although the extracts possessed weak CAA, this might be beneficial as appropriate intracellular ROS levels is essential to promote wound healing. The evidence obtained from this study support the traditional use of *E. coccinea* in treating skin wounds, while also highlighting its potential to be developed as a wound treatment agent.

In summary, *E. coccinea*, *E. pubescens*, and *E. sessilanthera* have great potential to be mined for bioactive compounds. The present work supports all conservation effort to preserve these ginger plants in their natural habitat. This is to ensure that they can be further studied in the future.

6.3 Recommendations for future work

Etlingerin has a potential to be used as a drug to treat oxidative stress-related diseases, however, further work should be conducted to assess its antioxidant activities using more cell lines as well as animal models. For example, the SH-SY5Y (neuroblastoma) cell line model can be employed, and this will provide evidence on etlingerin's neuroprotective ability (protection against neurodegenerative diseases). In addition, in vivo studies can also be conducted using mouse models.

Etlingerin also has a potential to be developed as an antimicrobial agent. Even so, it is essential that its mechanism is understood before it can be applied on humans. It was proposed that etlingerin exerts its effect by altering the bacterial membrane permeability. Future studies should confirm the interaction between etlingerin and the bacterial membrane. Electron microscopy can be done to assess the changes in the membrane structure while molecular docking studies could unveil the site where the compound binds to.

Current results have provided preliminary evidence on the wound healing property of *E. coccinea*. Thereafter, further in vivo assessments should be carried out using mouse models. Apart from this, it was also discussed that the weak CAA exhibited by *E. coccinea*'s extracts might be beneficial as they could promote the wound healing process. Future studies should investigate this interesting theory. The ROS levels could be monitored prior to, and after treatment with the extract. In parallel with this, molecular studies can be conducted to evaluate the correlation between the ROS levels and expression of genes associated with cell proliferation and migration.

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Appendix 1 – Access License from Sabah Biodiversity Centre

[redacted]

(Please quote your licence reference number and date if you have any queries)

SABAH BIODIVERSITY COUNCIL
(Majlis Biodiversiti Sabah)

[redacted]

LICENCE REF.NO. [redacted]

DATE OF APPROVAL : 9th SEPTEMBER 2014

ACCESS LICENCE

(Section 8(1) & 15 of Sabah Biodiversity Enactment 2000)

This is to certify that:
Delhousie Daniel Jambun

Passport No./ MyKad No.:
890905-12-5885

of
Monash University Malaysia

is authorized to access the following biological resources from the place(s) specified below, for **ACADEMIC PURPOSES**, upon terms and conditions hereinafter stipulated:

Type of Biological Resources

Common name	*Scientific name*	*Descriptions*	*Place(s) where access is permitted*
Tuhau	*Etlingera coccinea*	15 stalks each, including leaves, stems and roots/rhizomes	• Danum Valley Conservation Area • Kiansom
–	*Etlingera brevilabris*		
–	*Etlingera brevilabrum*		
–	*Etlingera pyramidosphaera*		
–	*Etlingera frimbriobracteata*		
–	*Etlingera velutina*		
–	*Etlingera sessilanthera*		

Terms and conditions

(Section 24 of Sabah Biodiversity Enactment 2000)

1. This licence is valid from **8th October 2014 for a period of 12 months.**
2. The holder must apply relevant pass from the State Immigration of Sabah.
3. This licence is not transferable. The holder must produce this licence for inspection by the Secretary or any person authorized by him.
4. Transfer of biological resources is not allowed without the written consent of the Sabah Biodiversity Council.
5. All biological resources collected shall be taken to, stored and preserved at:
   Universiti Malaysia Sabah
6. The holder must submit progress report and final research report to Sabah Biodiversity Council.

LICENCE REF.NO. : JKM/MBS.1000-2/2 (394)

DATE OF APPROVAL : 9th SEPTEMBER 2014

7. Application for patent or other intellectual property rights to the relevant authority in or outside Malaysia subject to first obtaining the prior written consent of the Sabah Biodiversity Council.

Secretary
and on behalf of Sabah Biodiversity Council

[redacted]

New: Renewal: *X* *(Dr. Abdul Fatah Amir)*

Extended to:
1. Pejabat Hal Ehwal Dalam Negeri & Penyelidikan, JABATAN KETUA MENTERI
2. Jabatan Imigresen Sabah (Bahagian Visa)

Appendix 2 – Export License from Sabah Biodiversity Centre

[redacted]

SABAH BIODIVERSITY COUNCIL
(Majlis Biodiversiti Sabah)

[redacted]

(Please quote your licence reference number and date if you have any queries)

LICENCE REF.NO. : [redacted]

DATE : **15TH DECEMBER 2014**

LICENCE FOR EXPORT OF BIOLOGICAL RESOURCES

(Section 8(2) and 28 of Sabah Biodiversity Enactment 2000)

This is to certify that:
Delhousie Daniel Jambun

Passport/ MyKad No.:
890905-12-5885

of
Monash University Malaysia

is authorized to export to:
Monash University Malaysia, Jalan Lagoon Selatan, 46150 Bandar Sunway, Selangor, Malaysia.

the quantities of biological resources stated hereunder for **ACADEMIC** purposes subject to the terms and conditions stated below:

Type of Biological Resources

Common name	*Scientific Name*	*Amount/number/volume (as appropriate)*	*No. of occasion or shipment*
Tuhau	*Etlingera coccinea*	15 stalks each, including leaves, stems and roots/rhizomes.	*One (1)*
–	*Etlingera brevilabris*		
–	*Etlingera brevilabrum*		
–	*Etlingera pyramidosphaera*		
–	*Etlingera frimbriobracteata*		
–	*Etlingera velutina*		
–	*Etlingera sessilanthera*		

Terms and conditions

1. This licence is valid until:
   14th December 2015
2. The biological resources or associated relevant knowledge or extracts thereof are exported for
   Preliminary research to determine the antimicrobial, anticancer and antioxidant properties of the sampled plants.
3. All biological resources exported shall be examined/analysed at
   Monash University Malaysia, Jalan Lagoon Selatan, 46150 Bandar Sunway, Selangor, Malaysia.

LICENCE REF.NO. : [redacted]

DATE : 15TH DECEMBER 2014

4. The licence holder shall comply with other applicable written laws governing the export of the said material from Sabah.

5. Application for patent or other intellectual property rights to the relevant authority in or outside Malaysia subject to first obtaining the prior written consent of the Sabah Biodiversity Council.

6. Export of the biological resources shall be undertaken in accordance with the terms and conditions of a statutory declaration dated:

26th May 2014

Secretary
and on behalf of Sabah Biodiversity Council

[redacted]

(Dr. Abdul Fatah Amir)

Extended to:
1. Jabatan Kastam Diraja Malaysia

Appendix 3 – Approval License from Danum Valley Management Committee

YS/DVMC/2014/49
24th March 2014

Danum Valley Management Committee

[redacted]

Delhousie Daniel Jambun
School of Science,
Monash Universiti Malaysia
Jalan Lagoon Selatan
46150 Bandar Sunway
Selangor

Dear Mr. Delhousie,

Permission to Conduct Research at Danum Valley Conservation Area: DVMC Approval No.: 2014/10 - Project No. 406

Your application to conduct research in Danum Valley Conservation Area (DVCA) entitled **"Antimicrobial, Antioxidant and Anticancer Properties of Some Etlingera Species from Sabah"** referred.

On behalf of the Danum Valley Management Committee (DVMC), we wish to inform you that we support your research at DVCA with condition that you had obtained approval from Sabah Biodiversity Council (SaBC) for Access License.

We have no objection for you enter DVCA and use our research facilities starting **March 2014 (1 year)** and would like to remind you of the following terms and conditions before you can commence your research:-

1) You must provide us the copy of you Access License from SaBC.

2) You must report to DVMC Secretary before proceeding to DVCA.

3) To report to Manager DVCA upon your arrival in Danum Valley Field Centre.

4) To comply with the research guidelines and procedures, especially those related to appointment of Research Assistants, collection and distribution of specimen and submission of research progress reports .

5) Must settle all costs incurred during your stay Danum Valley Field Centre upon leaving the studies centre.

6) To submit progress reports and final thesis/research report to DVMC Secretary.

7) To obtain approval from DVMC before submitting manuscripts for publication and submit to us copy of any publication arising out from your research.

Please provide copy of this letter to SaBC to support your application for Access License.

Thank you.

Yours sincerely,

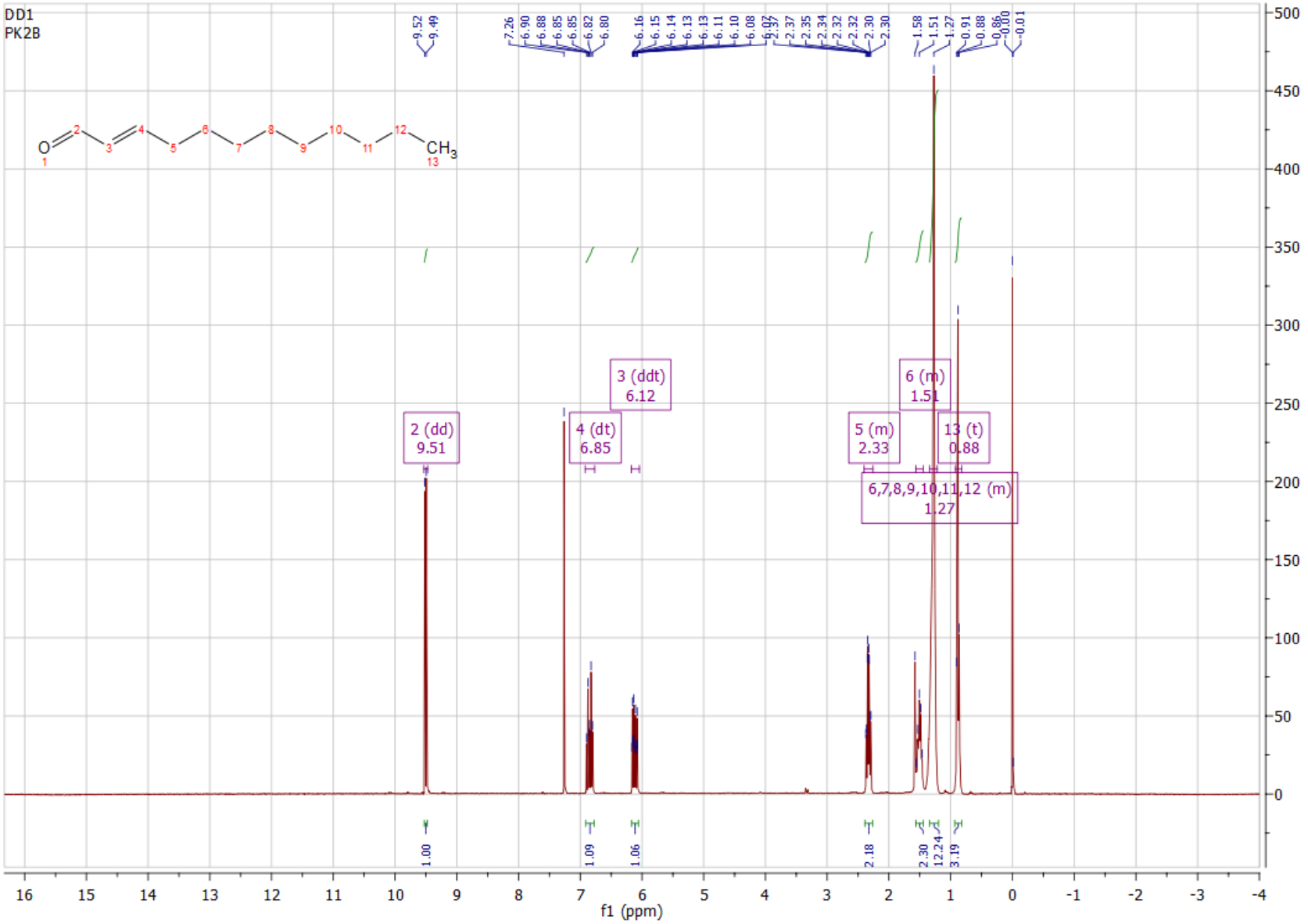
[redacted]

DR. WAIDI SINUN
Secretary
Danum Valley Management Committee

c.c.
- Chairman, DVMC
- Dr. Yap Sau Wai, Conservation & Environmental Management Division, Yayasan Sabah Group
- Manager, DVCA.....*Please ensure procedures/guidelines to do research in DVCA are comply with and, all payments are in order*
- Director, Sabah Biodiversity Centre, Chief Minister's Department

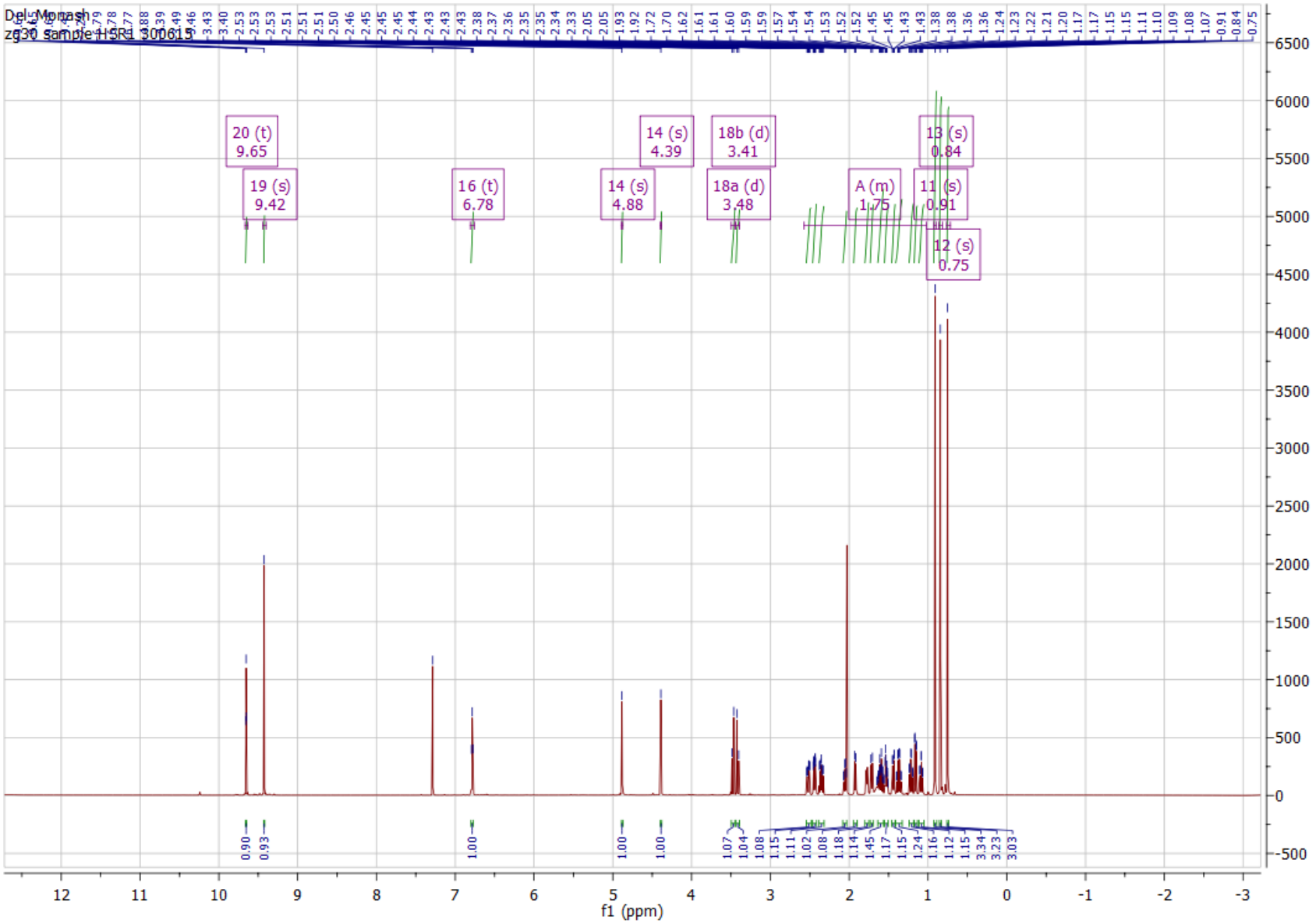
Appendix 4 – ^{1}H Nuclear Magnetic Resonance (NMR)

Assignment of *Trans*-2-dodecenal


DD1
PK2B
9.52
9.49
7.26
6.90
6.88
6.85
6.85
6.82
6.80
6.16
6.15
6.14
6.13
6.13
6.11
6.10
6.08
2.37
2.37
2.35
2.34
2.32
2.32
2.30
2.30
1.58
1.51
1.27
0.91
0.88
0.86
0.00
-0.01
O
CH3
2 (dd)
9.51
3 (ddt)
6.12
4 (dt)
6.85
6 (m)
1.51
5 (m)
2.33
13 (t)
0.88
6,7,8,9,10,11,12 (m)
1.27
1.00
1.09
1.06
2.18
2.30
12.24
3.19
f1 (ppm)

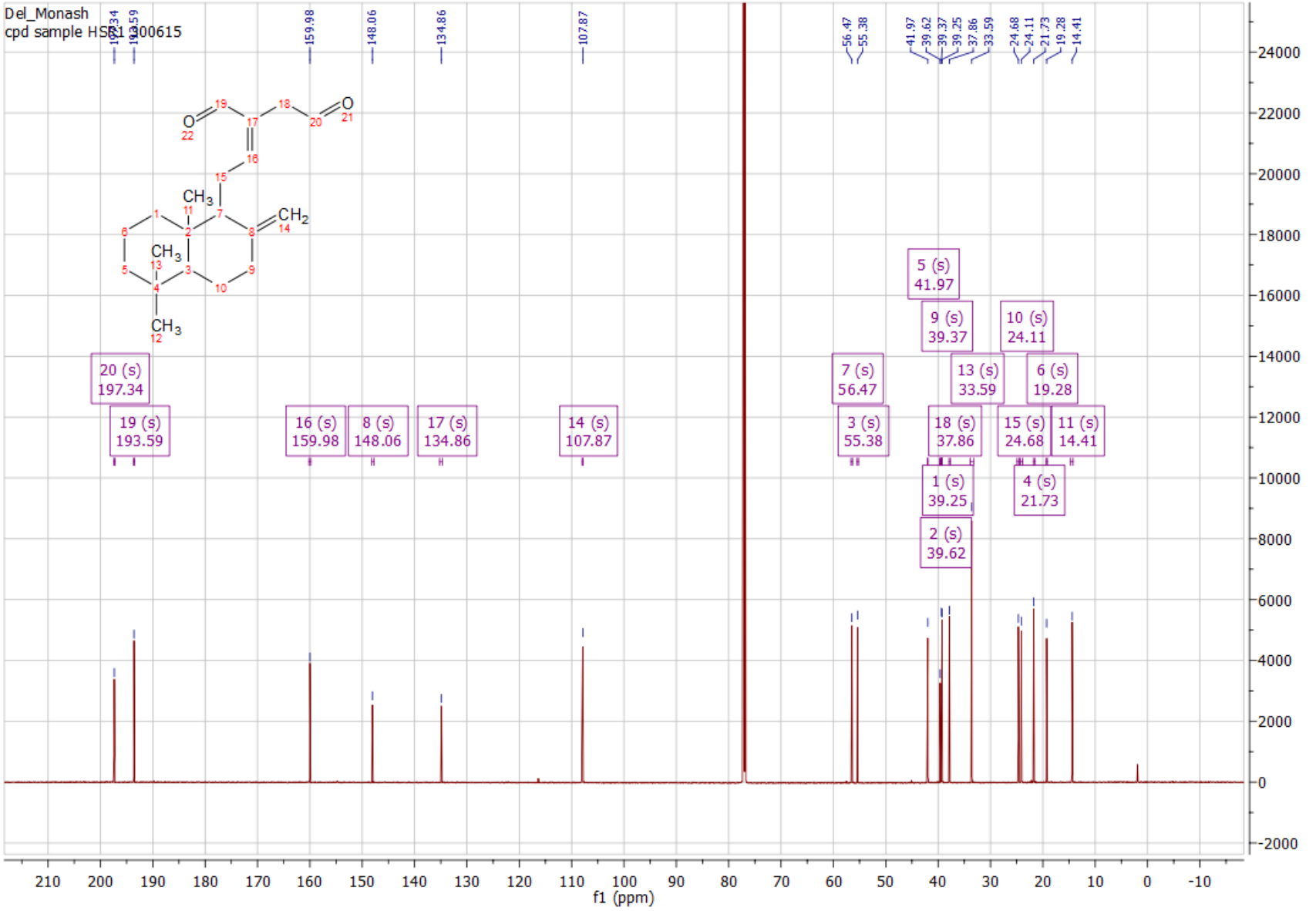
Appendix 5 – ^{1}H Nuclear Magnetic Resonance (NMR)

Assignment of 8(17),12-labdadiene-15,16-dial

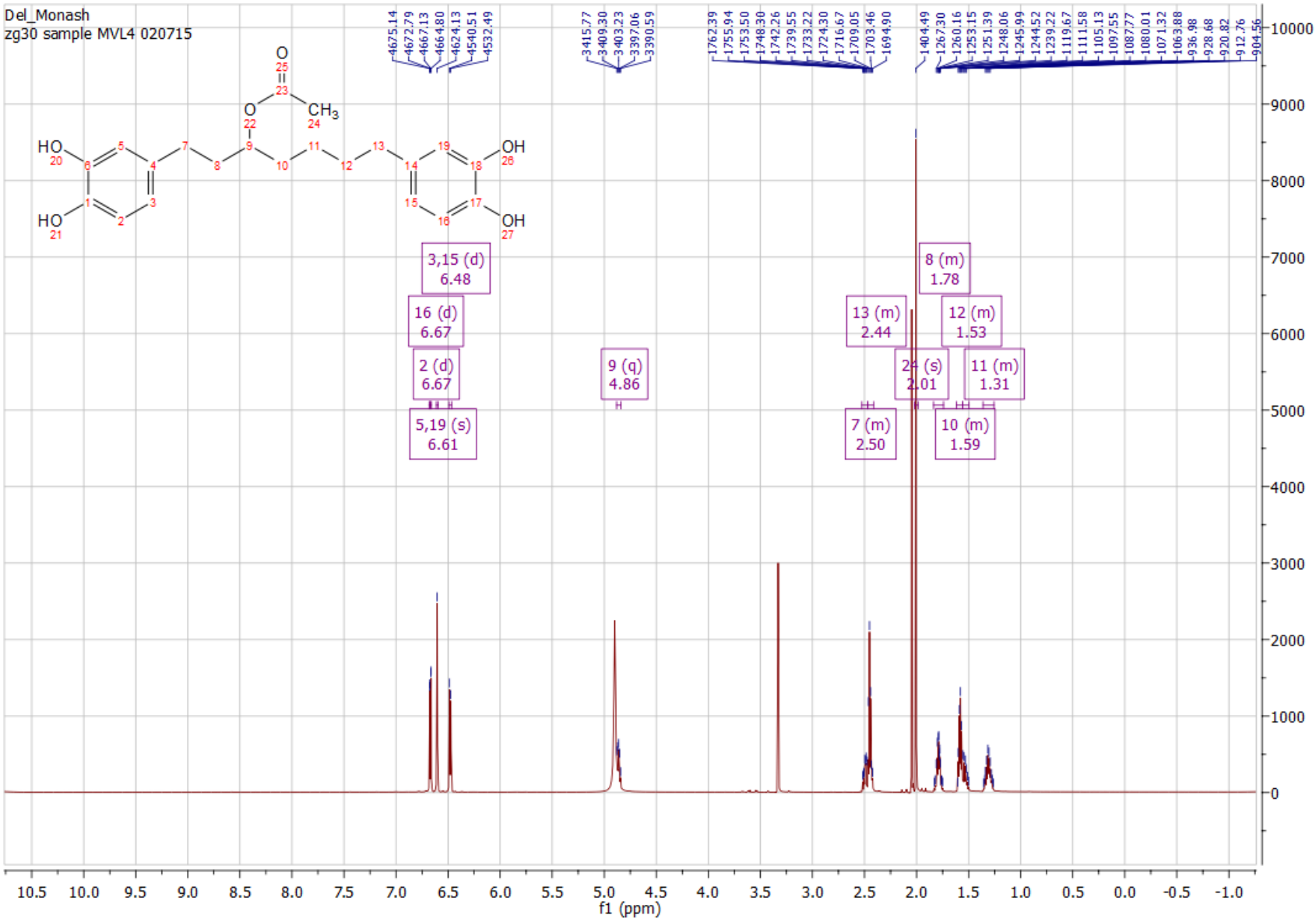

Del Monash
zg30 sample HSR1 300615
20 (t)
9.65
19 (s)
9.42
16 (t)
6.78
14 (s)
4.88
14 (s)
4.39
18a (d)
3.48
18b (d)
3.41
A (m)
1.75
11 (s)
0.91
13 (s)
0.84
12 (s)
0.75
f1 (ppm)

Appendix 6 – ^{13}C Nuclear Magnetic Resonance (NMR)

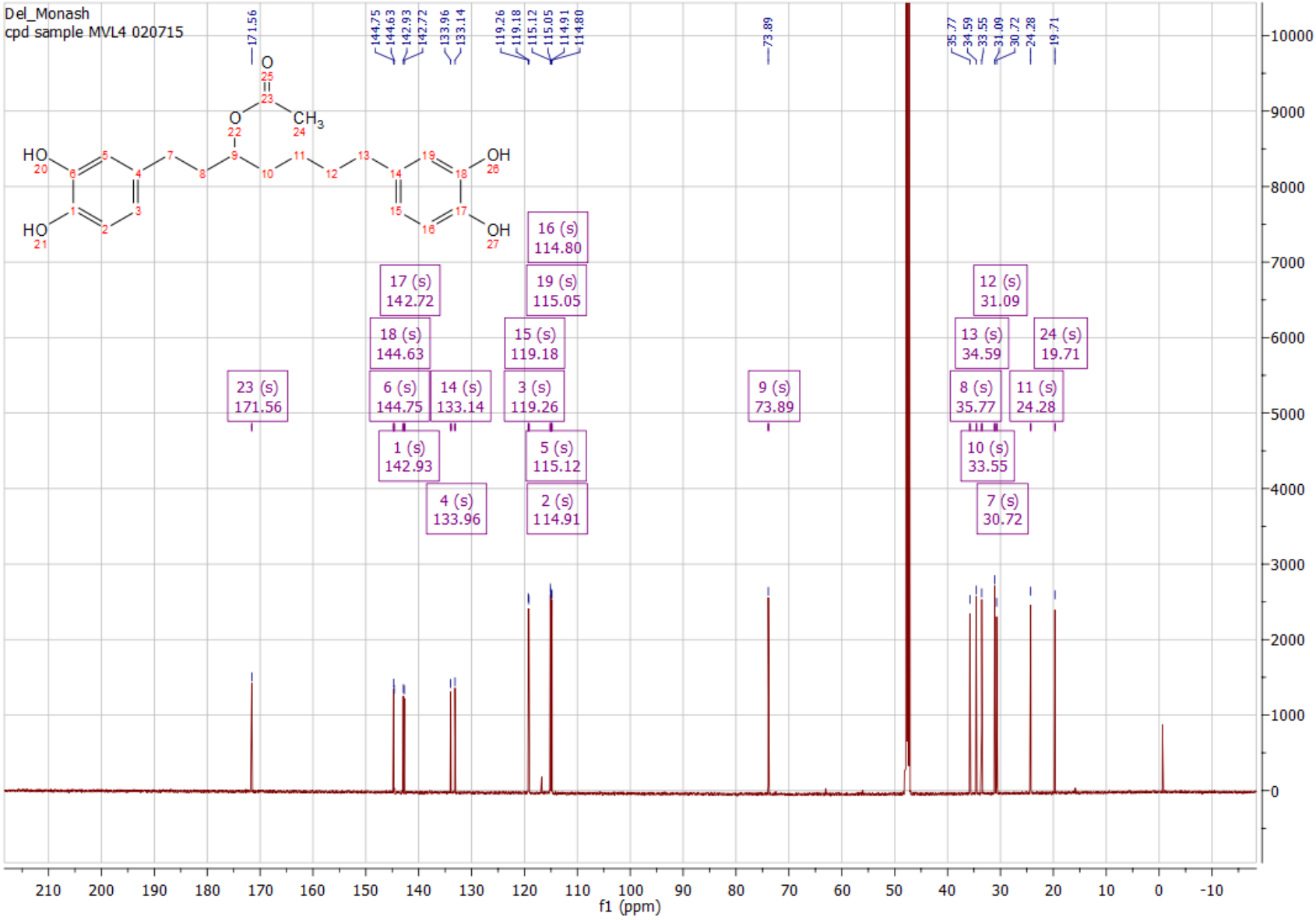
Assignment of 8(17),12-labdadiene-15,16-dial


Del_Monash
cpd sample HS21300615
197.34
193.59
159.98
148.06
134.86
107.87
56.47
55.38
41.97
39.62
39.37
39.25
37.86
33.59
24.68
24.11
21.73
19.28
14.41
20 (s) 197.34
19 (s) 193.59
16 (s) 159.98
8 (s) 148.06
17 (s) 134.86
14 (s) 107.87
7 (s) 56.47
3 (s) 55.38
5 (s) 41.97
9 (s) 39.37
1 (s) 39.25
2 (s) 39.62
18 (s) 37.86
13 (s) 33.59
10 (s) 24.11
15 (s) 24.68
4 (s) 21.73
6 (s) 19.28
11 (s) 14.41
f1 (ppm)

Appendix 7 – ^{1}H Nuclear Magnetic Resonance (NMR) Assignment of 1,7-bis(3,4-dihydroxyphenyl)heptan-3-yl acetate (etlingerin)


Del_Monash
zg30 sample MVL4 020715
3,15 (d)
6.48
16 (d)
6.67
2 (d)
6.67
5,19 (s)
6.61
9 (q)
4.86
13 (m)
2.44
8 (m)
1.78
12 (m)
1.53
24 (s)
2.01
11 (m)
1.31
7 (m)
2.50
10 (m)
1.59
f1 (ppm)

Appendix 8 – ^{13}C Nuclear Magnetic Resonance (NMR) Assignment of 1,7-bis(3,4-dihydroxyphenyl)heptan-3-yl acetate (etlingerin)


Del_Monash
cpd sample MVL4 020715
171.56
144.75
144.63
142.93
142.72
133.96
133.14
119.26
119.18
115.12
115.05
114.91
114.80
73.89
35.77
34.59
33.55
31.09
30.72
24.28
19.71
HO
OH
OH
HO
CH3
23 (s) 171.56
6 (s) 144.75
14 (s) 133.14
1 (s) 142.93
4 (s) 133.96
3 (s) 119.26
5 (s) 115.12
2 (s) 114.91
9 (s) 73.89
8 (s) 35.77
11 (s) 24.28
10 (s) 33.55
7 (s) 30.72
13 (s) 34.59
24 (s) 19.71
12 (s) 31.09
17 (s) 142.72
18 (s) 144.63
15 (s) 119.18
19 (s) 115.05
16 (s) 114.80
f1 (ppm)

Appendix 9 – Publication: Investigation on the antimicrobial activities of gingers (*Etlingera coccinea* (Blume) S.Sakai & Nagam and *Etlingera sessilanthera* R.M.Sm.) endemic to Borneo

Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Investigation on the antimicrobial activities of gingers (*Etlingera coccinea* (Blume) S.Sakai & Nagam and *Etlingera sessilanthera* R.M.Sm.) endemic to Borneo

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3 Conservation and Environmental Management Division, Kota Kinabalu, Sabah, Malaysia
4 Tropical Medicine and Biology, Monash University Malaysia, Bandar Sunway, Selangor, Malaysia

Keywords
(E)-2-dodecenal, 8(17),12-labdadiene-15, 16-dial, labdane diterpene, natural product, *trans*-2-dodecenal, Zingiberaceae.

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E-mail: [redacted]

2017/0972: received 18 May 2017, revised 24 June 2017 and accepted 4 July 2017

doi:10.1111/jam.13536

Abstract

Aims: To investigate the antimicrobial properties of *Etlingera coccinea* and *Etlingera sessilanthera* and to isolate and identify the antimicrobial compounds.
Methods and Results: Extracts were obtained via sequential solvent extraction method using hexane, dichloromethane, ethyl acetate, methanol and water. Antimicrobial activity testing was done using broth microdilution assay against 17 strains of bacteria. The leaf hexane extract of *E. coccinea* and rhizome hexane extract of *E. sessilanthera* showed best antimicrobial activities, with minimum inhibitory concentration (MIC) values ranging from 0·016 to 1 mg ml^{-1} against Gram-positive bacteria. From these active extracts, two antimicrobials were isolated and identified as *trans*-2-dodecenal and 8(17),12-labdadiene-15,16-dial with MIC values ranging from 4 to 8 μg ml^{-1} against *Bacillus cereus*, *Bacillus subtilis* and *Staphylococcus aureus*.
Conclusion: *Etlingera coccinea* and *E. sessilanthera* demonstrated good antimicrobial activities against clinically relevant bacteria strains. The antimicrobial compounds isolated showed low MIC values, hence suggesting their potential use as antimicrobial agents.
Significance and Impact of the Study: This study is the first to identify the potent antimicrobials from these gingers. The antimicrobials isolated could potentially be developed further for use in treatment of bacterial infections. Also, this study warrants further research into other *Etlingera* species in search for more antimicrobial compounds.

Introduction

Zingiberaceae is a large family of ginger plants commonly found throughout Southeast Asia. Some of its species are widely cultivated for consumption and traditional medicine purposes (Sirirugsa 1999). One example is the common ginger (*Zingiber officinale*). While being common as an ingredient in many Asian cuisines, it also has a wide range of medicinal uses such as treatment of asthma, rheumatism, stroke and toothache (Ali *et al.* 2008). The genus *Etlingera* is a member of Zingiberaceae family. Thus far, a total of 100 species of *Etlingera* have been identified with most being endemic to Borneo (Poulsen 2006; The Plant List 2013). Out of the 100 species, *Etlingera elatior* is the most well-known. The young flowers of *E. elatior* are commonly used as an ingredient for sour fish curry as well as some local Malaysian delicacies such as *asam laksa*, *nasi kerabu* and *nasi ulam* (Larsen *et al.* 1999; Wijekoon *et al.* 2011). In Borneo itself, the indigenous people use *Etlingera* plants as food, ornaments and traditional medicines (e.g. treatment for ailments such as stomach ache, wound, snake bite and others) (Poulsen 2006). This study focused on *Etlingera coccinea* and *Etlingera sessilanthera*.

Etlingera coccinea (Fig. 1) is a species endemic to Borneo. In Sabah, it is locally known as *tuhau* by the Kadazan/Dusun community and often eaten in the form of pickled vegetable (Poulsen 2006). It grows to an average height of about 2 m, and the strong coriander-like smell separates *E. coccinea* from other species of *Etlingera*. Its documented traditional medicinal uses include treatment of wounds, stomach ache, food poisoning and urine cleanser (Poulsen 2006; Vairappan *et al.* 2012). *Etlingera sessilanthera* (Fig. 1) is locally known as *tubu pelapad* by the Kelabit community, and this species of ginger grows up to 3 m tall. According to Poulsen (2006), it is characterized by purple tinges on the edge of the underside of the leaves. The stems of *E. sessilanthera* are usually eaten either raw or cooked as vegetable.

Antimicrobial resistance is known to be one of the most serious health threat in this century. The Centers for Disease Control and Prevention (CDC) reported that in the United States, at least two million people are infected with antimicrobial resistant bacteria and at least 23 000 died each year (CDC 2013). As the healthcare industry is running out of antimicrobials to treat infections by resistant bacteria, the CDC highly encourages the search and development of new antimicrobials. This emphasizes the importance of studying plants as newly discovered drugs could be used to address this issue.

The use of *E. coccinea* in treatment of wound and stomach ache drove our interest to study its antimicrobial property as these are often related to bacterial infections. Through literature search, only one published study had reported positive antimicrobial activity of *E. coccinea*. Although positive activity was found, Vairappan *et al.* (2012) only studied the essential oil extract from the plant's rhizomes. It is noteworthy that the indigenous people use the young shoots and stems for medicinal use (Poulsen 2006). In this research, the leaves, stems and rhizomes of *E. coccinea* were investigated, while the crude extracts were obtained via ultrasonic-assisted extraction using various solvents of different polarities. This provides a broader overview of the plant's antimicrobial property as different parts of plant as well as different polarities of phytochemicals were studied. As for *E. sessilanthera*, no published study was found on its antimicrobial property, thus highlighting the novelty of this research. Ginger plants are well-known for their antimicrobial property, and since other *Etlingera* species had been reported to exhibit good antimicrobial activities (Chan *et al.* 2007; Tadtong *et al.* 2009; Mahdavi *et al.* 2012; Hossan *et al.* 2013; Wijekoon *et al.* 2013), it was worthwhile to investigate whether *E. sessilanthera* possess the same property.

Materials and methods

Plant material

Etlingera coccinea and *E. sessilanthera* and specimens were sampled from Danum Valley, Sabah (GPS coordinates of 04°58′11·4″N, 117°48′41·5″E and 04°57′57·1″N, 117°48′3″E, respectively), with permission from the Sabah Biodiversity Centre (SaBC) and Conservation and Environmental Management Division, Yayasan Sabah (CEMD). Identification of the specimens was conducted by Mr. Bernadus Bala Ola, a local botanist from Danum Valley Conservation Area (DVCA), and Mr. John Sugau, from Forest Research Centre (FRC) Sabah. The leaves, stems and rhizomes were sampled haphazardly from mature plants (with heights approximately between 1 and 2 m tall). Voucher specimens were deposited at the Forest Department Herbarium Sandakan, Sepilok, 90000 Sandakan, Sabah, Malaysia, with the reference numbers 157373 (*E. coccinea*) and 157378 (*E. sessilanthera*).

Extraction of plant samples

Plant specimens were washed and dried using a freeze drier (Freezone 4.5 Plus; Labconco, Kansas City, Missouri). With aid of liquid nitrogen, the specimens were crushed into smaller parts. Sequential solvent extraction was carried out to extract the phytochemicals. Hexane was added to the crushed specimens and then subjected to sonication (35-kHz frequency; Elmasonic S130H; Elma, Fremont, California) for 15 min, followed by filtration of the solution. These steps were conducted three times. The filtered solutions were pooled and

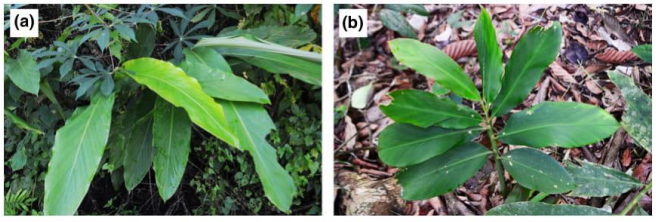

Figure 1 (a) *Etlingera coccinea* and (b) *Etlingera sessilanthera* plants in their natural habitat.

concentrated under reduced pressure using a rotary evaporator (N-1110; Eyela, Tokyo, Japan). With the same plant material, extraction was conducted again using dichloromethane, ethyl acetate, methanol and distilled water, sequentially. Finally, the extracts were dried using a freeze drier (Freezone 4.5 Plus; Labconco).

Bacterial strains

The following bacterial strains were purchased from American Type Culture Collection (ATCC): *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 10145, *Salmonella* Typhimurium ATCC 14028, *Shigella flexneri* ATCC 12022, *Enterococcus faecalis* ATCC 700802, *E. faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 43300, *Staph. aureus* ATCC 700699, *Staph. aureus* ATCC 6538P, *Staph. aureus* ATCC 29213, *Bacillus cereus* ATCC 14579, *Streptococcus pneumoniae* ATCC 49136, *Staphylococcus epidermidis* ATCC 12228, *B. subtilis* ATCC 8188, *Proteus vulgaris* IMR, *Proteus mirabilis* ATCC 12453, and *Salmonella* Ent ATCC 13076. All bacterial strains were maintained on tryptic soy agar (Merck, Darmstadt, Germany) at 37°C for 24 h and in 25% v/v glycerol at −80°C for long-term preservation.

Antimicrobial activity testing

Broth microdilution assay was conducted using 96-well flat-bottomed microtitre plates, as described by the Clinical and Laboratory Standards Institute (CLSI), with modifications (CLSI 2012). Plant extracts were prepared in 5% Tween 80 + 95% acetonitrile (for hexane, dichloromethane, ethyl acetate and methanol extracts) and diluted 10 times using TSB broth. Distilled water was used as solvent for the water extract. A twofold serial dilution was performed to prepare the required concentrations of plant crude extracts in the microtitre plates (4 to 0·032 mg ml^{-1} with final volume of 100 μl). Then, 100 μl of adjusted bacterial suspension (containing approximately 1×10^6 CFU per ml) was added into each well. The solvents used to dissolve the extracts were used as negative controls. Chloramphenicol with concentration of 200 μg ml^{-1} was used as positive control. After incubation at 37°C for 24 h, the minimum inhibitory concentration (MIC) was assessed. Clear wells with the lowest concentration of extracts were taken as MIC values.

Isolation and identification of compound

Thin layer chromatography

Thin layer chromatography (TLC) was performed to separate the components of the extracts with positive antimicrobial activities (leaf hexane extract of *E. coccinea* and rhizome hexane extract of *E. sessilanthera*); 10 mg ml^{-1} of extracts was prepared and spotted on a silica gel plate. The plate was then developed using HEX-EA (85:15) and visualized by heating the plate on a hot plate for 30 s after spraying with dilute sulphuric acid (0·1 N). The visible spots were grouped into separate regions (Fig. 2). Partial purification of the antimicrobial compounds was carried out through preparative TLC method. Five hundred microlitres of extracts with concentration of 50 mg ml^{-1} was prepared and introduced on a preparative silica gel plate (175–225 μm layer thickness). The plates were developed using the same mobile phase as stated previously. Each region (as determined using analytical TLC) was scrapped off from the plate and extracted using MeOH. The solutions obtained were subjected to sonication (35-kHz frequency; Elmasonic S130H, Elma), filtered, concentrated under reduced pressure using a rotary evaporator (N-1110; Eyela) and finally dried using freeze drier (Freezone 4.5 Plus).

High-performance liquid chromatography

Region 2 extracts of *E. coccinea* and *E. sessilanthera* showed positive antimicrobial activity. Therefore, high-performance liquid chromatography (HPLC) was employed to purify the antimicrobial compounds. The

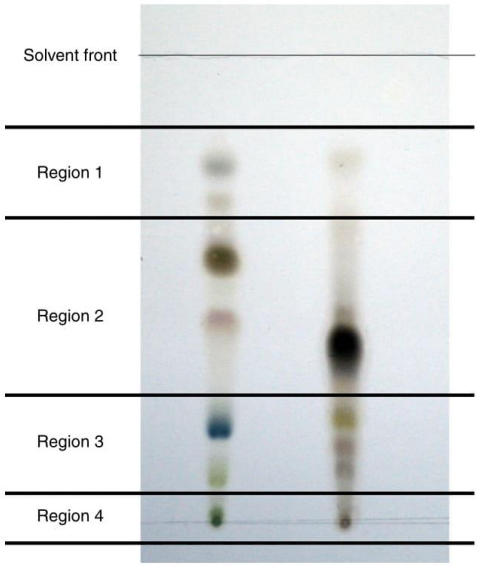

Figure 2 Images of developed thin layer chromatography plates (after sulphuric acid treatment) showing the components in (left) *Etlingera sessilanthera* rhizome hexane extract and (right) *Etlingera coccinea* leaf hexane extract.

partially purified extracts from preparative TLC were dissolved in acetonitrile and filtered through 0·22 μm polytetrafluoroethylene membrane. Pure *trans*-2-dodecenal from *E. coccinea* was obtained by preparative HPLC with the following conditions: Cosmosil C18 preparative column (20 × 250 mm, 5-micron particle); solvent system: A-water, B-methanol; 90% B for 13 min; flow rate: 20 ml min^{-1}; injection volume: 100 μl; concentration: 100 mg ml^{-1}. The compound was observed at t_R = 7·00 min. Pure 8(17),12-labdadiene-15,16-dial from *E. sessilanthera* was obtained by preparative HPLC with the following conditions: Cosmosil C18 preparative column (20 × 250 mm, 5-micron particle), solvent system: A-water, B-acetonitrile; 80% B for 6 min, 100% B for 4 min; flow rate: 30 ml min^{-1}; injection volume: 100 μl; concentration: 100 mg ml^{-1}. The compound was observed at t_R = 7·55 min.

LC-MS analysis

The accurate masses of *trans*-2-dodecenal and 8(17),12-labdadiene-15,16-dial were determined using positive ion mode through electrospray ionization–mass spectrometry. The following conditions were used to identify the mass of *trans*-2-dodecenal: Agilent 1290 Infinity LC instrument coupled to Agilent 6520 Accurate-Mass Q-TOF (Agilent, Santa Clara, CA, USA) Agilent Zorbax SB-C18 (2·1 × 150 mm, 3·5-micron particle); solvent system: A-0·1% formic acid, B-acetonitrile with 0·1% formic acid; 90% B for 15 min; flow rate: 0·5 ml min^{-1}; injection volume: 1 μl; concentration: 1 mg ml^{-1}. The compound was observed at t_R = 1·58 min. The following conditions were used to identify the mass of 8(17),12-labdadiene-15,16-dial: Acquity UPLC instrument (Waters, Milford, Massachusetts), Acquity UPLC BEH C18 column (2·1 × 50 mm, 1·7-micron particle); solvent system: A-0·1% formic acid, B-acetonitrile with 0·1% formic acid; 40–100% B over 2 min, 100% B for 0·5 min; flow rate: 0·5 ml min^{-1}; injection volume: 5 μl; concentration: 1 mg ml^{-1}. The compound was observed at t_R = 2·18 min.

Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) analyses were performed using Varian Unity Plus instrument (Bruker, Karlsruhe, Germany) to aid structure elucidation. ^{1}H NMR (300 MHz) experiment was done for the identification of trans-2-dodecenal while ^{1}H NMR (700 MHz) and ^{13}C (176 MHz) experiments were done for the identification of 8(17),12-labdadiene-15,16-dial.

8(17),12-labdadiene-15,16-dial

$[\alpha]_D^{25}$ = +44° (c = 0·04, ethanol). ^{1}H NMR (700 MHz, $CDCl_3$) δ 9·65 (t, J = 1·4 Hz, 1H), 9·42 (s, 1H), 6·78 (t, J = 6·6 Hz, 1H), 4·88 (s, 1H), 4·39 (s, 1H), 3·45 (dd, J = 43·2, 16·7 Hz, 2H), 2·52 (ddd, J = 16·8, 6·3, 2·9 Hz, 1H), 2·44 (ddd, J = 12·9, 4·2, 2·4 Hz, 1H), 2·39–2·32 (m, 1H), 2·08–2·03 (m, 1H), 1·92 (d, J = 10·9 Hz, 1H), 1·78 (ddt, J = 12·9, 5·0, 2·4 Hz, 1H), 1·71 (d, J = 12·4 Hz, 1H), 1·63–1·51 (m, 2H), 1·44 (dd, J = 13·2, 1·2 Hz, 1H), 1·37 (qd, J = 13·0, 4·3 Hz, 1H), 1·21 (td, J = 13·4, 4·0 Hz, 1H), 1·16 (dd, J = 12·6, 2·7 Hz, 1H), 1·09 (td, J = 12·8, 3·9 Hz, 1H), 0·91 (s, 3H), 0·84 (s, 3H), 0·75 (s, 3H). ^{13}C NMR (176 MHz, $CDCl_3$) δ 197·34, 193·59, 159·98, 148·06, 134·86, 107·87, 56·47, 55·38, 41·97, 39·62, 39·37, 39·25, 37·86, 33·59, 24·68, 24·11, 21·73, 19·28, 14·41. LCMS (ESI) *m/z* 302·22.

Trans-2-dodecenal

^{1}H NMR (300 MHz, $CDCl_3$) δ 9·51 (d, J = 7·9 Hz, 1H), 6·85 (dt, J = 15·6, 6·8 Hz, 1H), 6·12 (ddt, J = 15·6, 7·9, 1·5 Hz, 1H), 2·33 (ddd, J = 14·6, 7·0, 1·5 Hz, 2H), 1·51 (dt, J = 14·3, 7·3 Hz, 2H), 1·27 (s, 12H), 0·88 (t, J = 6·7 Hz, 3H). LCMS (ESI) *m/z* 182·17. ^{13}C NMR (Forbes *et al.* 2014) δ 194·1 (CH), δ 159·0 (CH), δ 132·9 (CH), δ 32·7 (CH_2), δ 31·8 (CH_2), δ 29·4 (CH_2), δ 29·3 (CH_2), δ 29·2 (CH_2), δ 29·16 (CH_2), δ 27·8 (CH_2), δ 22·6 (CH_2), δ 14·0 (CH_3).

Results

The antimicrobial activity of *E. coccinea* and *E. sessilanthera* crude extracts was tested against 17 bacteria strains using the broth microdilution assay, and the MIC values determined are summarized in Tables 1 and 2. In general, extracts of *E. coccinea* and *E. sessilanthera* showed inhibition activity against Gram-positive bacteria only.

The most active extracts from *E. coccinea* and *E. sessilanthera* originated from their leaves and rhizomes, respectively. More specifically, their hexane extracts were associated with lowest MIC values (ranging from 0·016 to 0·063 mg ml^{-1} and 0·125 to 1 mg ml^{-1}, respectively). Therefore, it was of our interest to isolate and identify the antimicrobial compounds from these extracts. Briefly, the components in the extracts were first separated and isolated through TLC, and following antimicrobial activity testing, only 'region 2' extracts from both *E. coccinea* and *E. sessilanthera* showed inhibition activities. These extracts were subjected to purification work via preparative HPLC. From this, two compounds exhibiting strong antimicrobial activities were isolated and identified as *trans*-2-dodecenal (from *E. coccinea*) and 8(17),12-labdadiene-15,16-dial (from *E. sessilanthera*). All spectroscopic data were consistent with previous literatures on these compounds (Itokawa *et al.* 1980; González *et al.* 2010; Forbes *et al.* 2014). Figures 3 and 4 illustrate the chemical structures of these compounds.

Table 1 Minimum inhibitory concentration (MIC) values of three different parts of *Etlingera coccinea* against 17 strains of bacteria

		Minimum inhibitory concentrations (mg ml^{-1})																
Part of plant	Solvent	*Staphylococcus aureus* ATCC 700699	*Staph. aureus* ATCC 43300	*Staph. aureus* ATCC 6538P	*Staph. aureus* ATCC 29213	*Staphylococcus epidermidis* ATCC 12228	*Bacillus subtilis* ATCC 8188	*Bacillus cereus* ATCC 14579	*Enterococcus faecalis* ATCC 29212	*E. faecalis* ATCC 700802	*Streptococcus pneumoniae* ATCC 49136	*Shigella flexneri* ATCC 12022	*Escherichia coli* ATCC 25922	*Pseudomonas aeruginosa* ATCC 10145	*Proteus vulgaris* IMR	*Proteus mirabilis* ATCC 12453	*Salmonella* Enteritidis ATCC 13076	*Salmonella* Typhimurium ATCC 14028
Leaf	HEX	0·031	0·031	0·031	0·031	0·016	0·016	0·016	0·063	0·063	0·016	>1	>1	>1	>1	>1	>1	>1
	DCM	0·063	0·063	0·063	0·063	0·031	0·031	0·031	0·031	0·031	0·031	>1	>1	>1	>1	>1	>1	>1
	EA	0·063	0·063	0·063	0·063	0·031	0·031	0·031	0·125	0·125	0·031	>1	>1	>1	>1	>1	>1	>1
	MeOH	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	H_2O	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
Stem	HEX	0·125	0·125	0·125	0·125	0·063	0·063	0·063	0·125	0·125	0·063	>1	>1	>1	>1	>1	>1	>1
	DCM	0·125	0·125	0·125	0·125	0·063	0·063	0·063	0·125	0·125	0·063	>1	>1	>1	>1	>1	>1	>1
	EA	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	MeOH	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	H_2O	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
Rhizome	HEX	0·5	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	DCM	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	EA	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	MeOH	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	H_2O	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1

MIC values were determined with three biological replicates. Concentration listed was based on final working concentration. HEX = hexane, DCM = dichloromethane, EA = ethyl acetate, MeOH = methanol, H_2O = water.

Table 2 Minimum inhibitory concentration (MIC) values of three different parts of *Etlingera sessilanthera* against 17 strains of bacteria

		Minimum inhibitory concentrations (mg ml^{-1})																
Part of plant	Solvent	*Staphylococcus aureus* ATCC 700699	*Staph. aureus* ATCC 43300	*Staph aureus* ATCC 6538P	*Staph aureus* ATCC 29213	*Staph epidermidis* ATCC 12228	*Bacillus subtilis* ATCC 8188	*B. cereus* ATCC 14579	*Enterococcus faecalis* ATCC 29212	*E. faecalis* ATCC 700802	*Streptococcus pneumoniae* ATCC 49136	*Shigella flexneri* ATCC 12022	*Escherichia coli* ATCC 25922	*Pseudomonas aeruginosa* ATCC 10145	*Proteus vulgaris* IMR	*Proteus mirabilis* ATCC 12453	*S.* Enteritidis ATCC 13076	*Salm.* Typhimurium ATCC 14028
Leaf	HEX	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	DCM	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	EA	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	MeOH	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	H_2O	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
Stem	HEX	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	DCM	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	EA	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	MeOH	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	H_2O	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
Rhizome	HEX	1	1	>1	>1	1	0·5	0·125	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	DCM	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	EA	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	MeOH	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	H_2O	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1

MIC values were determined with three biological replicates. HEX = hexane, DCM = dichloromethane, EA = ethyl acetate, MeOH = methanol, H_2O = water.

The antimicrobial activity of *trans*-2-dodecenal and 8 (17),12-labdadiene-15,16-dial were evaluated against *Staph. aureus* ATCC 43300, *Staph. aureus* ATCC 6538P, *B. cereus* ATCC 14579, *B. subtilis* ATCC 8188, *P. aeruginosa* ATCC 10145, *E. coli* ATCC 25922, *Salm.* Typhimurium ATCC 14028 and *P. mirabilis* ATCC 12453 and the outcome is shown in Table 3. Both compounds were able to inhibit the Gram-positive bacteria with MIC values ranging from 4 to 8 μg ml^{-1}.

Discussion

The ineffectiveness of the extracts towards Gram-negative bacteria could be due to the presence of lipopolysaccharide layer in their membrane which can act as a barrier that limits permeability of antimicrobials (Blair *et al.* 2015). This observation was also not surprising as other species of *Etlingera* were found to exhibit antimicrobial activity against Gram-positive bacteria only (Chandarana *et al.* 2005; Wong *et al.* 2006a,b; Chan *et al.* 2007). However, Vairappan *et al.* (2012) reported that the essential oil extract from *E. coccinea* rhizomes was able to inhibit Gram-negative bacteria. Although so, the differences between extraction protocols employed (extraction method affects type/amount of phytochemicals isolated), strains of bacteria used (different strains of the same bacteria species may possess different susceptibility towards the same antimicrobial) and location of plant specimens obtained (plants of the same species grown in different geographical area may contain different type/amount of phytochemical) could account for the differing observations (Silva *et al.* 1998; Wallaart *et al.* 2000; Crisóstomo *et al.* 2001). Not only that, since antimicrobial activity testing was done on crude extracts, there was a possibility of antagonism occurring between the extracts' constituents. For example, the combinations of carvacrol/thymol, carvacrol/eugenol and thymol/menthol were found to be ineffective against *Staph. aureus* and *E. coli* as compared with their individual counterparts (Gallucci *et al.* 2009). Regardless, the crude extracts were able to inhibit growth of wound-associated bacteria such as *Staph. aureus* and *Staph. epidermidis*, thus supporting their use in treatment of wounds by the indigenous community.

The antimicrobial compounds isolated exhibited MIC values that were comparable with many conventional antibiotics used to treat infections (1–8 μg ml^{-1}), thereby supporting their potential use in clinical settings (Patel *et al.* 2014).

Trans-2-dodecenal, or (E)-2-dodecenal, can be found in various sources such as culantro (*Eryngium foetidum*), coriander (*Coriandrum sativum*), in several bacteria species and even in the millipede, *Rhinocricus insulates* (Wheeler *et al.* 1964; Eyres *et al.* 2005; Blom *et al.* 2011). This study is the first to report the presence of this compound in the genus *Etlingera*. In terms of its antimicrobial activity, this compound was hypothesized to exert its killing effect through membrane disruption, as reported by Kubo *et al.* (2004). In addition, the work by Trombetta *et al.* (2002) on several 2E-alkenals suggested that apart from alterations in membrane permeability, these compounds could result in changes in other membrane functions as well as penetrate into the bacterial cells and interact with other cell constituents. As *trans*-2-dodecenal possesses α,β-unsaturated aldehyde structure, it can potentially act as an alkylating agent which could then interact with proteins and nucleic acids, thus affect bacterial growth (Witz 1989; Maillard 2002). Based on our results, *trans*-2-dodecenal was found to be inactive against the Gram-negative bacteria (Table 3). One plausible reason for this is related to the fact that Gram-positive bacteria utilizes the redox buffer bacillithiol, while the tripeptide glutathione is predominant in Gram-negative bacteria (Loi *et al.* 2015). Glutathiones have been associated with resistance to alkylating agents and therefore might explain the ineffectiveness of *trans*-2-dodecenal towards Gram-negative bacteria (Colvin *et al.* 1993). Contrariwise, Kubo *et al.* (2004) reported that the same compound was active against *Salmonella* Choleraesuis, a Gram-negative bacteria (Kubo *et al.* 2004). This could be due to the differences in experimental procedures such as bacterial strains and culture media used (different strains might have different susceptibility while culture media used can affect a compound's antimicrobial activity) (Crisóstomo *et al.* 2001; Ando *et al.* 2010). Nonetheless, its remarkably low MIC value against clinically relevant pathogens such as *Staph. aureus* warrants further work into its possible uses. One potential application of *trans*-2-dodecenal would be in wound dressings. As this compound is volatile (due to its strong smell), it might be able to exhibit its antimicrobial effect while in its vapour phase. This effect can be seen in cinnamon and clove essential oils (Goni *et al.* 2009).

Classified as a labdane diterpene, 8(17),12-labdadiene-15,16-dial can be found in other ginger plants such as *Alpinia nigra*, *Alpinia zerumbet*, *Zingiber ottensii* and *Siphonochilus aethiopicus* (Akiyama *et al.* 2006; Chompoo *et al.* 2011; Igoli *et al.* 2012; Ghosh *et al.* 2013). To date, no other studies have discovered the presence of labdane diterpenes in the *Etlingera* genus except for 6-hydroxy-labda-8(17),11,13-trien-15,16-olide which had been isolated from *E. elatior* (Mohamad *et al.* 2005). 8(17),12-labdadiene-15,16-dial is similar to *trans*-2-dodecenal in which both contain α,β-unsaturated aldehyde structures. Therefore, the former's potential mechanism of antimicrobial could be similar to the latter and this was discussed previously. Several studies have evaluated the

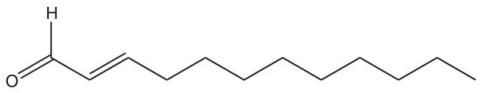

Figure 3 The chemical structure of *trans*-2-dodecenal.

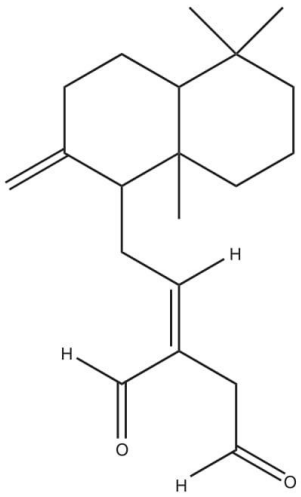

Figure 4 The chemical structure of 8(17),12-labdadiene-15,16-dial.

antimicrobial activity of 8(17),12-labdadiene-15,16-dial. One interesting finding was reported by Ghosh *et al.* (2013) in which 8(17),12-labdadiene-15,16-dial isolated from *Alpinia nigra* seeds was found to be active against both Gram-positive and Gram-negative bacteria with MIC values ranging from 12·5 to 25 μg ml^{-1}. In this study, no activity was found against the Gram-negative bacteria (Table 3). This might be due to the fact that 8(17),12-labdadiene-15,16-dial can exist as (+) and (−) stereoisomers and that different drug conformation can affect the efficacy of the said drug (Itokawa *et al.* 1980; McConathy and Owens 2003; González *et al.* 2010). Due to the site-specific properties of many drug receptors, different stereoisomers can exhibit different pharmacokinetics and pharmacological properties. For example, the (−) isomer of ofloxacin was found to be much more active in inhibiting bacterial growth than its (+) isomer (Tanaka *et al.* 1990). In another study, (+)-α-Pinene was found to be active against *Staph. aureus*, *E. coli*, *Candida albicans* and *Micrococcus luteus* while (−)-α-Pinene failed to inhibit any of the aforementioned pathogens. The optical rotation of 8(17),12-labdadiene-15,16-dial determined in this study was (+44) while in the work by Ghosh *et al.* (2013), the optical rotation was not mentioned. Therefore, further work should investigate the structure–activity relationship of 8(17),12-labdadiene-15,16-dial stereoisomers.

In terms of cytotoxicity, the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) concluded that there are no safety concerns on the use of *trans*-2-dodecenal (Joint FAO/WHO Expert Committee on Food Additives 2005). However, 8(17),12-labdadiene-15,16-dial was found to exhibit IC_{50} values (concentration which inhibit 50% of cells) of 13·1 ± 3·9 μg ml^{-1} and 19·5 ± 1·9 μg ml^{-1} against Vero and HeLa cell lines, respectively (González *et al.* 2010). The IC_{50} values of both compounds are higher than the MIC values determined against Gram-positive bacteria tested in this study, hence suggesting their potential to be used as antimicrobial agents (i.e. the compounds can inhibit bacterial growth while not being toxic to normal cells at the required concentration). However, further cytotoxicity studies should be performed to fully evaluate their safety for clinical use. There is also an opportunity for future work to modify the 8(17),12-labdadiene-15,16-dial to enhance its antimicrobial activity while reducing toxicity towards mammalian cells.

Table 3 Minimum inhibitory concentration (MIC) values of *trans*-2-dodecenal and 8(17),12-labdadiene-15,16-dial against several Gram-positive and Gram-negative bacteria

	Minimum inhibitory concentrations (μg ml^{-1})							
Compound	*Staphylococcus aureus* ATCC 43300	*S. aureus* ATCC 6538P	*Bacillus cereus* ATCC 14579	*B. subtilis* ATCC 8188	*Pseudomonas aeruginosa* ATCC 10145	*Escherichia coli* ATCC 25922	*Salmonella* Typhimurium ATCC 14028	*Proteus mirabilis* ATCC 12453
Trans-2-dodecenal	8	8	4	4	>1000	>1000	>1000	>1000
8(17),12-labdadiene-15,16-dial	8	4	4	4	>1000	>1000	>1000	>1000

MIC values were determined with three biological replicates.

In this study, *E. coccinea* and *E. sessilanthera* extracts were shown to possess good antimicrobial activities. The fact that *E. coccinea* extracts were able to inhibit growth of bacteria supports its use in treatment of wounds by the indigenous community. From the active extracts, two antimicrobial compounds exhibiting remarkable antimicrobial activities were isolated and identified as *trans*-2-dodecenal and 8(17),12-labdadiene-15,16-dial. Combined with the fact that these compounds are not toxic at the concentrations required to effectively inhibit bacterial growth, their potential to be used as antimicrobial agents should be further investigated.

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Conflict of Interest

No conflict of interest declared.

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Appendix 10 – Manuscript: Wound healing property of a ginger (*Etlingera coccinea* (Blume) S.Sakai & Nagam and *Etlingera sessilanthera* R.M.Sm.) used by the indigenous community in Sabah, Malaysia

Wound healing property of a ginger (*Etlingera coccinea* (Blume) S.Sakai & Nagam) commonly used by the indigenous community in Sabah, Malaysia

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Abstract

Ethnopharmacological relevance

The young shoots of *Etlingera coccinea* are traditionally used by the indigenous community in Sabah to treat wounds.

Aim of the study

To evaluate the effects of *E. coccinea* young shoots on the proliferation and migration of BEAS-2B, HaCaT, and NIH/3T3 cell lines, as well as to determine the major phytochemicals present.

Materials and Methods

Various concentrations of *E. coccinea* young shoots water extracts were used to treat the cells, and after 24 hours, methylthiazolyldiphenyl-tetrazolium (MTT) assay was performed to determine the cell viabilities. Enhancement of cell migration was evaluated via scratch assay, where the reduction in wound areas were determined at defined time points. Identification of the major phytochemicals present was achieved by mass spectrometry analyses.

Results

E. coccinea extracts enhanced proliferation of BEAS-2B, HaCaT, and NIH/3T3 cells with highest cell viabilities determined at 158.7 ± 3.3, 149.7 ± 2.4, and 136.5 ± 2.1 %, respectively. The extracts also resulted in increased reduction in wound areas with calculated percentage improvements

ranging from 1.6 ± 0.8 to 82.0 ± 22.5 %, as compared to the negative controls. Tannins (procyanidin B2 and C1) as well as other phenolic compounds were suggested to be present.

Conclusions

The traditional use of *E. coccinea* in treatment of wounds was supported by its ability to enhance cell proliferation and migration. The presence of tannins and other phenolic compounds could contribute to *E. coccinea*'s wound healing property.

Key words

Cell proliferation; cell migration; scratch assay; tannins; procyanidin; phenolic

1. Introduction

Plants are known to produce secondary metabolites that possess many important functions including protection against insect herbivory and microbial attack, as well as conferring resistance against photodamage (Bourgaud et al., 2001; Larson, 1988; Walton and Brown, 1999). The fact that these phytochemicals can interact with biological materials infer that they could be translated for use in human health. This is evidenced by the extensive application of plants in traditional medicine practices (Dhami, 2013). Plants have been used to treat various illnesses ranging from minor headaches and stomach aches to severe complications such as wounds and infections (Ali-Shtayeh et al., 1998; Kone et al., 2004; Muthu et al., 2006). Although plant-based traditional medicine is centuries old, their practices still exist today (Borchardt, 2002; Kaba, 2017; Nordin and Zakaria, 2016).

Plant-based traditional medicine is also widely practiced by the local indigenous communities in Borneo. For example, *Zingiber officinale*, *Senna alata*, *Sida rhombifolia*, *Etlingera elatior*, *Melastoma malabathricum*, and *Physalis minima* have been reported to treat muscle pains, stomach aches, diarrhea, headaches, measles, and hypertension, respectively (Ahmad and Holdsworth, 1995; Ahmad and Holdsworth, 2003; Kulip, 2003; Kulip et al., 2010; Poulsen, 2006). Among the various medicinal plants, we are particularly interested in *Etlingera coccinea*, which is one of the most utilized plants in Sabah. Locally known as 'tuhau' by the Kadazan/Dusun indigenous community, *E. coccinea* is easily identified by its strong coriander-like smell, while being frequently consumed as pickled vegetable (Poulsen, 2006). Its reported medicinal uses include treatment of stomach ache, gastric problems, and food poisoning (Poulsen, 2006; Vairappan et al., 2012). Interestingly, it can also be used to treat wounds whereby its young shoots are pounded (with a small amount of water) and pasted onto the affected area (Vairappan et al., 2012; Wong and Guntavid, 2000). Therefore, it was of interest to investigate the wound healing property of *E. coccinea*.

Wound healing is a complex and dynamic process that aims to restore the anatomical continuity and function of damaged tissues (Lazarus et al., 1994). Following a trauma that results in an open wound, platelets aggregate at the wound site to form fibrin clots that stop the bleeding (Diegelmann and Evans, 2004). These cells also produce various cytokines and inflammatory mediators that initiate the healing response, which can be categorized into three phases: inflammation, proliferation, and remodeling (Diegelmann and Evans, 2004; Guo and DiPietro, 2010). In the 'inflammation' phase, neutrophils, mast cells, and macrophages play important roles in removing foreign materials, bacteria, non-functional host cells, and damaged matrix components

(Diegelmann and Evans, 2004). The ‘proliferation’ phase mainly involves the migration and proliferation of fibroblasts, which are responsible for producing collagen needed to form extracellular matrix (ECM) (Diegelmann and Evans, 2004). At the same time, other cells such as keratinocytes will also proliferate and adhere to the newly formed ECM (O'toole, 2001). Lastly, in the ‘remodeling’ phase, modification of collagen occurs due to the actions of collagenases and proteases, therefore restoring the skin structure (Diegelmann and Evans, 2004).

Impaired wound healing could result in long treatment duration, hence increasing treatment cost, which in turn causes financial burden to patients (Sen et al., 2009). A recent report estimated that about six million individuals from the United States suffer from wound injuries and it was expected that the demand for wound care would exceed 50 billion USD annually (Fife and Carter, 2012). In Wales, United Kingdom, the total cost of managing patients with chronic wounds exceeded 300 million pounds (Phillips et al., 2016). Nonhealing wounds can also instigate functional limitations (gait changes and difficulty ambulating), infections, and malignant transformation, which in turn cause physical burdens (Menke et al., 2007).

Plants are widely used as herbal remedies to treat wounds (Agyare et al., 2016; Jarić et al., 2017). In this study, *E. coccinea* young shoots were assessed for their effects on cell proliferation and migration. As previously mentioned, these properties are essential in the wound healing process. Identification work was also performed to reveal the major phytochemicals present that could contribute to wound healing effects.

2. Materials and Methods

2.1 Plant materials

E. coccinea specimens were obtained from Danum Valley, Sabah (GPS coordinates of 04°58'11.4" N, 117°48'41.5" E), with permission from the Sabah Biodiversity Centre (SaBC) and Conservation and Environmental Management Division (CEMD) of Yayasan Sabah. Identification of the specimens was conducted by Mr. Bernadus Bala Ola from Danum Valley Conservation Area (DVCA) and Mr John Sugau from Forest Research Centre (FRC) Sabah. The young shoots were sampled haphazardly, and voucher specimens were deposited at the Forest Department Herbarium Sandakan, Sepilok, 90000 Sandakan, Sabah, Malaysia, with the reference number 157373.

2.2 Extraction of plant materials

The young shoots were washed to remove any remaining dirt, blotted with paper towels and air dried for 30 minutes at room temperature. Two types of extraction procedures were employed to obtain extracts for use in subsequent assays: fresh water extract (FE) and dried water extract (DE). The former was done to imitate the plant's traditional use. The intention of preparing DE was to preserve the bioactive compounds for further identification as drying process can preserve phytochemicals (Julkunen-Tiitto and Sorsa, 2001). Also, accurate preparation of required concentrations for further analyses could be done on DE.

2.2.1 Preparation of fresh water extract (FE)

The air-dried samples (375 g) were pounded using mortar and pestle with aid of liquid nitrogen and placed into glass jars. Ultrapure water (1.85 L) was added in just enough to cover the samples. The jars were subjected to sonication for 15 minutes using an ultrasonic bath (35 kHz frequency,

Elmasonic S130H, Elma, Singen, Germany). The solutions were filtered using Whatman Grade 1 filter papers (Whatman, Buckinghamshire, United Kingdom) and aliquoted in 1.5 mL microcentrifuge tubes. All tubes containing the extracts were stored in −80 ºC until further use.

2.2.2 Preparation of dried water extract (DE)

Similar procedure was repeated as described in section 2.2.1. The addition of ultrapure water, sonication and filtration were performed three times. The solutions were then pooled together, concentrated under reduced pressure using a rotary evaporator (EYELA OSB-2100, Japan), and dried using a freeze-drier (FreeZone 4.5 Plus, Labconco, United States). All dried extracts were kept at −80 °C until further use.

2.3 Wound healing assays

2.3.1 Cell culture

BEAS-2B (human lung epithelial), HaCaT (human keratinocyte), and NIH/3T3 (mouse fibroblast) cell lines were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2 mM L-glutamine, 1 % (w/v) sodium pyruvate, 10 % of fetal bovine serum (FBS) and 1 % penicillin-streptomycin (10,000 U/mL). The cells were cultivated in either T25 (25 cm^2) or T75 (75 cm^2) tissue culture flasks at 37 °C with 5% CO_2 supply in a biological incubator.

2.3.2 Methylthiazolyldiphenyl-tetrazolium (MTT) cell viability assay

MTT assay was performed to determine cell viability following treatment with the crude extracts. The method described by Sylvester (2011) was adopted with modifications. Approximately 5,000 cells in 150 µL culture media were loaded into the wells of a 96 well microtiter plate (SPL 30196,

BioLab, Singapore). After 24 hours incubation, the old culture media was removed, and new media containing various concentrations of *E. coccinea* crude extract was added back into the wells. Following an additional 24 hours incubation, the cells were exposed to 0.5 % MTT solution (Nacalai Tesque, Kyoto, Japan) for two to three hours, and the purple formazans formed were dissolved with 150 µL dimethyl sulfoxide (DMSO). Absorbance values were measured at 590 nm and dose response curves were constructed. Cell viability values were calculated as follow: cell viability = 100 % × (absorbance of treated cells / absorbance of untreated cells).

2.3.3 Scratch assay

Scratch assay was conducted as described by Liang et al. (2007) with modifications. Approximately 20,000 cells (in 500 µL) were loaded into each well of a 24-well microtitre plate (Thermo Fisher Scientific, Massachusetts, United States), followed by incubation at 37°C with 5% CO_2 supply. After a confluent cell monolayer was formed, a p200 pipette tip (200 µL Universal Fit Bevel Point Pipette Tip, Labcon, California, United States) was used to create a straight scratch in the middle of the cell monolayers. The wells were then rinsed with phosphate buffered saline (PBS) twice to remove unbound cells, followed by addition of fresh media containing *E. coccinea* crude extracts at appropriate concentrations. Ten ng/mL epidermal growth factor (EGF) (Thermo Fisher Scientific, Massachusetts, United States) and 1 % ultrapure water were used as positive and negative controls, respectively. Images of the scratch in each well were captured at t = 0, 12, and 24 hours for BEAS-2B and HaCaT cells, and t = 0, 6, and 12 hours for NIH/3T3 cell, using Nikon TS-100 (Nikon, Tokyo, Japan) inverted phase-contrast microscope. The area of each scratch was measured by using NIS Element Basic Research software (Nikon Instruments, New York, United

States) and the area of reduction was calculated according to the formula: Reduction in wound area ($\mu metre^2$) = wound area at 0 hour – wound area after 6, 12 or 24 hours.

2.4 Identification of major phytochemical constituents in *E. coccinea* young shoot water extract

2.4.1 High performance liquid chromatography (HPLC) analysis

HPLC analysis was first performed to analyze the peaks present in the extract. The following parameters were used to obtain the chromatogram: Agilent 1200 Infinity HPLC instrument (Agilent Technologies, California, United States), Cosmosil C18 preparative column (2.1 × 250 mm, 5-micron particle) (Nacalai Tesque, Kyoto, Japan); solvent system: A-3% formic acid, B-methanol with 3% formic acid; 10 to 60% B in 40 min, 60 to 95% B in 5 min, 95% B for 5 min; flow rate: 1 mL min/mL; injection volume: 50 µL; concentration: 2 mg/mL; detection wavelength: 280 nm. The major peaks were observed at t_R = 2 to 5 min (group A), t_R = 8 to 18 min (group B), and t_R = 24.09 min, 25.13 min, 25.60 min, 26.50 min, and 27.79 min (group C).

2.4.2 Liquid chromatography mass spectrometry (LCMS) and tandem mass spectrometry (MS^2) analysis

The accurate masses of compounds found in the major peaks observed via HPLC analysis were determined using negative ion mode electrospray ionization – mass spectrometry (ESI-MS). The following conditions were used: Agilent 1290 Infinity LC instrument coupled to Agilent 6520 Accurate-Mass Q-TOF (Agilent, California, United States), Agilent Zorbax 300SB-C18 column, 2.1 × 150 mm, 5-micron particle (Agilent, California, United States); solvent system: A-0.1% formic acid, B-methanol with 0.1% formic acid; 10 to 60% B in 50 min, 60 to 95% B for 10 min, 95 to 100% B in 5 min; flow rate: 0.5 mL min/mL; injection volume: 2 µL; concentration: 1

mg/mL. The major peaks were observed at t_R = 0.5 to 1.5 min (group A), t_R = 2 to 10 min (group B), and t_R = 13.85 min, 14.30 min, 16.01 min, 18.86 min, 19.18 min, and 19.97 min (group C). Tandem mass spectrometry (MS^2) analysis was achieved using similar parameters as described for LCMS with the following modifications: injection volume: 5 µL; collision energies: 10, 20, and 40V. Identification of major phytochemicals was achieved by comparing the fragmentation patterns observed with known compounds from the following mass spectrometry databases: METLIN (http://metlin.scripps.edu), MassBank (http://www.massbank.jp) and ReSpect (http://spectra.psc.riken.jp/).

2.5 Statistical analysis

Statistical analysis was conducted using IBM SPSS Statistics Version 21.0. The significance of results for different assays were performed using either independent sample *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's Honest Significant Difference (Tukey's HSD) test at the level of significance, $\alpha = 0.05$. The differences with probability value $p \leq 0.05$ were considered statistically significant.

3. Results and Discussion

3.1 Effects of *E. coccinea* young shoots crude water extracts on proliferation and migration of BEAS-2B, HaCaT, and NIH/3T3 cell lines.

The effects of the crude water extracts (FE and DE) on the proliferation of BEAS-2B, HaCaT, and NIH/3T3 cells were determined by the MTT cell viability assay. The results are presented in Figure 1.

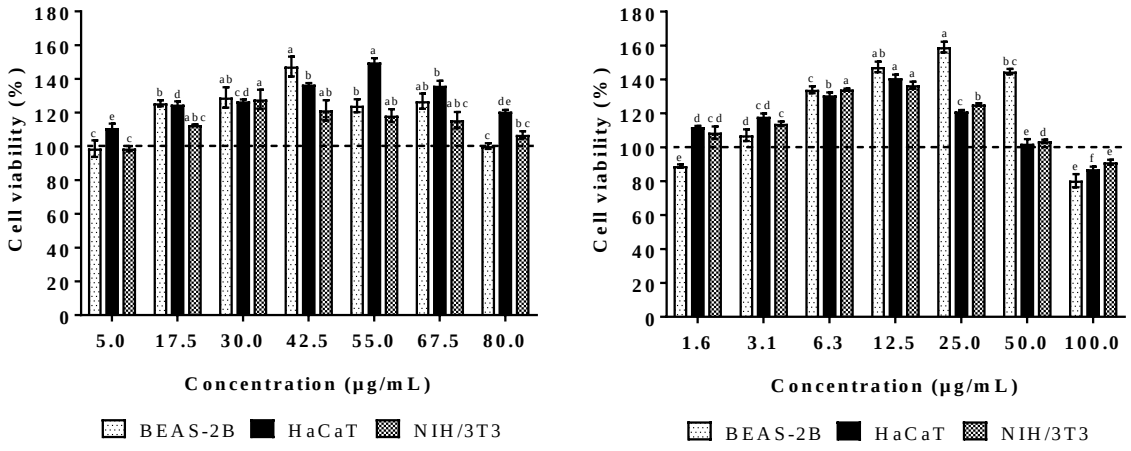

Figure 1: Cell viabilities of BEAS-2B, HaCaT, and NIH/3T3 cells following treatment with various concentrations of *E. coccinea* young shoots fresh water extract (FE) (left) and dried water extract (DE) (right). Assays were performed with three independent replicates and bar heights are reported as mean ± standard error. Different alphabets indicate significant differences between the mean cell viabilities for the same cell line at $\alpha = 0.05$ (using independent sample *t*-test).

Dose-dependent effects were observed when the cells were treated with various concentrations of FE and DE (Figure 1). Comparatively, highest cell viability values for each cell line for each treatment is as follows: treatment with 42.5, 55, and 30 µg/mL FE resulted in cell viability values of 147.3 ± 5.8, 149.7 ± 2.4, and 127.6 ± 5.9 % on BEAS-2B, HaCaT, and NIH/3T3 cell lines, respectively. Also, treatment with 25, 12.5, and 12.5 µg/mL DE resulted in cell viability values of 158.7 ± 3.3, 140.8 ± 2.0, and 136.5 ± 2.1 % on BEAS-2B, HaCaT, and NIH/3T3 cell lines, respectively. At higher concentrations, the cell viability values were observed to decrease in a dose-dependent manner. This could be due to the presence of compounds that are harmful at higher concentrations, hence causing cytotoxicity. Conversely, at lower concentrations, the bioactive compounds might not be sufficient to effectively enhance cell proliferation. It was also noted that

the cell viability of BEAS-2B cells treated with 1.6 µg/mL DE was lower than 100 %, and this might be due to the presence of cytotoxic compounds. Similar to this study, a study by Annan and Houghton (2008) showed that the extracts of *Gossypium arboretum* and *Ficus asperifolia* enhanced proliferation of 142BR fibroblast cells in a dose-dependent manner, with 50 µg/mL being the optimum concentration determined for both extracts, after which cytotoxic effects were observed (at higher concentrations).

Apart from cell proliferation, FE and DE were also evaluated for their effects on cell migration. This was achieved via scratch assay in which the reduction in wound areas at different time points were compared. The optimum concentrations that resulted in highest viabilities were used: 42.5, 55, and 30 µg/mL FE, as well as 25, 12.5, and 12.5 µg/mL DE, on BEAS-2B, HaCaT, and NIH/3T3 cell lines, respectively. An example of the wound area assessment conducted is shown in Figure 2.

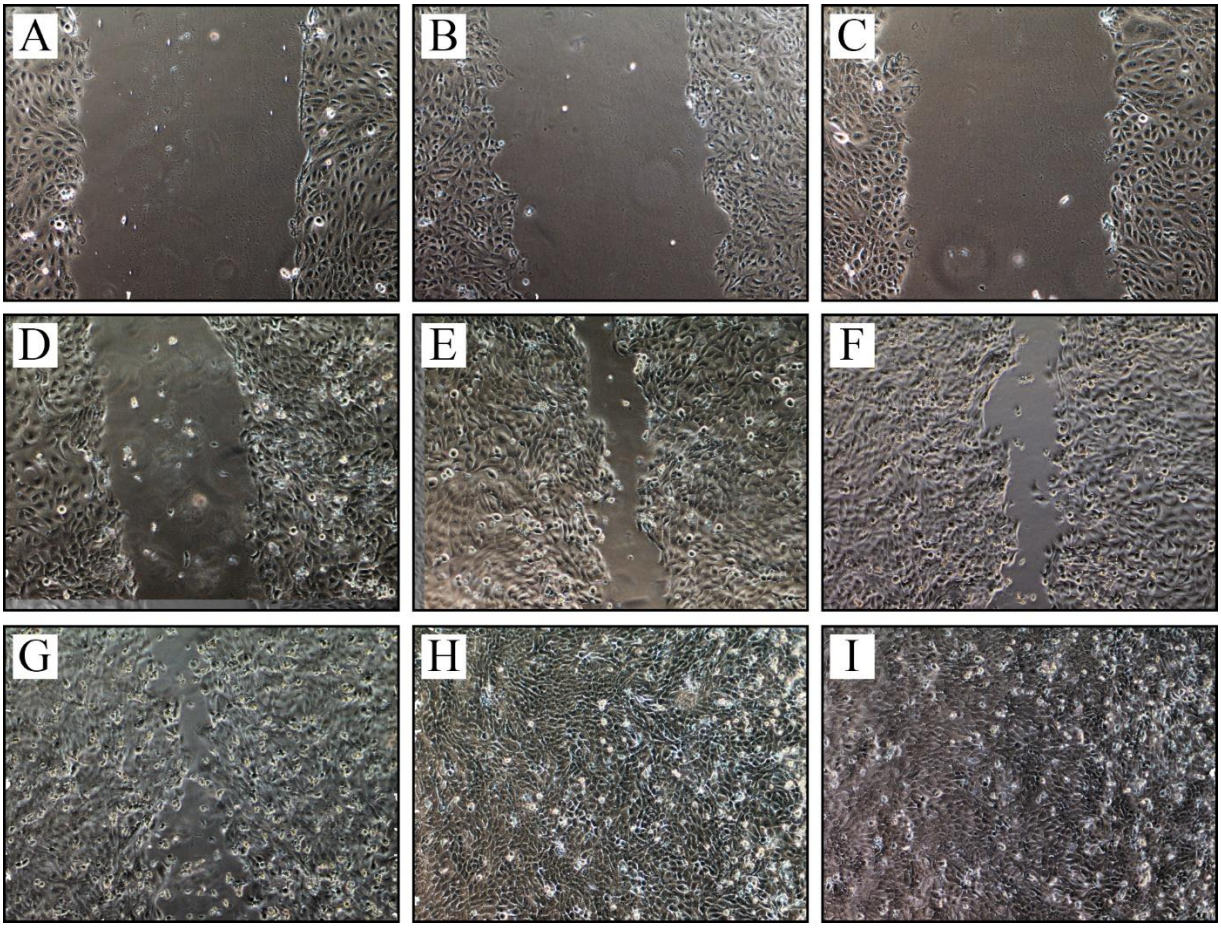

Figure 2: Images of HaCaT cells with wound areas at t = 0 hour (A, B, and C), 12 hours (D, E, and F), and 24 hours (G, H, and I) following treatment with 1 % ultrapure water (negative control) (A, D, and G), 10 ng/mL epidermal growth factor (EGF) (positive control) (B, E, and H), and 55 µg/mL *E. coccinea* young shoots fresh water extract (FE) (C, F, and I).

As shown in Figure 2, when compared with HaCaT cells treated 1 % ultrapure water, treatment with 10 ng/mL EGF resulted in better wound closure at t = 12 hours, while complete closure was observed at t = 24 hours. This supports the use of EGF as a positive control. Similar observations were made for the cells treated with 55 µg/mL FE. Images of three independent replicates were obtained for each treatment on the three cell lines, and the reductions in wound area were calculated and statistically analyzed. A summary of the results is presented in Figure 3.

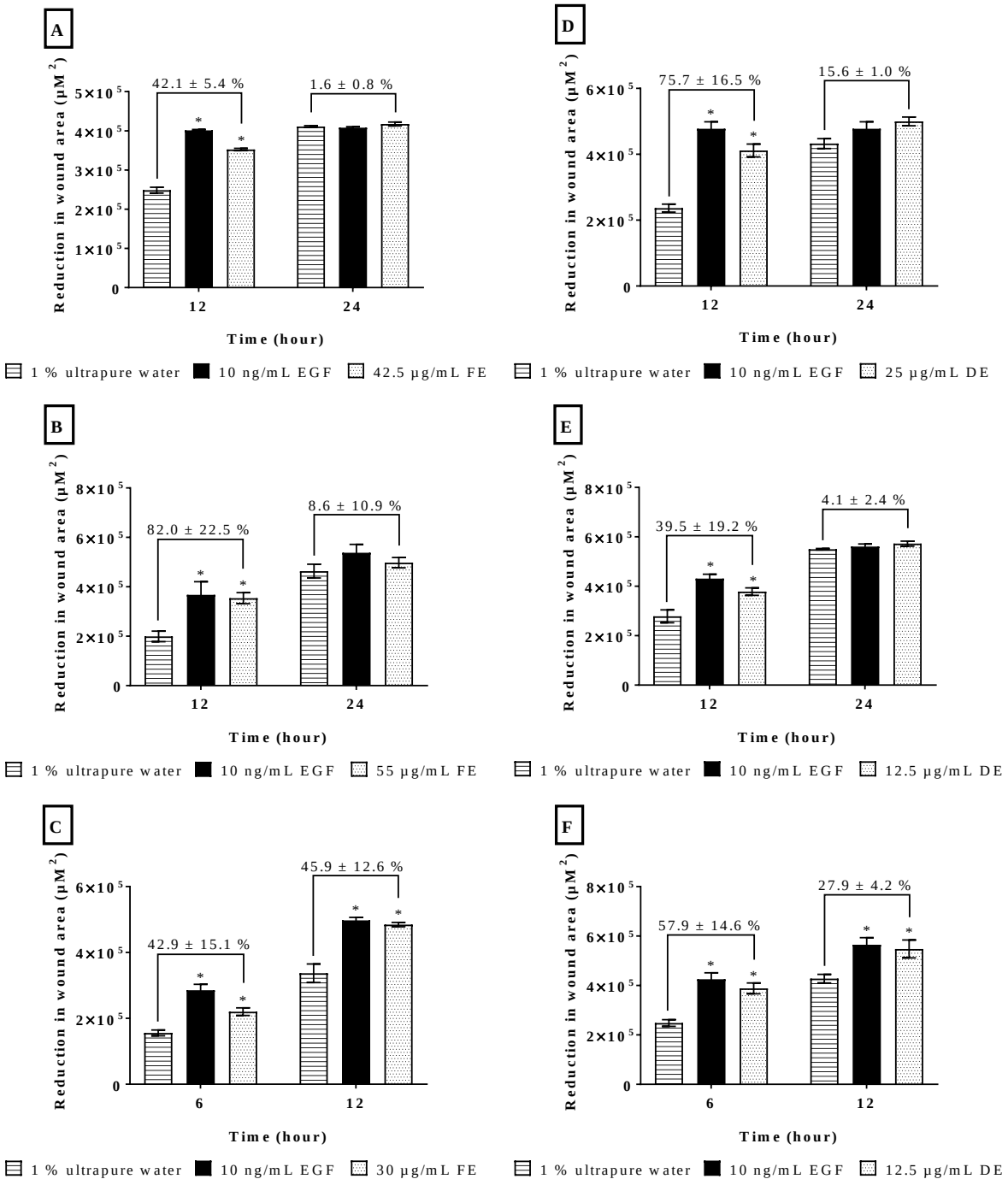

Figure 3: Reduction in wound areas measured at different time points for BEAS-2B (A and D), HaCaT (B and E), and NIH/3T3 (C and F) cell lines, following treatments with *E. coccinea* young shoots fresh water extract (FE) (A, B, and C), and dried water extract (DE) (D, E, and F). One % ultrapure water and epidermal growth factor (EGF) were used as negative and positive controls,

respectively. Assays were performed with three independent replicates and error bars are reported as mean ± standard error. Asterisks indicate significant difference between treatment and negative control at $\alpha = 0.05$ (using independent sample *t*-test). Values above bars represent the percentage improvement of reduction in wound areas associated with *E. coccinea* extracts as compared to their respective negative controls.

In Figure 3, treatment with 10 ng/mL EGF significantly enhanced wound closure at $t = 6$ hours for NIH/3T3 cell, and at $t = 12$ hours for BEAS-2B and HaCaT cells (see graphs A to F). This proved the effectiveness of EGF as a promoter of cell migration. EGF is known to stimulate cell migration by binding to the EGF receptor (EGFR), which subsequently triggers several mechanisms linked to cell motility such as PLCγ-dependent pathway, mitogen-activated protein (MAP) kinases, and calcium-dependent proteases (calpains) (Jorissen et al., 2003; Peplow and Chatterjee, 2013; Wells, 1999). Treatment with FE and DE on BEAS-2B, HaCaT, and NIH/3T3 cells also resulted in increased reduction in wound areas as compared to their respective negative controls, with percentage improvements determined ranging from 1.6 ± 0.8 to 82.0 ± 22.5 %.

It is noteworthy that *E. coccinea* young shoots water extracts could enhance proliferation and migration of the three cell lines used. This is because different types of cells play different roles in the wound healing process. The rapid migration and proliferation of fibroblasts during the early phase of wound healing are essential as these cells deposit new extracellular matrices needed to repair the tissue injury (Diegelmann and Evans, 2004). On the other hand, epithelial cells are known to migrate from the wound edges within a few hours of wounding, and subsequently proliferate over the provisional matrix below them, hence closing the wound (Velnar et al., 2009).

The migration and proliferation of keratinocytes are also important as they are responsible for stimulating fibroblasts to synthesize growth factors, as well as cause differentiation of fibroblasts into myofibroblasts (Werner et al., 2007). Myofibroblasts contract to bring the margins of open wounds together during the later stages in wound healing (Grinnell, 1994). Therefore, the improvement of proliferation and migration shown by *E. coccinea* extracts on different cell types infer that the wound healing process could be hastened. Thus, our results support its traditional application in treatment of wounds.

Apart from providing evidence for its traditional use, we are also interested to compare the wound healing ability of *E. coccinea* with those of other plants (see Table 1). Only DE was used for comparison due to the similarity in extraction methods: the extracts obtained from other studies were also dried, hence their concentrations were expressed in µg extract/mL (as opposed to FE in which its concentrations were expressed in µg plant material/mL).

Table 1: The effects of *E. coccinea* young shoot dried water extract (DE) on the cell proliferation and migration of BEAS-2B, HaCaT, and NIH/3T3 cell lines in comparison with the reported wound healing effects of several other plant species.

Plant species	Effective concentration (µg/mL)	Cell line used	Reported wound healing effect	Reference
Aloe vera	25	L929 fibroblast	137.15 % (cell proliferation) 19.7 ± 2.63 % (increased cell migration)	(Manoj et al., 2009)
Bridelia ferruginea	5	FS5 fibroblast	133.3 ± 9 % (cell proliferation)	(Adetutu et al., 2011)
Calendula officinalis	10	Swiss 3T3 fibroblast	70.53 ± 2.64 % (cell migration)	(Fronza et al., 2009)
Etlingera coccinea	25	BEAS-2B epithelial	158.7 ± 3.3 % (cell proliferation) 75.7 ± 16.5 % (cell migration)	This study
	12.5	HaCaT keratinocyte	140.8 ± 2.0 % (cell proliferation) 39.5 ± 19.2 % (cell migration)	
	12.5	NIH/3T3 fibroblast	136.5 ± 2.1 % (cell proliferation) 57.9 ± 14.6 % (increased cell migration)	
Parkia biglobosa	30	FS5 fibroblast	131.3 ± 12 % (cell proliferation)	(Adetutu et al., 2011)

The enhancement of cell proliferation shown by *E. coccinea* was comparable to other plant species that are known for their wound healing effects, as displayed in Table 1. For example, the cell viabilities of fibroblast cells treated with *Aloe vera*, *Bridelia ferruginea* and *Parkia biglobosa* extracts range from 131 to 137 %, while *E. coccinea* extract improved NIH/3T3 fibroblast proliferation with cell viability determined at 136.5 ± 2.1 %. In terms of cell migration, *E. coccinea*

also showed comparable activity with *Calendula officinalis* and *A. vera* (57.9 ± 14.6 % against 70.53 ± 2.64 and 19.7 ± 2.63 %, respectively). It is worth noting that evidence from in vitro, in vivo, as well as clinical studies supported the efficacy of *A. vera* and *C. officinalis* as wound treatment agents (Duran et al., 2005; Feily and Namazi, 2009; Leach, 2008; Preethi and Kuttan, 2009; Surjushe et al., 2008). The fact that *E. coccinea* showed comparable enhancement of cell proliferation and migration activities further suggest its effectiveness as a wound healing treatment. However, in vivo wound healing studies are needed to provide more evidence on its efficacy.

3.2 Major phytochemicals present in *E. coccinea* young shoot water extract

The crude water extracts of *E. coccinea* young shoots showed enhancement of cell proliferation and migration, thus suggesting their wound healing ability. Thereafter, it was of our interest to determine the major compounds present. Identification work was done on DE due it being in powder form, hence enabled us to accurately prepare the required concentrations for HPLC and LCMS analyses. Also, the absence of water could reduce the risks of the bioactive compounds being degraded over time. The contents of DE were first analyzed via analytical HPLC and the chromatogram obtained is presented in Figure 4.

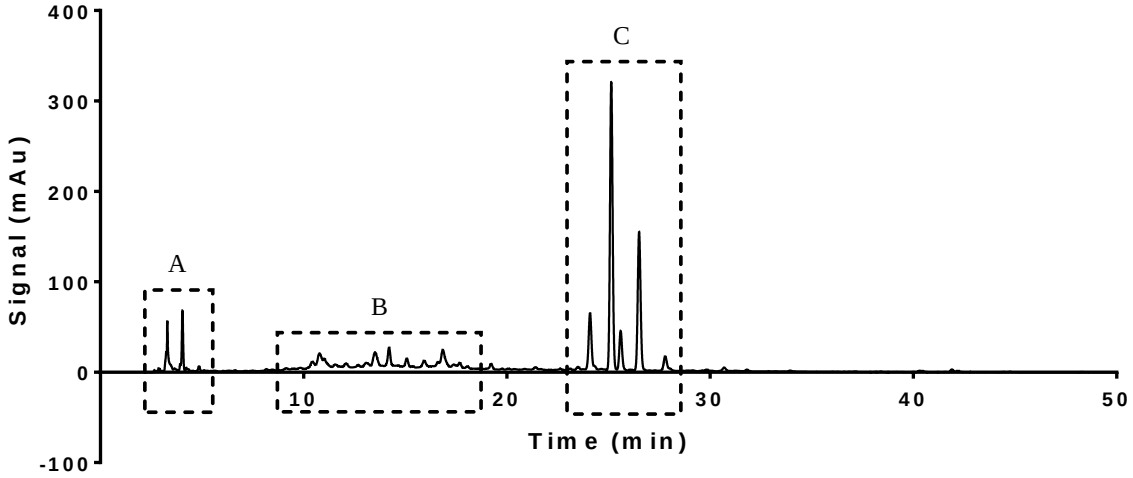

Figure 4: High performance liquid chromatography (HPLC) chromatogram of *E. coccinea* young shoots dried water extract (DE) showing peaks detected at 280 nm. Three major regions of compounds were observed as indicated by boxes A, B, and C.

As displayed in Figure 4, the major peaks found in DE can be grouped into three regions (A, B, and C). Although only two peaks can be observed in region A, it is important to note that this region contains highly polar compounds that do not have strong interaction with the hydrophobic C18 column. Thus, this region could contain many compounds that elute simultaneously. In region B, the peaks appear unresolved at t_R = between 8 to 18 minutes. As for region C, five major peaks were observed at t_R = 24.09, 25.13, 25.60, 26.50, and 27.79 minutes. The peaks in these three regions were subjected to LCMS and MS^2 analyses to determine the major compounds' accurate masses as well as their fragmentation patterns. Table 2 lists the compounds' accurate masses, ion fragments, and their identities based on database search.

Table 2: Identities of the major compounds present in *E. coccinea* young shoots water extract as determined via liquid chromatography mass spectrometry (LCMS) and tandem mass spectrometry (MS^2).

Group	t_R (min)	Accurate mass of [M] (m/z)	MS^2 ion fragment mass (m/z)	Compound identification
A	2-5	612.1532	128.0359, 160.0098, 254.0819, 272.0911, 304.0596, 306.0779, 307.0845, 338.0499, 611.1459, 612.1513	Glutathione (oxidized) [M] 612.1520 m/z
		196.0597	57.0346, 59.0146, 61.9867, 68.9968, 71.0154, 75.0086, 83.0108, 87.0068, 99.0090, 129.0173	Gluconic acid [M] 196.0583 m/z
		134.0227	115.0046, 116.0081, 133.0155, 134.0184, 135.0285	Malic acid [M] 134.0215 m/z
		192.0284	101.0266, 111.0108, 111.1028, 112.0143, 129.021, 131.0014, 154.9993, 173.0111, 191.0220, 192.0253	Citric acid [M] 192.0270 m/z
		174.0164	59.0134, 60.9963, 67.0191, 68.9993, 85.0307, 88.9884, 107.3141, 111.0091, 130.0839	Aconitic acid [M] 174.0164 m/z
B	8-18	578.1439	125.0243, 161.0260, 287.0572, 289.0733, 290.0768, 407.0791, 408.0820, 425.0891, 451.1053, 577.1384	Procyanidin B2 [M] 578.1425 m/z
		866.2064	287.0565, 425.0877, 575.1201, 577.1352, 578.1380, 695.1409, 713.1502, 865.2000, 866.2030, 867.2085	Procyanidin C1 [M] 866.2058 m/z
C	24.09	310.0704	115.0033, 117.0341, 134.0374, 135.0415, 149.0604, 178.0267, 193.0512, 194.0543, 195.0554	Unidentified
	25.13	620.1402	115.0034, 133.0143, 193.0509, 194.0537, 287.0540, 309.0607, 331.0444, 332.0482, 409.0910, 525.1041	Unidentified
	25.60	623.1281/ 624.1352	121.0268, 151.0014, 178.9981, 300.0283, 301.0354, 302.0390, 303.0440, 321.0830, 623.1258, 624.1293, 625.1323	Unidentified
	26.50	478.0688	113.0234, 151.0035, 178.9984, 301.0353, 301.1829, 302.0375, 303.0391, 477.0654, 478.0728	Quercetin-3-glucuronide [M] 478.0750 m/z
		608.1400	113.0239, 163.0031, 229.0507, 285.0413, 286.0445, 287.0460, 321.0835, 322.0863, 607.1316, 608.1346, 609.1379	Unidentified
	27.79	610.1531	Undetected	Unidentified

The major phytochemicals that can be found in region A are organic acids: gluconic, malic, citric, and aconitic acids. They are commonly found in plants and play important roles in metabolism (Bennet-Clark, 1933; Vickery and Pucher, 1940). Apart from organic acids, glutathione was also suggested to be present, though in oxidized form, which could be due to oxidation by air molecules. Glutathione is an essential component to many organisms including bacteria, plants, and animals as it functions in oxidative stress response mechanisms (Frova, 2003; Lushchak, 2011).

Although region B appeared unresolved, only two major compounds with the masses 578.1439 and 866.2064 m/z were found. These compounds were identified as procyanidin B2 and procyanidin C1, respectively, as suggested by their matching fragmentation patterns with those reported in databases. In region C, each of the five peaks (as shown in Figure 4) corresponded to one parent compound, except for the peak at t_R = 26.50 minutes, which contains two parent compounds. Their corresponding accurate masses and MS^2 ion fragments detected are listed in Table 2. In this region, quercetin-3-glucuronide was suggested to be present along with four other compounds with masses 310.0704, 620.1402, 624.1352, and 608.1400 m/z. The identities of the latter four were unable to be determined as no matching fragmentation patterns were found on the databases. However, the compounds with masses 310.0704, 624.1352, and 608.1400 might consist of ferulic acid ([M] 194.0579 m/z), quercetin ([M] 302.0427 m/z), and fisetin ([M] 286.0477 m/z) moieties, respectively. The fragmentation patterns of these moieties matched with those reported in databases, hence suggesting that they could be part of their respective parent compounds. Further identification work via nuclear magnetic resonance (NMR) spectroscopy are warranted to determine the identities of the compounds. The MS^2 ion fragments for the compound with mass 610.1531 m/z were unidentified and this could be due to its low abundance, or its inability to be

fragmentized. The chemical structures of the major compounds and possible moieties are displayed in Figure 5.

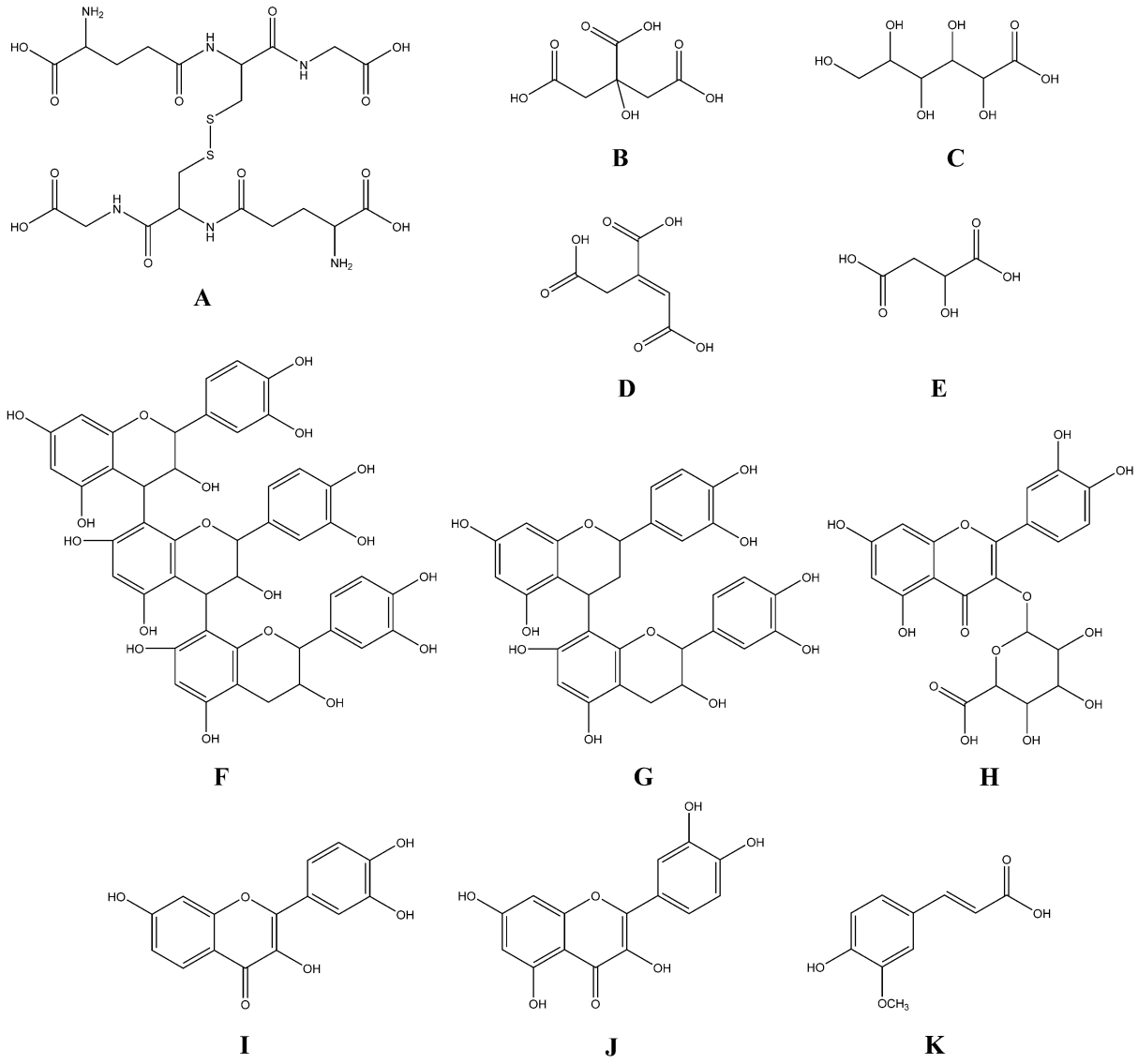

Figure 5: The chemical structures of compounds identified from *E. coccinea* young shoots water extract as suggested by mass spectrometry analyses: glutathione (oxidized) (A), citric acid (B), gluconic acid (C), aconitic acid (D), malic acid (E), procyanidin C1 (F), procyanidin B2 (G), and quercetin-3-glucuronide (H). Several other unidentified compounds were also present which possibly possess the following moieties: fisetin (I), quercetin (J), and ferulic acid (K).

Procyanidin B2 and procyanidin C1 (see Figure 5 for their chemical structures) are classified as tannins, which are a diverse group of high molecular weight phenolic compounds (ranging from 500 to more than 3000 Da) (Hassanpour et al., 2011; Khanbabaee and van Ree, 2001). Tannins play a role in a plant's defense mechanism against mammalian and insect herbivores because: 1) they have a bitter taste, thus acting as feeding deterrents, and 2) they possess protein and metal ion-binding abilities which can reduce their nutritive values when ingested (Robbins et al., 1987; War et al., 2012). Although tannins could be harmful when consumed, past research had shown that they have beneficial effects such as anticarcinogen, antimutagen, antibacterial, and antiviral activities (Chung et al., 1998; Yang et al., 2013). Additionally, wound healing has also been attributed to tannins and tannin-rich plant extracts (Agyare et al., 2009; Bueno et al., 2014; Deters et al., 2001; Hupkens et al., 1995; Li et al., 2011; Lopes et al., 2005; Su et al., 2017).

Many studies have linked tannins with improved wound healing, although the exact mechanisms are still not well understood. Nevertheless, several theories have been proposed. Firstly, tannins can promote capillary vasoconstriction, causing a local anti-inflammatory effect. This reduces the build-up of inflammatory exudates, and thus prevent growth of microorganisms at the wound site (Edwin et al., 2008; Lopes et al., 2005). This anti-inflammatory effect is also important as prolonged inflammation can lead to increased levels of proteases, which are enzymes that can degrade ECM and growth factors (Edwards and Harding, 2004; Guo and DiPietro, 2010; Menke et al., 2007). Some studies suggest that tannins might improve wound healing by preventing the build-up of excessive edema that can retard cicatrization (scar tissue formation process) (Lopes et al., 2005; Sanchez Neto et al., 1993). Procyanidins in particular, have been associated with upregulation of vascular endothelial growth factor (VEGF) in keratinocytes (Khanna et al., 2001).

VEGF is known for its role in angiogenesis (generation of new blood vessels) (Bernatchez et al., 2002). This process is important as the new vasculature delivers essential nutrients as well as remove wastes from the developing tissues (Pettet et al., 1996).

Another mechanism is related to the fact that tannins and other phenolic compounds, such as those suggested to be present in this study (quercetin-3-glucuronide as well as ferulic acid, quercetin, and fisetin moieties), possess good antioxidant activities. In the 'inflammation' phase, neutrophils, macrophages, and fibroblasts produce high levels of reactive oxygen species (ROS) to combat bacterial invasion (Auf Dem Keller et al., 2006). ROS molecules also play other critical roles such as modulation of cellular processes including proliferation, metabolism, differentiation, and survival (Ray et al., 2012). However, when present at large amounts, they can cause damage to DNA, protein, and lipid molecules (Buonocore et al., 2010). When this occurs, tissues that are otherwise healthy could be damaged, consequently resulting in impaired wound healing. Tannins and other phenolic compounds can prevent this from occurring by acting as antioxidants to lower ROS levels at wound site, hence preventing cellular damage (Süntar et al., 2012).

As opposed to being antioxidants, tannins and other phenolic compounds can also exhibit pro-oxidant activities (generation of ROS molecules) at higher concentrations (Fukumoto and Mazza, 2000; Khan et al., 2000; Shao et al., 2003). Though high ROS levels can cause cellular damage, past studies have linked low ROS levels with improvement in wound healing due to their ability to promote cell adhesion, proliferation, and migration (Huo et al., 2009; Pan et al., 2011). Therefore, the tannins and other phenolic compounds present in water extracts of young *E. coccinea* shoots might have enhanced cell proliferation and migration through this mechanism.

Although several theories have been discussed, further studies are required to explore the wound healing mechanism by *E. coccinea*.

The fact that tannins and other phenolic compounds possess pro-oxidant properties could also explain the dose-dependent effects observed in Figure 1, whereby cells treated with the extracts showed decreasing viabilities at higher concentrations. As previously discussed, high levels of ROS can be detrimental to cells. Thus, high concentrations of the extracts might have resulted in high ROS levels, which led to decreased cell viability. Curcumin is a well-known example of a phenolic compound that can kill cells via generation of ROS, at high concentrations (Lee et al., 2016).

4. Conclusion

The young shoots of *E. coccinea* were shown to enhance cell proliferation and migration, hence supporting the plant's traditional use as a wound treatment by the indigenous community in Sabah. Its observed effects were also comparable to other plants known for their wound healing effects such as *C. officinalis* and *A. vera*. The major phytochemicals present in the extract include tannins (procyanidins) and other phenolic compounds. Past studies have shown that these groups of compounds can contribute to the wound healing process by reducing inflammation, promoting cell proliferation and migration, as well as reducing the levels of ROS. Their exact mechanisms are yet to be understood, thus further molecular and gene expression work could be done to investigate the effects of the treatments on cellular responses.

Conflict of interest

The authors declare no conflict of interest.

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Author's contributions

The research project was designed by SML and LWL, and reviewed by YYL, JBLT and SWY. Plant specimen collection and identification were conducted by SWY. DDJ and LSL prepared the plant crude extracts as well as performed the bioassays (MTT cell viability and scratch assay). Liquid chromatography mass spectrometry and tandem mass spectrometry analyses were carried out by DDJ. DDJ drafted the manuscript. All authors were involved in writing and giving feedback on the manuscript. The final manuscript was approved by all authors.

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