APPLICATION OF LIGHT-EMITTING DIODES ON *Eurycoma longifolia* HAIRY ROOT CULTURES: A STRATEGY FOR ENHANCED PRODUCTION OF ANTIPARASITIC COMPOUNDS TARGETING *Blastocystis* sp.

MAHMOUD ALI KHALAF ABUSHATTAL

UNIVERSITI SAINS MALAYSIA

2025

APPLICATION OF LIGHT-EMITTING DIODES ON *Eurycoma longifolia* HAIRY ROOT CULTURES: A STRATEGY FOR ENHANCED PRODUCTION OF ANTIPARASITIC COMPOUNDS TARGETING *Blastocystis* sp.

by

MAHMOUD ALI KHALAF ABUSHATTAL

Thesis submitted in fulfilment of the requirements.
for the degree of
Doctor of Philosophy

March 2025

ACKNOWLEDGEMENT

First and foremost, I offer my deepest gratitude to the Almighty Allah for His endless blessings, guidance, and inspiration throughout my life journey. I am deeply grateful to my supervisor, Dr. Mohamad Fadhli bin Mad' Atari, for his invaluable guidance, mentorship, and unwavering support throughout my PhD journey. His encouragement and dedication have been crucial to my growth and success. I also express my deep gratitude to my co-supervisor, Professor Dr. Sreeramanan Subramaniam, for his insightful contributions, which were helpful in shaping this research. I also want to thank Dr. Farah Haziqa Meor Termizi and Dr. Nor Hasnida Hassan for their assistance.

Special appreciation to Dr. Mohamad Nurul Azmi bin Mohamad Taib for his expertise and guidance in the chemistry aspects of this research. I also extend my heartfelt gratitude to Dr. Arutchelvan Rajamanikam for his expert support with the *Blastocystis* sp assay. His assistance was critical to the successful completion of the assay work. I also want to express my sincere thanks to my cousin, Dr. Saqer Abushattal, for his encouragement and support from the beginning to the end of this project. Additionally, my sincere gratitude goes to Dr. Lance Ong for his invaluable assistance.

I am forever grateful to my parents, especially to my late father, Mr. Ali (may his soul rest in peace), and my mother, whose prayers and love have always been my source of strength. I owe an enormous debt of gratitude to my brother Hasan, who took on the significant responsibility in our family in my father's absence. His unwavering commitment and sacrifices allowed me to fully focus on my research without worry. His leadership, financial support, and emotional strength have been a cornerstone in

my PhD journey. A special thank you also goes to Hossam for his constant support, as well as to my other siblings for their encouragement. May Allah reward them abundantly, and may this work be a source of everlasting charity for them.

I would also like to extend my deep gratitude to my colleagues, particularly Dr. Sani Sale, and my PTC lab journal club members. I am thankful to the NPSO family: Tahir, Idris, Ibrahim, Mahdi, Solehin, and others for their friendship and support, as well as to the EP1 UM and Vet Parasitology lab members. A special mention goes to my nephew Ahmad Ali, my dear friend Mohammad Bassam Alshawabkeh, and all those who have motivated and inspired me along this journey. Your prayers and support have been critical to the success of my PhD program.

Thank you, everyone. May Allah bless you all.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

1D	One-Dimensional
2D-NMR	Two-Dimensional Nuclear Magnetic Resonance Spectroscopy
AI	Activity Index
AO	Acridine Orange
BPC	Bee Pollen Compound
BSA	Bovine Serum Albumin
CC	Column Chromatography
$CDCl_3$	Deuterated chloroform
COSY	Homonuclear correlation spectroscopy
DAMD	Directed Amplification of Microsatellite DNA Regions
DCM	Dichloromethane Fraction
DMSO	Dimethyl sulphoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	Dry Weight
ELHRCs	*E. longifolia* Hairy Root Cultures
FECT	Formalin Ethyl Acetate Technique
FT-IR	Fourier Transform Infrared
FW	Fresh Root Weight
GC-MS	Gas Chromatography Mass Spectrometry
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High Performance Liquid Chromatography
HSQC	Heteronuclear Single Quantum Correlation
IBS	Irritable Bowel Syndrome
LED	Light-Emitting Diode
LOD	Limit of Detection

LOQ	Limit of Quantification
MeOH	Methanol
MIC	Minimum Inhibitory Concentration
MTZ	Metronidazole
NMR	Nuclear Magnetic Resonance
NPs	Natural Products
OLE	Olive Leaf Extract
PAR	Photosynthetically Active Radiation
PCR	Polymerase Chain Reaction
PLBs	Oncidium Protocorm-Like Bodies
RAPD	Random Amplified Polymorphic DNA
SD	Standard Deviation
SPAR	Single Primer Amplification Reaction
SSC	Soluble Sugar Content
SSU-rDNA	Small Subunit Ribosomal Deoxyribonucleic Acid
SSU-rRNA	Small Subunit Ribosomal Ribonucleic Acid
ST	Subtypes
TLC	Thin Layer Chromatographic
TMPSMX	Metronidazole, Nitazoxanide, Trimethoprim-Sulfamethoxazole
TMS	Traditional Medicinal Systems
UV	Ultraviolet
XIVC	Xenic *in Vitro* Culture

LIST OF APPENDICES

APLIKASI DIOD PEMANCAR CAHAYA PADA KULTUR AKAR RERAMBUT *Eurycoma longifolia*: STRATEGI UNTUK MENINGKATKAN PENGELUARAN SEBATIAN ANTIPARASIT YANG MENSASARKAN *Blastocystis* sp.

ABSTRAK

Eurycoma longifolia (juga dikenali sebagai Tongkat Ali) ialah tumbuhan ubatan bernilai tinggi yang sering digunakan dalam perubatan tradisional di Malaysia. Alkaloid dan kuasinoidnya dilaporkan mempunyai kesan antiparasit. Namun, kesan rawatan spektrum cahaya diod pancaran cahaya (LED) terhadap sintesis sebatian bioaktif, ciri pertumbuhan, kandungan karbohidrat dan protein keseluruhan, aktiviti antioksidan, serta histologi dalam kultur akar rerambut *E. longifolia* (ELHRCs) belum diterokai sepenuhnya, begitu juga kesan antiparasitnya terhadap *Blastocystis* sp., iaitu protozoa parasit usus yang lazim. Dalam kajian ini, ELHRCs telah didedahkan kepada penyinaran cahaya putih, biru, merah, kombinasi biru dan merah (1:1), hijau pudina, dan keadaan gelap sebagai kawalan secara *in vitro*. Variasi kandungan sebatian bioaktif dinilai menggunakan kromatografi cecair berprestasi tinggi (HPLC) untuk mengukur 9-hydroxycanthin-6-one, 9-methoxycanthin-6-one, dan eurycomanone. Selain itu, ekstrak mentah diklorometana (DCM) dikaji melalui proses pengasingan, penulenan, dan penjelasan struktur, yang menghasilkan dua sebatian yang dikenali, iaitu canthin-6-one dan 9-methoxycanthin-6-one. Struktur kimia sebatian ini dijelaskan dan dicirikan menggunakan kaedah spektroskopi termasuk resonans magnet nuklear (NMR) 1D dan 2D, inframerah transformasi Fourier (FT-IR), serta spektrometri jisim kromatografi gas (GC-MS), yang kemudiannya dibandingkan dan disahkan dengan rujukan literatur. Kesan antiparasit ekstrak mentah dan sebatian yang diasingkan

selepas 10 minggu kultur terhadap *Blastocystis* sp. dinilai melalui kultur parasit secara *in vitro*, dan kepekatan perencatan minimum (MIC_{90}) diukur. Berbanding dengan metronidazole (MTZ), ekstrak mentah dan sebatian yang diasingkan daripada ELHRCs menunjukkan aktiviti antiprotozoa tertinggi (0.1 mg/ml) terhadap *Blastocystis* sp. ST3. Analisis molekul menggunakan DAMD dan ISSR didapati berkesan dalam mengkaji kestabilan genetik. Keputusan ini menunjukkan bahawa penyinaran LED mampu mengubah komposisi bioaktif untuk menghasilkan agen antiparasit yang lebih tinggi daripada ELHRCs. Oleh itu, penyinaran LED berpotensi sebagai agen antimikrob pelengkap dan alternatif.

APPLICATION OF LIGHT-EMITTING DIODES ON *Eurycoma longifolia* HAIRY ROOT CULTURES: A STRATEGY FOR ENHANCED PRODUCTION OF ANTIPARASITIC COMPOUNDS TARGETING *Blastocystis* sp.

ABSTRACT

Eurycoma longifolia (also known as Tongkat Ali) is a valuable medicinal plant that is frequently used in traditional medicine in Malaysia. Its alkaloids and quassinoids reportedly confer antiparasitic effects. The effects of different light-emitting diode (LED) spectral treatments on bioactive compounds synthesis, growth characteristics, total carbohydrate and protein content, antioxidant activity, and histological in *E. longifolia* hairy root cultures (ELHRCs) have not been explored, and the consequent antiparasitic effects on *Blastocystis* sp., a common intestinal protozoan parasite, have not been determined. In this study, ELHRCs were irradiated with white, blue, red, blue plus red (1:1) mint green light and dark as control *in vitro.* The variation in bioactive compound content was assessed by using high-performance liquid chromatography (HPLC) were employed to quantify 9-hydroxycanthin-6-one, 9-methoxycanthin-6-one, and eurycomanone. In addition, the dichloromethane (DCM) crude extract was studied through the isolation, purification, and structural elucidation processes, resulting in the discovery of two known compounds, namely canthin-6-one and 9-methoxycanthin-6-one. The chemical structures of these compounds were elucidated and characterised by using spectroscopic methods consisting of 1D and 2D nuclear magnetic resonance (NMR) in combination with Fourier transform infrared (FT-IR) and gas chromatography mass spectrometry (GC-MS) analysis, which were later compared and confirmed with the literature. Subsequently, the antiparasitic effect

of crude extracts and isolated compounds after 10 weeks of culture on *Blastocystis* sp. was assessed via *in vitro* parasite culture, and the minimum inhibitory concentration (MIC_{90}) was measured. Moreover, compared with metronidazole (MTZ), the crude extraction and isolated compounds of ELHRCs exhibited the highest anti-protozoal activity (0.1 mg/ml) against *Blastocystis* sp. ST3. Molecular analysis using DAMD and ISSR was found effective in studying the genetic stability. These results indicate that LED irradiation alters bioactive composition and is promising for obtaining higher yields of antiparasitic agents from ELHRCs and thus, can be considered a potential complementary and alternative antimicrobial agent.

CHAPTER 1

INTRODUCTION

1.1 General introduction

In recent years, many plant-based products have been introduced to the commercial market and are now used in many regions of the world, especially for the treatment of various diseases and for maintaining health (Chaachouay & Zidane, 2024). These types of products are generally well received by consumers as they assume that herbal products are much safer and have fewer harmful side effects compared to synthetic medicines (Newman & Cragg, 2020). There is a growing interest among pharmaceutical companies and other industries worldwide, in commercializing parts or the entire plant of medicinally valuable species. This trend highlights the increasing recognition of the therapeutic potential of these plants, including their bioactive compounds, which can be used for various health applications (Abdallah *et al*., 2023).

Eurycoma longifolia Jack (known as Tongkat Ali), a popular plant in traditional herbal medicine, is native to Malaysia and Indonesia (Rehman *et al*., 2016). *E. longifolia* is a thin, shrub-like, tall and slow-growing tree that is widely recognised for its medicinal properties (Chen *et al.,* 2019). In Southeast Asia, *E. longifolia* is known as a traditional medicine with aphrodisiac and anti-fever properties (malaria). Many traditional medicinal systems (TMS) are used worldwide to provide a modern rationale for their use in the treatment infectious disease (Alam *et al.,* 2021). Numerous studies have demonstrated the continued importance of natural chemicals and structures in the development of novel medicines (Newman, 2019). These chemicals have potent anti-

allergic, anti-inflammatory, anti-tumour, anti-proliferative and anti-cancer properties that help in maintaining health and treating diseases (Kumar & Pandey, 2013).

The roots and stems of *E. longifolia* contain a variety of important phytochemical compounds, such as quassinoids, squalene-type triterpenes, canthin-6-on alkaloids, tirucallane-type triterpenes, polyphenols, saponins, high-molecular-weight polysaccharides, and glycoproteins (Rehman *et al*., 2016). Alkaloids were only found in the chloroform, ethyl acetate, and petroleum ether extracts of the stem (Khanam *et al*., 2015; Bräuer *et al*., 2019; Tsai *et al*., 2020). A total of twelve known isolates and four new phenolic acids were found in a 70% ethanol extract from the roots (Ruan *et al*., 2019). Quassinoids and other compounds have a wide range of biological effects with a variety of biological functions and were discovered as a result of phytochemical research on this *E. longifolia* (Yang *et al*., 2020). Antioxidant activity has been observed for flavonoids, alkaloids, phenols, and glycosides identified in *E. longifolia* (Sanyoto & Noor, 2020). Lee *et al*. (2019) investigated the effect of a quassinoids namely, pasakbumin A isolated from *E. longifolia* on *in vitro* tuberculosis.

E. longifolia and its medicinal constituents have anti-cancer activity and therefore have the potential to be used as a complementary medicine in the treatment of a variety of human cancers (Thu *et al*., 2017). In addition to fifteen known alkaloids, the roots of *E. longifolia* yielded two new canthin-6-one alkaloids: 4,9-dimethoxy-5-hydroxycanthin-6-one and 9-methoxy-5-(1-hydroxyethyl)-canthin-6-one were examined in lipopolysaccharide (LPS)-stimulated RAW264.7 cells (Zhang *et al*., 2020).

Furthermore, one study found that the alkaloid 9-methoxycanthin-6-one, isolated from the ELHRCs, inhibited the development of ovarian, breast, colon, and skin cancers (Yunos *et al*., 2022). The most abundant quassinoids in *E. longifolia* roots is eurycomanone, a phytochemical specifically identified in *E. longifolia* extracts (Rehman *et al*., 2017). *E. longifolia* roots were extracted to test the antiprotozoal activity of four quassinoids: eurycomanone, 13,21-dihydroeurycomanone, 13-epoxyeurycomanone, eurycomalactone, and an alkaloid called 9-methoxycanthin-6-one (Chan *et al*., 2004). These chemicals were tested for their ability to kill chloroquine-resistant Gombak, and among the chemicals tested, *E. longifolia* root extract showed exceptional efficacy against *Plasmodium falciparum* malaria isolates (Mohd Ridzuan *et al*., 2005).

Nowadays, intestinal parasitic infection remains a major health problem worldwide. *Blastocystis* sp. is one of the most prevalent protozoan parasites found infecting the intestine (Ahmed & Karanis, 2019). There are up to about 38 subtypes (ST), and ST1 to ST10 are isolated predominantly from humans (Maloney *et al*., 2023). *Blastocystis* sp. infection is often associated with non-specific gastrointestinal symptoms such as diarrhoea, bloating, flatulence, and abdominal cramps (Tan, 2008). Recent studies have also associated infection with this organism with irritable bowel syndrome (Ragavan *et al*., 2015; Cifre *et al*., 2018) and colorectal cancer. However, its pathogenicity is uncertain. The conventional treatment for *Blastocystis* sp. is metronidazole (MTZ) (Stensvold *et al*., 2010; Adao & Rivera, 2018). It has been shown to exhibit low efficacy in some studies (Rajamanikam *et al*., 2019), and resistance has been already reported (Raman *et al*., 2016; Rajamanikam *et al*., 2019). There is a consistent need for an alternative anti-protozoal agent, and developing new

therapeutic options focusing mainly on medicinal plants is gaining ground due to their availability, lower toxicity, and broad spectrum of action compared to synthetic drugs.

To ensure a continuous supply of bioactive compounds, especially from roots (Gutierrez-Valdes *et al*. 2020; Morey & Peebles 2022), much attention has recently been paid to the culture of hairy roots to achieve continuous, enhanced, and stable production of specialised metabolites under controlled conditions (Pedreño & Almagro 2020; Shi *et al*., 2021). *E. longifolia* hairy root cultures (ELHRCs) were established by transformation with *Agrobacterium rhizogenes* strain A4 (Nazirah *et al*., 2018). It has been reported that the efficiency of hairy root productivity in terms of secondary metabolites can be increased by optimising growth conditions, including growth media, elicitation (biotic, bacteria, or abiotic, nanoparticles), and few of these techniques have been applied to *E. longifolia* (Sale *et al*., 2023). Therefore, there is still a need to search simple and effective strategies that can enhance the accumulation of medicinally important compounds in ELHRCs, which will contribute to promoting the industrial application process of this *in vitro* platform as plant factory in pharmaceutical fields.

Light-emitting diodes (LEDs) have gained significant attention in recent years due to their ability to provide specific light spectra that can effect plant metabolism (Dutta Gupta & Jatothu, 2013). LEDs are energy-efficient, produce less heat, and offer precise control over light quality, making them ideal for use in controlled environment agriculture (Neo *et al*., 2022). In plant tissue culture systems, where precise control over environmental conditions is necessary, LEDs provide an unequalled level of control over light quality, intensity, and duration (Azizi *et al*., 2024). It has been reported that LED light treatment regardless of colours was found beneficial for root growth and effective for enhancing phytochemical accumulation in *Astragalus*

membranaceous (Gai *et al.*, 2023) and *Isatis tinctoria* L (Jiao *et al*., 2023). While previous studies have demonstrated the general benefits of LED treatments for plant growth and phytochemical enhancement, little is known about their specific impact on ELHRCs. Moreover, the potential of LED-induced phytochemical changes to enhance antiparasitic activity, particularly against *Blastocystis* sp., remains unexplored. Therefore, this research uniquely investigates the effects of different LED spectral treatments on ELHRCs to optimise the production of bioactive compounds with antiparasitic properties. This study bridges the gap in understanding the intersection of LED technology, secondary metabolite enhancement, and its application in addressing parasitic infections, offering novel insights into the role of LEDs in medicinal plant biotechnology.

1.2 Problem statement

E. longifolia, commonly known as Tongkat Ali, is a well-known medicinal plant native to Southeast Asia, particularly Malaysia. It has been traditionally used for its aphrodisiac, anti-malarial, anti-cancer, and anti-inflammatory properties. Despite its extensive traditional use and recognised pharmacological potential, the biosynthesis and optimisation of its valuable compounds remain underexplored. Recent advances in plant biotechnology, such as hairy root cultures, offer promising strategies to enhance the production of secondary metabolites. However, the application of such technologies to *E. longifolia* has not been thoroughly investigated. One of the major challenges is the limited understanding of how different wavelengths of LED treatments affect the growth, morphological, biochemical, and molecular properties of ELHRCs. While various studies have highlighted the benefits of LED treatments in other plant species, there is a significant lack of research focussing on *E. longifolia*.

The potential to increase the production of bioactive compounds through LED treatments in ELHRCs is largely untapped.

Furthermore, the compounds produced by ELHRCs under LED treatments may have significant therapeutic potential, particularly against infectious diseases such as *Blastocystis* sp. Existing treatments for *Blastocystis* sp. infections, such as metronidazole, have limitations, including resistance and side effects. There is an urgent need to develop new, more effective treatments with less side effects. By optimising LED treatments to increase the production of secondary metabolites in ELHRCs, it could be used for various purposes and with promising anti-*Blastocystis* sp activity. Therefore, this study aims to fill the knowledge gap by investigating the effects of different LED treatments on growth, histological properties, biochemical and antioxidant activity, and synthesis of bioactive compounds in ELHRCs. Subsequently, to evaluate the anti-*Blastocystis* sp activity of the crude extract and isolated compounds from these cultures, potentially offering new approaches for effective treatments against *Blastocysti*s sp infections.

1.3 Significance of study

This study will significantly improve the understanding of the potential benefits of LED treatments to optimise the production of bioactive compounds in ELHRCs. By exploring the effects of LED treatments on ELHRCs, this research will provide valuable insights and practical applications in several key areas. Firstly, it will highlight the potential of *E. longifolia*, an indigenous plant with well-documented medicinal properties, and promote its conservation and sustainable utilisation. Secondly, the study aims to increase the yield of valuable secondary metabolites through optimal LED treatments, leading to more efficient and cost-effective processes

for the extraction of important therapeutic agents. Thirdly, by isolating and characterising bioactive compounds with potential anti-*Blastocystis* sp activity, the study could lead to the development of new antiparasitic drugs that have fewer side effects and are more effective than current treatments. In addition, increasing the production of bioactive compounds from cultures of ELHRCs may open up new economic opportunities, particularly in regions where the plant is native, and improve therapeutic agents that address important health problems, leading to better health outcomes and lower healthcare costs.

1.4 Research objectives

The objectives of this research are as follows:

i) To determine the effect of LED treatments on the morphological, histological, biochemical, antioxidant activity, and growth characteristics of *E. longifolia* hairy root culture

ii) To investigate how LED treatments enhance the synthesis of bioactive compounds in *E. longifolia* hairy root culture.

iii) To isolate and characterise compounds from *E. longifolia* hairy root culture using chromatographic and spectroscopic methods.

iv) To assess the effects of LED treatments on genetic stability of *E. longifolia* hairy root culture through DNA molecular analyses.

v) To evaluate the anti-*Blastocystis* sp activity of the crude extract and isolated compounds from *E. longifolia* hairy root culture under LED treatments.

CHAPTER 2

LITERATURE REVIEW

2.1 The *Eurycoma longifolia*

2.1.1 The characteristics of *E. longifolia*

Eurycoma longifolia Jack, commonly known as Tongkat Ali in Malaysia, Pasak Bumi in Indonesia, Cay ba Binh in Vietnam, and Ian-don in Thailand, belongs to the Simaroubaceae family (Meng *et al*., 2014). The genus *Eurycoma* consists of a few species and is native to the tropical rainforests of Southeast Asia, especially Malaysia and Indonesia. This plant is often referred to as "Malaysian ginseng" or a "national treasure" due to its revered status and medicinal properties (Mohamed *et al.*, 2015).

Tongkat Ali is a slow-growing evergreen tree that can tolerate nutrient-poor soils but thrives well-sunlit environments. It can reach heights of 12–15 meters (Figure 2.1) and produces green, ovoid fruits that mature to a reddish hue after 2–3 years of cultivation. Full maturity of the plant may take up to 25 years, but its roots are typically harvested for commercial use after 4 years. The leaves, which are 5–20 cm long, are pinnately compound and spirally arranged, with numerous obovate-lanceolate leaflets. The plant produces both male and female flowers, which are small and densely clustered (Bhat & Karim, 2010). Due to its protected status in Malaysia, the harvesting of Tongkat Ali is highly restricted. However, its root extracts are widely commercialised as a natural health supplement, often used as an alternative to tea, coffee, and carbonated drinks for promoting general well-being.

Below is the scientific classification of *E. longifolia*:

Kingdom:	Plantae
Division:	Magnoliophyta
Class:	Magnoliopsida
Order:	Sapindales
Family:	Simaroubaceae
Genus:	*Eurycoma*

Figure 2.1 The *E. longifolia* tree at the main campus of Universiti Sains Malaysia. Photo courtesy of the author.

2.1.2 Traditional uses of *E. longifolia*

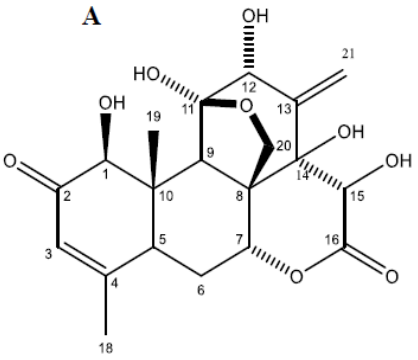
E. longifolia is a medicinal plant highly valued in Southeast Asia for its extensive therapeutic applications. It has been traditionally used to treat a variety of ailments, including sexual dysfunction, malaria, hyperglycemia, dysentery, schistosomiasis, chronic fever, mouth ulcers, and headaches (Kuo *et al*., 2004). Various parts of the plant, such as the bark, roots, seeds, and stem core, have been utilized for both medicinal and nutritional purposes, with the roots being especially prized for their potent therapeutic properties. In Malaysia and Indonesia, *E. longifolia* roots are commonly believed to possess aphrodisiac effects that enhance male sexual performance. Typically, the roots are boiled to create a tea, often sweetened with sugar or honey to offset its natural bitterness. In addition to the roots, the leaves of *E. longifolia* have been noted for their anti-ulcer, antimalarial, gum-protective, and venereal disease-treating properties (Bhat & Karim, 2010). Today, the plant is widely commercialized, with more than 200 products available on the market, including capsules, tablets, and beverages such as tea and coffee (Rehman *et al*., 2016).

2.1.3 The compounds isolated from *E. longifolia* extracts

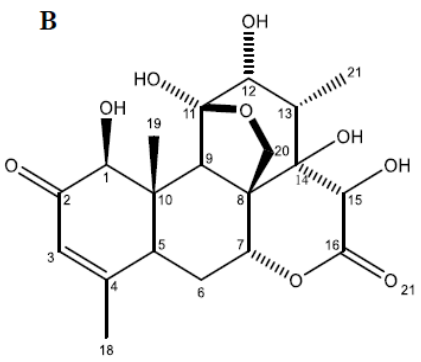
Phytochemical studies on *E. longifolia* have led to the isolation of numerous bioactive compounds from different parts of the plant. These compounds include quassinoids, canthin-6-one alkaloids, squalene derivatives, tirucallene-type triterpenes, biphenyl-neo-lignans, anthraquinones, and polyacetylenes (Teh *et al*., 2010; Farag *et al*., 2022). To date, over 65 compounds have been identified from various parts of the plant and tested for their pharmacological potential, with the roots, stems, leaves, and bark being particularly rich in bioactive constituents (Kuo *et al*., 2004).

2.1.3(a) Quassinoids

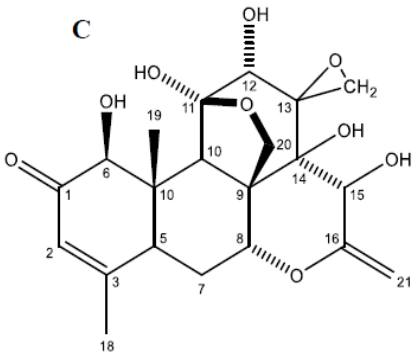
Quassinoids, a class of naturally occurring degraded triterpenes, are the most prominent bioactive compounds found in *E. longifolia*. They are classified according to the number of carbon atoms in their fundamental structure (C18, C19, and C20). Rehman *et al*. (2016) identified several quassinoids from *E. longifolia*, including C-18 compounds like laurictone A, eurycolactone B, and eurycolactone D. C-19 quassinoids, such as 6α-hydroxyeurycomalactone, 7α-hydroxyeurycomalactone, eurycomalide A, and eurycolactone E, have demonstrated a variety of biological activities, including antiplasmodial, antimalarial, antileukemic, antitubercular, and antiviral effects. The C-20 quassinoids, including eurycomanone, eurycomanol, and 13α(21)-dihydroeurycomanone, are the most widely studied and are known for their broad range of biological activities. Figure 2.2 illustrates the chemical structures of the four major quassinoids in *E. longifolia*.



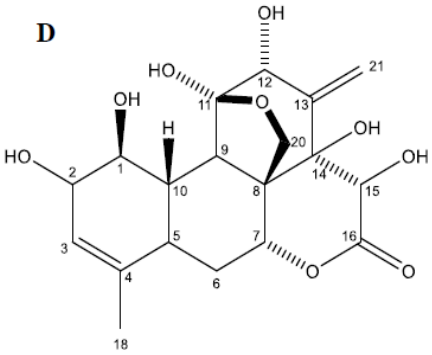
Eurycomanone (EN)



13α, 21-Dihydroeurycomanone (DHY)



13α(21)-Epoxyeurycomanone (EP)



Eurycomanol (EL)

Figure 2.2 Chemical structure of four major quassinoid in *E. longifolia*: (A) eurycomanone, (B) 13α, 21-dihydroeurycomanone, (C) 13α (21)-epoxyeurycomanone and (D) eurycomanol (Rehman *et al*., 2016).

2.1.3(b) Alkaloids

Alkaloids are nitrogen-containing organic compounds commonly found in plants and are known for their significant pharmacological properties, including antimalarial, anti-inflammatory, and antioxidant activities. Common alkaloids include morphine, quinine, nicotine, and ephedrine. Figure 2.3 provides an overview of the chemical structures of four major alkaloids found in *E. longifolia*.

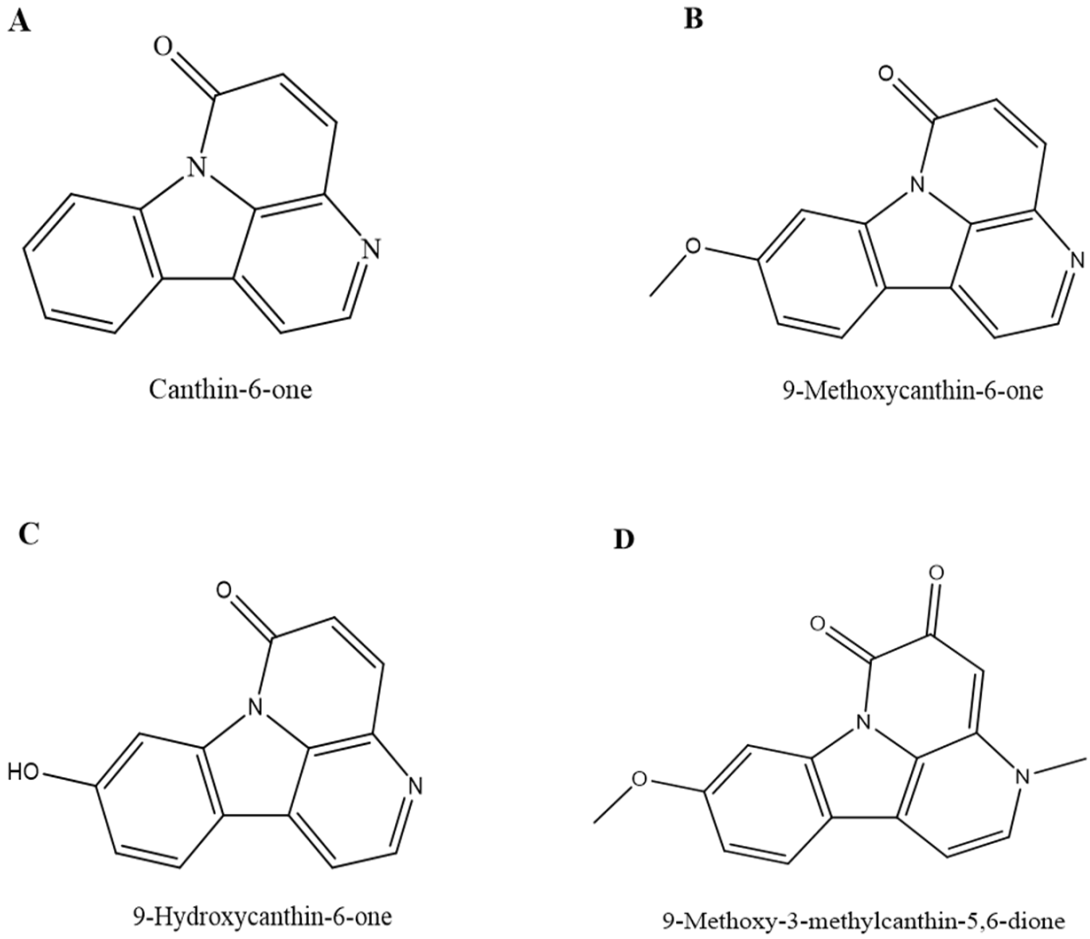


Figure 2.3 Chemical structure of four major alkaloids in *E. longifolia*: (A) Canthin-6-one, (B) 9-Methoxycanthin-6-one, (C) 9-Hydroxycanthin-6-one and (D) 9-Methoxy-3-methylcanthin-5,6-dione (Rehman *et al*., 2016).

Kuo *et al*. (2004) reported the extraction of approximately 65 compounds from the roots of *E. longifolia*, with their structures elucidated using advanced spectroscopic techniques such as 1-D and 2-D nuclear magnetic resonance (NMR) spectroscopy, in addition to mass spectrometry data. Recent studies have identified novel quassinoid diterpenoids, including eurycomalide A, eurycomalide B, 5α-14β, 15β-trihydroxyklaineanone, and 13β, 21-dihydroxyeurycomanol (Serag *et al*., 2023). Furthermore, Park *et al*. (2014) identified five new quassinoids—eurylactone E, eurylactone F, eurylactone G, eurycomalide D, and eurycomalide E—along with ten

previously known quassinoids extracted from the plant's roots (Tung *et al*., 2017). Additionally, Zhang *et al*. (2020) reported two previously unknown canthin-6-one alkaloids, 4,9-dimethoxy-5-hydroxycanthin-6-one and 9-methoxy-5-(1-hydroxyethyl)-canthin-6-one, from *E. longifolia* roots.

2.2 Pharmacological properties of compounds isolated from *E. longifolia*

Numerous scientific studies have validated the traditional uses of *E. longifolia* by exploring its pharmacological properties. Research has primarily focused on four key areas: aphrodisiac activity, antimalarial and anticancer properties, anti-diabetic effects, and antimicrobial activities.

2.2.1 Aphrodisiac activities

The aphrodisiac properties of *E. longifolia* have garnered widespread attention. Zanoli *et al*. (2009) found that administering root powder from *E. longifolia* increased sexual activity in male rats previously displaying sexual inactivity. The treated rats exhibited reduced ejaculation latency, increased mounting frequency, and shorter post-ejaculatory intervals compared to the control group. Furthermore, the study noted that daily administration of 500 mg of root powder per kilogram of body weight over six days resulted in a significant increase in testosterone levels in the treated rats.

2.2.2 Antimalarial and anticancer properties of *E. longifolia*

Malaria is a disease caused by the parasite *Plasmodium* sp. and is transmitted by the bites of infected mosquitoes. Multiple studies have reported that resistance of *Plasmodium* sp. to antimalarial medications, such as azithromycin and chloroquine, is the primary reason for the failure of antimalarial therapy (Zhou *et al*., 2017). Therefore,

the identification of suitable antimalarial medications is crucial in reducing mortality rates.

E. longifolia has shown promise as a potential antimalarial treatment (Wernsdorfer *et al.*, 2009). Hout *et al*. (2006) demonstrated that water extracts of *E. longifolia* were highly effective in inhibiting the growth of chloroquine-resistant *Plasmodium falciparum* (strain W2), with an IC_{50} value of less than 4 μg/ml. In addition, compounds such as eurycomanone, 13α(21)-epoxyeurycomanone, 13,21-dihydroeurycomanone, and 9-methoxycanthin-6-one, isolated from the plant's roots, have demonstrated efficacy against chloroquine-resistant *Plasmodium* isolates (Segaran *et al*., 2021). Furthermore, 1,15-di-O-isovaleryleurycomanone, 1,15-di-O-benzoyleurycomanone, and 1,15-di-O-(3,3-dimethylacryloyl)-eurycomanone exhibited significant cytotoxicity and hindered the growth of Gombak A parasites that are resistant to chloroquine (Low *et al*., 2005).

Studies have also explored the anticancer potential of *E. longifolia*. Tee & Azimahtol, (2005) reported that methanolic extracts of the plant's roots significantly inhibited the proliferation of breast cancer cells (MCF-7) with an IC_{50} of 7.80 ± 0.45 μg/ml, while demonstrating lower toxicity to normal breast cells (MCF-10A). Kaewpiboon *et al.* (2012) found that dichloromethane and ethanol extracts from *E. longifolia* roots induced apoptosis in several human cancer cell lines, including lung (A549), breast (MDA-MB-231), cervical (KB3-1), and colon (SW480) cancers, with the greatest cytotoxicity observed in the MDA-MB-231 breast cancer cell line. In addition, the plant's root extract demonstrated substantial cytotoxicity against the human promyelocytic leukemia cell line HL-60, with an IC_{50} value of 15.2 μg/ml (Al-Salahi *et al*., 2012).

Further *in vivo* studies confirmed the anticancer properties of *E. longifolia*. Al-Salahi *et al*. (2014) evaluated the antiproliferative activity of methanolic root extracts in mice injected with chronic myelocytic leukemia cells (K-562). The treatment significantly reduced tumor size by 85% compared to the control group. Key bioactive compounds responsible for these effects include quassinoids such as eurycomanone and eurycomalactone, along with alkaloids like 9-methoxycanthin-6-one and β-carboline, which have been scientifically proven to inhibit cancer growth (Kardono *et al*., 1991; Jiwajinda *et al*., 2002; Chuen & Pihie, 2004); Nurhanan *et al*., 2005; Zakaria *et al*., 2009; Miyake *et al*., 2010; Al-Salahi *et al*., 2014).

2.2.3 Anti-diabetic properties

The consumption of Tongkat Ali leaves and roots is traditionally believed to aid in regulating blood glucose levels (Bhat & Karim, 2010). A study by Panjaitan & Astuti (2021) explored the antidiabetic potential of *E. longifolia* leaf extract in a streptozotocin-nicotinamide-induced diabetic rat model. Their research aimed to assess the effectiveness of ethanol extracts from *E. longifolia* leaves in managing diabetes. The study demonstrated that administering the ethanol extract at a dosage of 176.4 mg per 200 g of body weight significantly reduced both blood glucose and malondialdehyde levels in diabetic rats, comparable to the effects observed with glibenclamide, administered at 0.09 mg per 200 g of body weight. Husen *et al*. (2004) further hypothesized that the therapeutic effects of *E. longifolia* develop gradually over time and require consistent consumption. They also concluded that patients with diabetes who suffer from sexual dysfunction can safely use *E. longifolia* alongside antidiabetic medications, such as rosiglitazone, potentially enhancing the treatment's efficacy.

2.2.4 Antimicrobial activities

Several studies have evaluated the antimicrobial properties of extracts from various parts of *E. longifolia*, including the leaves, stems, and roots. Research by Farouk & Benafri (2007) and Danial *et al*. (2013) examined methanol, ethanol, acetone, and water extracts of *E. longifolia* against a range of Gram-positive and Gram-negative bacteria. Their findings revealed that alcoholic and acetone extracts derived from the leaves and stems exhibited antibacterial activity against most tested bacterial strains, with the exception of *Escherichia coli* and *Salmonella typhi* (Gram-negative). In contrast, the root extracts did not demonstrate significant antibacterial activity against either Gram-positive or Gram-negative bacteria. However, aqueous leaf extracts were effective against *Staphylococcus aureus* and *Serratia marcescens*.

Further studies by Khanam *et al*. (2015) assessed the antimicrobial efficacy of root and stem extracts from wild *E. longifolia* plants. Their results showed that all extracts exhibited dose-dependent antimicrobial properties, with the highest antibacterial effects seen against Gram-positive bacteria. Particularly, stem extracts demonstrated greater potency than root extracts, particularly against *Bacillus cereus* and *Staphylococcus aureus*. The ethyl acetate extract of the stem also displayed moderate activity against the Gram-negative bacterium *Pseudomonas aeruginosa* and exhibited substantial antifungal activity against *Aspergillus niger*.

In a study by Kuspradini *et al*. (2019), ethanol extracts from different roots, including *E. longifolia*, were tested for antimicrobial potential. The study found that *E. longifolia* exhibited the highest phenol content and an activity index (AI) of 0.96 at a concentration of 1000 µg. This suggests that *E. longifolia* holds promise as an antimicrobial agent against oral pathogens such as *Candida albicans*, *S. aureus*,

Streptococcus mutans, and *Streptococcus sobrinus*. Liu *et al*. (2022) further explored the antibacterial mechanism and antibiofilm effects of the dichloromethane fraction (DCM) of *E. longifolia* against *S. aureus*, highlighting its potential as a powerful antimicrobial agent.

2.3 Hairy root cultures: background and establishment

The concept of "hairy root cultures" refers to the extensive proliferation of roots in an *in vitro* culture, which has been modified due to infections caused by the bacterium *Agrobacterium rhizogenes*. Numerous studies have documented the successful establishment of hairy root cultures (HRCs) from a diverse array of plant species, encompassing both dicotyledonous and monocotyledonous plants. Ono & Tian (2011) reported that hairy root cultures have been produced from over 400 different plant species.

Plant tissue culture has long been recognized as a valuable alternative source for producing physiologically active compounds (Łuczkiewicz & Kokotkiewicz, 2005). Since the early 20th century, two plant diseases hairy root and crown gall have significantly impacted agricultural industries, particularly affecting winegrowers. Consequently, extensive research has been conducted to understand these diseases and the mechanisms by which they are caused (Sena, 2015).

Hairy root disease in dicotyledonous plants is induced by the *A. rhizogenes* bacterium (Sevón & Oksman-Caldentey, 2002). However, plant is infected by this bacterium, the T-DNA from the Ri-plasmid is transferred into the plant nuclear genome, leading to the formation of hairy roots a beneficial by-product in biotechnology (Chilton *et al*., 1982; Georgiev *et al*., 2007). One of the advantages of the hairy roots are highly branched, fast-growing structures that can proliferate on a

substrate without the need for added phytohormones. In addition , hairy root cultures are genetically and biochemically stable, making it capable of consistently producing a varied range of secondary metabolites (Kittipongpatana *et al*., 1998).

Hairy root cultures have become a valuable tool in biotechnology for the production of high-value compounds. According to Georgiev *et al*. (2007), the induction of hairy roots is achieved by culturing wounded plant explants under aseptic conditions using *A. rhizogenes* suspensions. Several critical factors are essential for the successful establishment of a hairy root culture system, including the selection of an appropriate *A. rhizogenes* strain, the use of effective antibiotics to eliminate residual bacteria, and the optimisation of the culture medium (Hu & Du, 2006).

One significant advantage of plant cell suspension culture, which can be applied in hairy root cultures, is its rapid expansion and resilience to seasonal and geographical variations. However, it is important to recognise that plant suspension cells often lack differentiation, which results in unstable and low production of secondary metabolites (Zhao, 2014). Hairy root cultures, however, overcome these limitations, offering a stable and efficient platform for secondary metabolite production.

Figure 2.4 *E. longifolia* hairy root cultures. Photo courtesy of the author.

2.3.1 Beneficial attributes of hairy root cultures system

Hairy root cultures can be grown easily and cultivated in limited areas (Danial *et al*., 2011a). These roots have the potential to regenerate entire plants while maintaining high genetic stability. Most transformed explants, when cultured on phytohormone-free media, exhibit altered phenotypes such as hairy root syndrome, dwarfism, altered flowering patterns, wrinkled leaves, and increased branching, traits that can be advantageous for ornamental breeding and other plant improvement programs (Giovannini *et al*., 1997; Hu & Du *et al*., 2006). Moreover, transformed

explants offer a significant advantage over untransformed plant cell cultures, which are often genetically and biochemically unstable and produce low yields of secondary metabolites (Kittipongpatana *et al*., 1998; Hu & Du *et al*., 2006).

In recent years, hairy root cultures have also been used to produce recombinant therapeutic proteins, particularly those that are challenging to express in traditional systems such as bacteria, yeast, and mammalian cells (Ono & Tian, 2011). Numerous studies have utilized hairy root cultures for a range of purposes, including phytochemical production (Bourgaud *et al*., 2001; Georgiev *et al*., 2007; Ochoa-Villarreal *et al*., 2016), phytoremediation (Flocco & Giulietti, 2007), molecular breeding (Giri & Narasu, 2000; Casanova *et al*., 2005; Christensen & Müller, 2009), and metabolic engineering (Hu & Du, 2006; Mehrotra *et al*., 2010). These systems have also been employed for the design and optimisation of bioreactors (Uozumi, 2004; Sivakumar, 2006; Srivastava & Srivastava, 2007; Mishra & Ranjan, 2008).

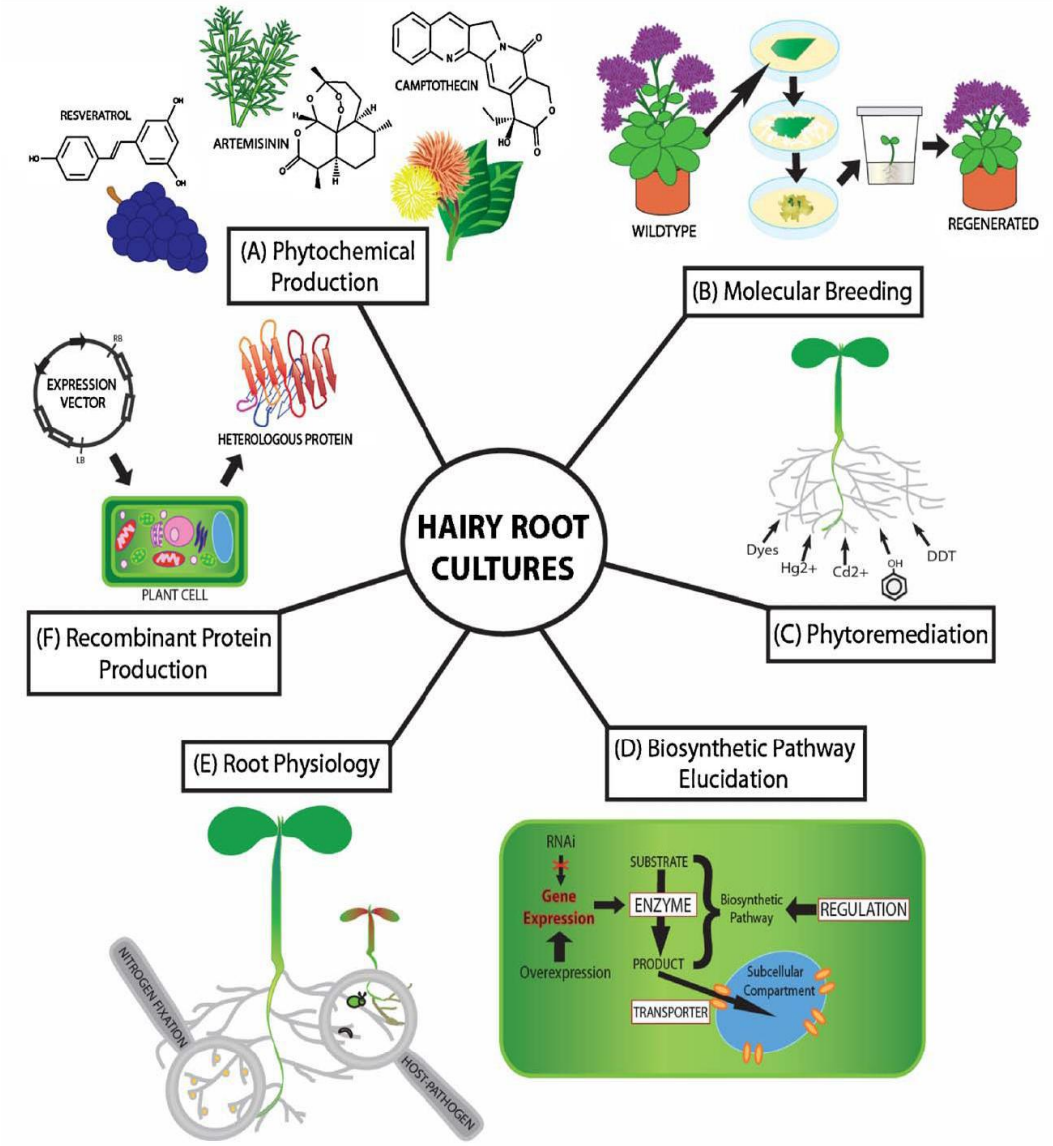


Figure 2.5 The many beneficial uses of hairy root cultures which include (A) Phytochemical production, (B) Molecular breeding, (C) Phytoremediation studies, (D) Biosynthetic pathway elucidation, (E) Root physiology studies and (F) Recombinant protein production (Adapted from: Ono & Tian, 2011).

2.3.2 Phytochemicals production in hairy root cultures

Hairy root cultures are an excellent platform for the production of key phytochemicals, including terpenoids, alkaloids, and phenolics (Li & Wang, 2021). Plants have the inherent ability to synthesize a variety of industrially and pharmaceutically relevant compounds from simple precursors (Ono & Tian, 2011).

However, extracting phytochemicals from conventional plant tissues is often inefficient due to environmental stress and the destructive nature of the extraction process (Ono & Tian, 2011). Hairy root cultures overcome these challenges by providing a stable, consistent source of phytochemicals, due to their genetic stability and enhanced biosynthetic capabilities (Hu & Du, 2006). Additionally, these cultures often accumulate higher concentrations of phytochemicals than cell or callus cultures (Ono & Tian, 2011).

Biotic and abiotic stimulation, either alone or in combination, can increase phytochemical synthesis from hairy root cultures (Guillon *et al*., 2006). Abiotic stress has been employed to increase desired products in *Salvia miltiorrhiza* and *Pisum sativum* hairy root cultures (Kaimoyo *et al*., 2008; Wu & Shi, 2008). Elevated osmotic stress was induced in *S miltiorrhiza* hairy root cultures by mixing high sorbitol concentrations with a yeast elicitor, resulting in synergistic effects on diterpenoid tanshinone production up to 100-fold higher in fed-batch cultures than in controls (Wu & Shi, 2008).

Both biotic and abiotic elicitors can be employed to enhance phytochemical production in hairy root cultures (Guillon *et al*., 2006). For example, in *Salvia miltiorrhiza*, elevated osmotic stress induced by high sorbitol concentrations combined with a yeast elicitor resulted in a 100-fold increase in diterpenoid tanshinone production (Wu & Shi, 2008). Similarly, *Pisum sativum* hairy root cultures treated with sub-lethal doses of electric current saw increased accumulation of pisatin, a process hypothesized to suppress secondary metabolite degradation and initiate a signaling cascade that activates production genes and enzymes (Kaimoyo *et al*., 2008). Electric current elicitation has proven effective across a range of plant species and culture

systems without contaminating the produced compounds, making it a promising method for enhancing industrial phytochemical production (Ono & Tian, 2011).

However, the injection of an electric current may affect the integrity of the cellular membrane, resulting in the release of endogenous elicitors or a signalling cascade that activates secondary metabolite production genes and enzymes (Cuello & Yue, 2008). Furthermore, the extensive application of electric current to elicit phytochemicals has resulted in phytochemical accumulation in a number of plant species, tissue types, and culture systems (Kaimoyo *et al*., 2008). The elicitation with electric current does not contaminate the system or the compounds produced. In addition, repeated rounds of electric current elicitation can be applied to the hairy root culture, enhancing phytochemical output. This benefits the industry since, combined with the potential to promote penetration to the roots cells of hairy root cultures, electric current elicitation is an appealing method (Ono & Tian, 2011).

2.3.3 Plant secondary metabolites production through hairy root cultures

Transformed roots from numerous plant species have been thoroughly investigated for secondary metabolite synthesis *in vitro* (Mukundan *et al*., 1998). Hairy roots provide potential for the continuous and standardised synthesis of secondary metabolites under regulated conditions (Hu & Du, 2006). Hairy roots generate secondary metabolites throughout numerous generations while retaining genetic and metabolic stability (Mukundan *et al*., 1998). This trait can be employed in genetic engineering to increase the plant biosynthetic potential.

Furthermore, secondary metabolite synthesis in hairy roots is regulated by nutrition and environmental factors. According to Narayanaswamy (1994), the biomass and composition of the needed secondary metabolites are the most essential