THE EFFECTS OF PHOTOPERIODS AND LIGHT EMITTING DIODES (LEDs) ON CELL SUSPENSION AND HAIRY ROOT CULTURES OF *Eurycoma longifolia* JACK

SALE SANI

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by

SALE SANI

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2,4-D	2,4-Dichlorophenoxyaceticdichlorophenoxyacetic acid
AR	Adventitious Roots
BAP	Benzylaminopurine
COX-2	Cyclooxygenase 2
CS	Cell Suspension
E. longifolia	Tongkat Ali
FAA	Formalin-Aceto-Alcohol
FRIM	Forest Research Institute Malaysia
GUS	β-glucuronidase
HCl	hydrochloric acid
HMDS	Hexamethyldisilazane
HPLC	High performance liquid chromatography
HR	Hairy Roots
iNOS	Inducible Nitric Oxide Synthase
LED	Light Emitting Diodes
MeJa	Methyl Jasmonate
MS	Murashige and Skoog
NAA	Naphthaleneacetic Acid
NaOH	Sodium Hydroxide
NO	Nitric Oxide
PEC	Pectin
PGR	Plant Growth Regulators
ROS	Reactive Oxygen Species
RSA	Radical Scavenging Capacity
SA	Salicylic Acid

SE	Somatic Embryos
SEM	Scanning electron microscopy
SI	Similarity Indices
VAL	Valine
YE	Yeast

KESAN FOTO PERIOD DAN DIOD PEMANCAR CAHAYA (LED) PADA KULTUR AMPAIAN SEL DAN KULTUR AKAR RERAMBUT *Eurycoma longifolia* JACK

ABSTRAK

Eurycoma longifolia Jack, biasanya dikenali sebagai Tongkat Ali, ialah pokok perubatan pelbagai guna di Asia Tenggara yang digunakan untuk merawat pelbagai penyakit, termasuk cirit-birit, leukemia, dan malaria, serta penyakit kronik seperti diabetes dan kanser. Tongkat Ali mendapat permintaan tinggi terutamanya untuk sifat afrodisia, tetapi pengeluarannya terhad disebabkan tempoh eram yang panjang dan pergantungan kepada populasi liar. Sistem penanaman *in vitro*, seperti kultur ampaian sel dan kultur akar rerambu, menyediakan alternatif yang menjanjikan untuk pengeluaran yang mampan. Kajian ini bertujuan untuk meningkatkan pertumbuhan dan pengeluaran metabolit bioaktif, termasuk eurycomanone, 9-hydroxycanthin-6-one, dan 9-methoxycanthin-6-one, dalam kultur ampaian sel dan kultur akar rerambut *E. longifolia* dengan menggunakan fotokala dan rawatan diod pancaran cahaya (LED). Kultur ampaian diperoleh daripada eksplan ruas, dan akar rerambu diperoleh daripada Institut Penyelidikan Perhutanan Malaysia (FRIM). Kultur ampaian sel didedahkan di bawah cahaya berterusan selama 24 jam dan kitaran cahaya/gelap 16/8 jam sebagai kawalan. Seperti di atas, kultur akar rerambu didedahkan kepada cahaya selama 24 jam, cahaya/gelap 16/8 jam dan kegelapan 24 jam sebagai kawalan. Kedua-dua kultur juga dirawat dengan lampu LED biru, merah dan biru+ merah, dengan LED putih sebagai kawalan. Morfologi pertumbuhan, sintesis metabolit bioaktif, jumlah kandungan flavonoid dan aktiviti antioksidan telah dinilai. Keputusan menunjukkan bahawa kultur ampaian sel di bawah cahaya selama 24 jam mempunyai berat kering

yang jauh lebih banyak iaitu 0.13 g/50 mL selepas 15 hari dan menghasilkan paras eurycomanone (8.96 μg/g DW) dan 9-hydroxycanthin-6-one (0.29 μg/g DW) yang lebih tinggi selepas 18 hari. Selain itu, DPPH dan ABTS+ kebolehan pemusnahan radikal, masing-masing memuncak pada 44.8% dan 47.8%, selepas 21 hari, di samping jumlah kandungan flavonoid sebanyak 28.6 QE/g DW. LED biru dan putih meningkatkan biojisim dengan ketara pada hari 21 dan 24, dengan berat kering masing-masing 0.23 dan 0.22 g/50 mL. Rawatan LED juga mempengaruhi sintesis sebatian bioaktif, dengan LED putih menghasilkan eurycomanone yang lebih banyak (8.72 μg/g DW) pada 21 hari, dan LED biru+ merah menghasilkan 9-hydroxycanthin-6-one (0.61 μg/g DW) dan 9-methoxycanthin-6 one (0.87 μg/g DW) paling banyak. LED biru juga meningkatkan DPPH dan ABTS+ RSA masing-masing kepada 64.3% dan 72.8%, bersama dengan jumlah kandungan flavonoid sebanyak 64.4 QE/g DW. Cahaya berterusan meningkatkan kandungan eurycomanone, manakala kegelapan menggalakkan pengeluaran 9-hydroxycanthin-6-one dan 9-methoxycanthin-6-one, masing-masing mencapai 14.05, 10.97, dan 7.78 μg/g DW, selepas 4 minggu dalam kultur akar rerambu. Walaupun fotokala tidak menjejaskan aktiviti antioksidan dengan ketara, kegelapan berterusan dan kitaran cahaya/gelap 16/8 jam meningkatkan kandungan flavonoid kepada 92 QE/g DW. Kombinasi LED biru dan merah menghasilkan berat kering terbanyak (0.39 g/50 mL) selepas 8 minggu, diikuti oleh LED merah (2.8 g/50 mL). Kombinasi LED ini juga memaksimumkan hasil eurycomanone, 9-hydroxycanthin-6-one, 9-methoxycanthin-6-one, dan jumlah kandungan flavonoid selepas 8 minggu, dengan nilai 25.83, 2.16, 13.13 μg/g DW, dan 139.4 mg QE/g DW, masing-masing. Kesimpulannya, kedua-dua fotokala dan LED boleh digunakan untuk memaksimumkan produktiviti *E. longifolia* melalui sistem *in vitro*.

THE EFFECTS OF PHOTOPERIODS AND LIGHT EMITTING DIODES (LEDs) ON CELL SUSPENSION AND HAIRY ROOT CULTURES OF *Eurycoma longifolia* JACK

ABSTRACT

Eurycoma longifolia Jack, commonly known as Tongkat Ali, is a Southeast Asian multipurpose medicinal tree used for treating various ailments, including diarrhea, leukemia, and malaria, as well as chronic diseases like diabetes and cancer. Tongkat Ali is of high demand especially for its aphrodisiac properties, but production is limited by long gestation periods and dependence on wild populations. *In vitro* cultivation systems, such as cell suspension and hairy root cultures, provide promising alternatives for sustainable production. This study aims to enhance the growth and production of bioactive metabolites, including eurycomanone, 9-hydroxycanthin-6-one, and 9-methoxycanthin-6-one, in cell suspension and hairy root cultures of *E. longifolia* using photoperiods and light-emitting diodes (LEDs) treatments. Cell suspensions were derived from internodal explants, and hairy roots were obtained from the Forest Research Institute Malaysia (FRIM). The cell suspension cultures were subjected to 24-hour continuous light and a 16/8-hour light/dark cycle as a control. Similarly, the hairy root cultures were exposed to 24-hour light, 16/8-hour light/dark, and 24-hour darkness as a control. Both cultures were also treated with blue, red, and blue+ red LED lights, with white LED as control. Growth morphology, bioactive metabolite synthesis, total flavonoid content and antioxidant activity were assessed. The results have shown that cell suspension cultures under 24-hour light have significantly higher dry weight of 0.13 g/50 mL after 15 days and produced higher levels of eurycomanone (8.96 µg/g DW) and 9-hydroxycanthin-6-one (0.29 µg/g DW) after 18 days. Additionally, DPPH and ABTS+ radical scavenging abilities peaked at

44.8% and 47.8%, respectively, after 21 days, alongside a total flavonoid content of 28.6 QE/g DW. Blue and white LEDs significantly enhanced biomass on days 21 and 24, with dry weights of 0.23 and 0.22 g/50 mL, respectively. LED treatments also influenced bioactive compound synthesis, with white LED producing higher eurycomanone (8.72 µg/g DW) at 21 days, and blue+ red LED yielding the highest 9-hydroxycanthin-6-one (0.61 µg/g DW) and 9-methoxycanthin-6-one (0.87 µg/g DW). Blue LED also increased DPPH and ABTS+ RSA to 64.3% and 72.8%, respectively, along with a total flavonoid content of 64.4 QE/g DW. In hairy root cultures, continuous light increased eurycomanone content, while darkness promoted 9-hydroxycanthin-6-one and 9-methoxycanthin-6-one production, reaching 14.05, 10.97, and 7.78 µg/g DW, respectively, after 4 weeks. Although photoperiods did not significantly affect antioxidant activity, continuous darkness and 16/8-hour light/dark cycle increased flavonoid content to 92 QE/g DW. The combination of blue and red LEDs yielded the highest dry weight (0.39 g/50 mL) after 8 weeks, followed by red LED (2.8 g/50 mL). This LED combination also maximized the yields of eurycomanone, 9-hydroxycanthin-6-one, 9-methoxycanthin-6-one, and total flavonoid content after 8 weeks, with values of 25.83, 2.16, 13.13 µg/g DW, and 139.4 mg QE/g DW, respectively. In conclusion, both photoperiods and LEDs can be utilized to maximize the productivity of *E. longifolia* via *in vitro* systems.

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Eurycoma longifolia Jack is a versatile tree from the Simaroubaceae family, popularly known as the Quassia family. It belongs to the genus *Eurycoma* along with two other species, namely *Eurycoma apiculata* A. W. Benn. which is found in Malaysia and Indonesia, and *Eurycoma harmandiana* Pierre which grows along the axis between Thailand and Laos. Although these species share some similarities in terms of chromosome number (Zulfahmi et al., 2018) and basic phytochemicals (Chaingam et al., 2022), *E. longifolia* is more widespread and contains numerous bioactive compounds (Chaingam et al., 2022) and uses. It is native to Southeast Asia and is mainly found in Indonesia, Malaysia, Singapore and Thailand. It can also be found in Brunei Darussalam, Cambodia, Laos, Vietnam, southern Myanmar and the Philippines (Bhat & Karim, 2010; Chua et al., 2005; Wizneh & Asmawi, 2014). *E. longifolia* has various local names depending on regions, such as Tongkat Ali in Malaysia, Pasak Bumi in Indonesia, Ian-don in Thailand, Cây bách bệnh in Vietnam and Tho nan in Laos (Hidayati et al., 2021).

Eurycoma longifolia is a medium-sized tree that typically grows to a height of 15 to 18 meters and is commonly found as undergrowth in forests (Nordin, 2014). Historically, wild populations of this tree were the sole source of Tongkat Ali products. However, as demand for its medicinal properties increased, pressure on these natural resources intensified. This rising demand led to the establishment of commercial plantations to ensure a more sustainable supply of Tongkat Ali (Jian & Jian, 2013).

Eurycoma longifolia is considered one of the most valuable medicinal plants. In Vietnam, it is listed in the pharmacopoeia and known locally as “chy ba binh”, which literally means tree that cures hundreds of diseases (Le-Van-Thoi & Nguyen-Ngoc-Suong, 1970). It is considered a national treasure in Malaysia (Mohamed et al., 2015) and a popular medicinal plant in Indonesia (Susilowati et al., 2019). *E. longifolia* is used in traditional herbal medicine as well as in modern pharmaceutical applications. Additionally, the plant extract is employed in dietary supplements and in the cosmetics industry. The variety of applications has helped to raise the status of *E. longifolia* in both the commercial and scientific fields.

Traditionally, various parts of the *E. longifolia* tree have been used to treat many diseases, including skin itching, dysentery, stomach worms, diarrhoea and fever (Kuo et al., 2004). The root extract of *E. longifolia* is highly valued for its aphrodisiac properties and its effectiveness in the treatment of chronic diseases such as cancer and diabetes (Tsai et al., 2020). It is also used for diseases such as leukaemia, syphilis, fever and osteoporosis (Abdul Rahman et al., 2018). It is also used as an antibiotic, helps to slow down the ageing process and helps to reduce stress and anxiety. It is also used for gynaecological diseases (Abdul Rahman et al., 2018).

Scientific experiments with *in vitro* systems, animal models and clinical studies have demonstrated the antimalarial (Chan et al., 2005), cytotoxic, anticancer (Nguyen-Pouplin et al, 2007; Nurhanan et al, 2005; Ye et al, 2022), antidiabetic, aphrodisiac, proandrogenic and antimicrobial effects (Ang et al, 2001; Farouk, 2007; Khanam et al, 2015) of *E. longifolia*. In addition, its efficacy in the treatment of male sexual dysfunction (Thu et al., 2017) and osteoporosis (Mohd Effendy et al., 2012) has been confirmed. In addition, clinical studies have shown that eurycomanone, the main bioactive molecule in *E. longifolia*, is effective against lung, breast, stomach and colon

cancer (Thu, Hussain, et al., 2017). It also shows wound-healing properties (Al-Bayati et al., 2022) and efficacy against bacteria, fungi, protozoa (Liu et al., 2022; Thu et al., 2018), dengue (He et al., 2023) and coronavirus (Choonong et al., 2022).

Eurycoma longifolia has been extensively researched for its phytochemicals and bioactive compounds isolated from its root, leaf and stem (Rehman et al., 2016). These compounds form a group of quassinoids/degraded triterpenoids (Izzati et al., 2020), including eurycomalactone, eurycomanone, eurycomanol and others (Darise et al., 1982; Miyake et al., 2009). Abubakar et al. (2017) reported over 70 bioactive compounds from different parts of *E. longifolia* in their review. In the last ten years, numerous new bioactive compounds have been reported, which include eurycomanone (pasakbumin-A), eurycomanols, pasakbumin-B, hydroxyklaineanones, eurycomalactones, eurycomadilactones, eurylactones, laurycolactones, longilactones, and hydroxyglaucarubol, 5,9-dimethoxycanthin-6-one, 9,10-dimethoxycanthin-6-one, 11-hydroxy-10-methoxycanthin-6-one, 10-hydroxy-9-methoxycanthin-6-one, and 9-methoxy-3-methylcanthin-5,6-dione, 9-hydroxycanthin-6-one (He et al., 2023; Izzati et al., 2020; Meng et al., 2014; Ngoc et al., 2015; Park et al., 2014; Ruan et al., 2019; Yang et al., 2020, 2021; Zhang et al., 2020a).

Eurycoma longifolia products are gaining public acceptance worldwide and hundreds of products have been registered by the relevant authorities. The Malaysian Ministry of Health estimates the total value of *E. longifolia* at USD 1.7 billion and expects annual growth of 15% (Brinckmann & Brendler, 2019). This lucrative market has attracted considerable interest, fuelling concerns about product adulteration and highlighting the need for product authentication (Mutschlechner et al., 2018; Serag et al., 2023).

The authorised products are already on international markets (Sambandan et al., 2006), leading to increased pressure on resources and prompting the implementation of government legislation to ensure sustainability (Brinckmann & Brendler, 2019). Ensuring sustainability in the utilisation of plant resources can be achieved through effective propagation techniques.

In the case of *E. longifolia*, numerous research reports focus on *in vitro* propagation and various strategies to improve the synthesis of bioactive compounds through chemical or biochemical elicitors. For example, studies on the effects of plant growth regulators (PGRs) in cell suspension cultures of *E. longifolia* have demonstrated that MS medium supplemented with 0.5 mg/L NAA and 0.25 mg/L 2,4-D significantly increases the production of canthin-6-one alkaloids (Siregar et al., 2009). Similarly, research on carbon and nitrogen sources shows that glucose and potassium nitrate (KNO_3) enhance cell growth and protein content in suspension cultures (Lim et al., 2011), while eurycomanone production improves with 1.2 mg/L NAA and 1.0 mg/L kinetin (Nhan & Loc, 2017).

Moreover, various elicitation techniques using biotic and abiotic factors have been explored. For instance, chitosan at 100–150 g/L promotes biomass and canthin-6-one alkaloid production, while casein hydrolysate stimulates canthin-6-one synthesis (Siregar et al., 2009), and UV irradiation further increases canthin-6-one content (Natanael et al., 2014). Additionally, biotic elicitors such as yeast extract (YE), methyl jasmonate (MeJa), salicylic acid (SA), pectin (PEC), and valine (VAL) have been reported to enhance the synthesis of eurycomanone and other bioactive compounds (Nhan & Loc, 2018; Kwan et al., 2021). However, there is a need for alternative and more specific, efficient and novel approaches to produce bioactive compounds in *E. longifolia*.

The use of specific light spectra to target specialised plant photoreceptors such as cryptochromes and phytochromes that respond to blue and red light, respectively, represents a new and innovative approach (Habibah et al., 2024; Lian et al., 2019; Jiao et al., 2023). This research is motivated by the unique abilities of these photoreceptors in plant tissues to respond differently to different light spectra. This, in turn, may lead to different effects on metabolic pathways, potentially having a positive impact on the synthesis of bioactive compounds in *E. longifolia*.

The effects of photoperiods and LEDs on *in vitro* cultures of *E. longifolia* remain unexplored, leaving a significant gap in our understanding of how light conditions impact this medicinal plant's growth and bioactive compound production. By investigating these factors, this research aims to provide crucial insights that could optimize both the cultivation process and the yield of valuable compounds in *E. longifolia*. These findings will not only advance scientific knowledge but also offer practical applications for enhancing the efficiency of its commercial and medicinal use.

1.2 Problem Statement

To produce high quality products from *E. longifolia* (Tongkat Ali), roots from 4- to 7-year-old mature trees are required. This long maturation period coupled with the overwhelming global demand for these products is a significant challenge. Furthermore, current harvesting practises that rely on wild roots are not only time consuming but also environmentally unsustainable. The task becomes even more complicated when specific metabolites are required for pharmaceutical and nutraceutical products. Therefore, there is an urgent need to develop a more efficient and sustainable method for the production of *E. longifolia* products.

In the search for a viable alternative, *in vitro* systems such as hairy roots (HR) and cell suspension (CS) cultures have proven to be promising solutions. Previous research has focused on optimising the growth conditions of these *in vitro* systems and using chemical elicitation technique to increase the production of metabolites. However, this approach has limitation as it only puts the cultured tissues under arbitrary stress, which in turn may lead to producing more phytochemicals as a defence mechanism. To achieve improved synthesis of specific and valuable *E. longifolia* metabolites such as eurycomanone, 9-hydroxycanthin-6-one and 9-methoxycanthin-6-one, an innovative approach is required. This research, therefore, focuses on light factors as a new and novel approach to enhancing the synthesis of bioactive compounds in *E. longifolia*.

Light stands out as the most important factor capable of stimulating specific changes in the metabolic pathways for the synthesis of phytochemicals. Even more promising is monochromatic LED light, which can trigger specific photoreceptors in plants, leading to precise changes in gene activity and thus in the metabolic pathways responsible for the synthesis of the bioactive target metabolites.

1.3 Objectives of the Study

i. To determine the effects of photoperiods and light emitting diodes (LEDs) on growth, morphology of cell suspension and hairy root culture of *E. longifolia*.

ii. To investigate the effects of photoperiods and LED on synthesis of eurycomanone, 9-hydroxycanthin-6-one and 9-methoxycanthin-6-one in cell suspension and hairy root culture of *E. longifolia*.

iii. To evaluate the antioxidant potential of cell suspension and hairy root cultures subjected to photoperiods and LEDs treatments.

iv. To investigate the effects of photoperiods and LEDs on genetic fidelity of cell suspension and hairy roots of *E. longifolia*.

CHAPTER 2

LITERATURE REVIEW

2.1 Taxonomic Classifications of *E. longifolia*

Eurycoma longifolia, widely known as Tongkat Ali, is an important medicinal tree native to Southeast Asia. Its taxonomic classification provides a systematic understanding of its botanical identity within the plant kingdom as follows (Perry, 1980):

Kingdom:	Plantae
Phylum:	Tracheophyta
Class:	Magnoliopsida
Order:	Sapindales
Family:	Simaroubaceae
Genus:	*Eurycoma*
Species:	*Eurycoma longifolia*

2.2 Botanical Description

Eurycoma longifolia is a medium-sized deciduous tree that typically reaches a height of 15 to 18 metres and has a monopodial branch structure (Nordin, 2014). Its leaves are imparipinnate, spirally arranged, mainly at the tips of the trunks, and reach a length of up to 100 cm. Each ovate-oblong leaflet has a pointed tip and measures 5–20 by 1–6 cm (Mustaqim et al., 2021), and convex abaxial surface and blunt tip margin (Lee et al., 2015). The inflorescence, which forms a panicle, appears at the same time as the leaves and is attached to stems about 7 cm long. The dioecious flowers, which vary in colour from greenish to red or purple, are usually pollinated by insects and ripen into 1-5 fruits (Mustaqim et al., 2021). The ripe fruits, which are initially greenish

in colour, develop into red to maroon shades and are about 10–20 by 5–12 mm in size (Mustaqim et al., 2021).

2.3 Ecology and Distribution

Eurycoma longifolia finds its ecological niche in the living landscapes of Southeast Asia, especially in Indonesia, Malaysia and Thailand. Outside of these primary regions, it thrives in a variety of ecosystems, including Brunei Darussalam, Cambodia, Laos, Vietnam, Myanmar and the Philippines (Bhat & Karim, 2010; Tan et al., 2015; Wizneh & Asmawi, 2014). In its natural habitat, *E. longifolia* is frequently found in coastal vegetation, often as undergrowth alongside other trees (Nordin, 2014).

Eurycoma longifolia thrives in temperatures ranging from 27 to 30°C, with relative humidity levels of 60% to 90.8%, and a light intensity of 1.136 klx. It is typically found at elevations between 280 and 700 meters above sea level, growing in red-yellow podsolic soils with textures ranging from clay to sandy clay (Susilowati et al., 2019; Kartikawati et al., 2014)

2.4 Medicinal Uses of *E. longifolia*

2.4.1 Traditional medicinal practices

Traditionally, different parts of the *E. longifolia* tree have been used in the treatment of a variety of diseases in different places. In particular, a decoction of the leaves is used to relieve skin itching. The fruits are used to treat dysentery and the stem bark is used to treat stomach worms, diarrhoea and fever (Kuo et al., 2004). The roots are used to treat blood pressure.

The root extract is proving to be a cornerstone in the treatment of various diseases, including cancer, constipation, diabetes, leukaemia, syphilis, fever and osteoporosis (Tsai et al., 2020). It is used as an antibiotic, stress and anxiety reliever and appetite stimulant. The extract from the root of *E. longifolia* is known for its aphrodisiac properties. In addition, *E. longifolia* is used as a blood tonic and dietary supplement (Kuo et al., 2004) and is also useful in the treatment of female diseases (Rahman et al., 2018). The rich traditional use of *E. longifolia* emphasises its importance in holistic health practises.

2.4.2 Antimicrobial properties

Eurycoma longifolia shows robust antimicrobial activity against both Gram-positive and Gram-negative bacteria. The strain extracts have shown remarkable activity, especially against strains such as *Staphylococcus aureus* and *Bacillus cereus*, as highlighted in the study by Khanam et al. (2015). In addition, the ethanolic extract from the roots of *E. longifolia*, has shown positive results. In particular, it shows efficacy against Gram-negative strains such as *Salmonella typhi*, with inhibition zone values exceeding those of positive controls (Faisal et al., 2015). This emphasises the potency of *E. longifolia* in combating a broad spectrum of bacteria.

The antimicrobial effect against *Streptococcus mutans, Streptococcus sobrinus* and *Lactobacillus* was also confirmed (Alloha et al., 2019; Kuspradini et al., 2019; Ramzi et al., 2021). Overall, these results emphasise the diverse and strong antibacterial properties of the bioactive compounds of *E. longifolia*. This reaffirms the role of *E. longifolia* as a natural reservoir of compounds with significant antibacterial potential.

2.4.3 Antifungal properties

Eurycoma longifolia has remarkable antifungal properties and shows a positive effect against various pathogenic fungi. When used with various solvents, the extracts have shown positive activity as documented in studies by Khanam et al. (2015) and Faisal et al. (2015). In particular, Khanam et al. (2015) emphasise the efficacy of the extracts against *Aspergillus niger*, showing promising antifungal activity. Similarly, Faisal et al. (2015) address the positive results observed against *Aspergillus fumigatus* and emphasise the broad-spectrum antifungal potential of *E. longifolia*.

Candida albicans, an extensively tested fungal species, stands as evidence for the bioactivity of *E. longifolia* extracts. Several studies, including those by Kuspradini et al. (2019), Alloha et al. (2019) and Ramzi et al. (2021), confirm the consistently positive response of *Candida albicans* to *E. longifolia* extracts. Overall, these results emphasise the robust antifungal properties of *E. longifolia* and position it as a valuable natural resource against pathogenic fungi.

2.4.4 Antiviral properties

Numerous studies have reported the antiviral potential of *E. longifolia*, with significant efficacy against various viruses. George et al. (2019) reported on the anti-dengue potential of standardised *E. longifolia* extract using mice as a model. The results showed the efficacy of the extract in inhibiting all four serotypes of the virus, with particularly strong effects against DENV-2. Post-treatment analysis revealed reduced weight loss, lower viral load and increased platelet levels, indicating its promising anti-dengue properties. In addition, the compound 6α-hydroxyeurycomalactone isolated from *E. longifolia* showed potent anti-DENV-2 activity by effectively inhibiting viral replication He et al. (2023).

In addition, the bioactive compounds extracted from *E. longifolia* were tested for their antiviral activity against COVID-19 viruses, including HCoV-OC43 and SARS-CoV-2. The results revealed a very low IC50 range of 0.32–0.51 μM, emphasising the efficacy of these compounds as potential candidates for COVID-19 (Choonong et al., 2022). These results have shown the potential of *E. longifolia* as a promising candidate for the development of antiviral therapies.

2.4.5 Antiparasitic properties

The ethanolic extract of the dried roots of *E. longifolia* was tested for its antimalarial activity against *P. falciparum* stain 3D7 and K1. The results showed a very low IC50 of 2.16 μg/ml and 1.79 μg/ml, respectively, indicating a strong antimalarial activity (Katib et al., 2015). In another study by Wijayanti et al. (2021), the *in vitro* Heme Polymerisation Inhibition Assay (HPIA) was used to evaluate the ability of the isolated compounds from *E. longifolia* to inhibit β-hematin formation in *P. falciparum*. The results showed significant effects on both the FCR3 and D10 strains of *P. falciparum*. In addition to antimalarial activity, extracts of *E. longifolia* have also shown potent antiprotozoal activity against blastocysts (Girish et al., 2015). This is clear evidence of the antiparasitic activity of *E. longifolia*.

2.4.6 Anti-inflammatory properties

Eurycoma longifolia appears as a noteworthy subject in various studies, which consistently show its strong anti-inflammatory properties in different contexts. In a study by Han et al (2016), extracts of *E. longifolia* were found to alleviate pain and inflammation by inactivating the NF-κB signalling pathway. Hendra et al. (2017) confirmed these findings and reported that the root extract of *E. longifolia* not only

lowered triglyceride levels but also inhibited the formation of paw oedema in mice, indicating its efficacy in relieving inflammation. In addition, Tran et al. (2018) emphasised the inhibitory effect of 9-methoxycanthin-6 - a component of *E. longifolia*— - on the proinflammatory cytokines IL-6 and TNF-α in various cell types. Various extracts and compounds from *E. longifolia* have shown suppression of pro-inflammatory mediators, including nitric oxide (NO), inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2), along with downregulation of pro-inflammatory genes (Chaingam et al., 2022; Chau et al., 2020; Hien et al., 2019).

In addition, Subhawa et al. (2023) drew attention to the multiple anti-inflammatory effects of the ethanolic extract of *E. longifolia* Jack by demonstrating its ability to reduce inflammation of the ears and paws while preventing gastric ulcers. These results emphasise the robust potential of *E. longifolia* as a natural anti-inflammatory agent.

2.4.7 Aphrodisiac properties

Eurycoma longifolia emerges as a promising aphrodisiac, which is supported by convincing research results from various fields. In a clinical study by Udani et al. (2014) with men aged 20 to 65 years, significant improvements in erectile function, sexual performance, erection hardness and mood were observed in the group receiving *E. longifolia* extract compared to the placebo group. This not only emphasises its potential as a means of improving male sexual function, but also indicates its positive effects on general sexual well-being.

In addition to these results, Vejayan et al. (2020) investigated the physiological effects of *E. longifolia* in chickens. The study found increased testosterone levels and improved testicular histology, suggesting its potential as a testosterone booster. This

dual benefit of increasing testosterone levels and improving reproductive histology is consistent with the aphrodisiac properties traditionally associated with *E. longifolia.* This aphrodisiac potential is further supported by a systematic review and meta-analysis by Leisegang et al (2022). This comprehensive analysis of the relevant literature not only supports the herb's role in improving testosterone levels, but also confirms its effectiveness as a natural intervention to improve overall sexual health. By integrating these various findings, *E. longifolia* proves to be a holistic solution with multiple benefits for men's sexual well-being, offering both physiological and psychological improvements.

2.4.8 Anticancer activities of *E. longifolia*

Eurycoma longifolia has been extensively researched for its anticancer potential, and various studies have produced promising results. In one particular approach, Rahman et al. (2020) performed a computational analysis suggesting that the quassinoids of *E. longifolia* have the potential to activate caspase-9 activity and trigger apoptotic mechanisms in prostate cancer. This in silico study provides valuable insights into the molecular interactions that contribute to the anti-cancer effects of *E. longifolia*.

In an experimental study, the efficacy of the ethanolic root extract of *E. longifolia* was demonstrated against nasopharyngeal carcinoma cell lines with low IC50 values (Kajahmohideen et al., 2021) and its effect on human lymphocytes with a dose-dependent reduction in metaphase cells and various chromosomal aberrations (Chaiphech et al., 2022). Kwan et al. (2021) reported the cytotoxic effect against human colon cancer, while Yang et al. (2021) identified novel compounds with potent

antiproliferative activity against human leukaemia, further substantiating its anticancer potential.

Eurycomanone, a major bioactive compound in E. longifolia, has demonstrated effectiveness against lung cancer by inducing cell cycle arrest and apoptosis. It has also shown a multifaceted effect against colon cancer by inhibiting cell proliferation, colony formation, and angiogenesis, while suppressing autophagy and activating the mTOR signaling pathway (Ye et al., 2022). In addition, 9-methoxycanthin-6-one, another valuable compound, was shown to have a significant inhibitory effect against ovarian, breast, colon, skin and cervical cancer cells, with low IC_{50} values and a concentration-dependent induction of apoptosis (Yunos et al., 2022). Overall, these studies present *E. longifolia* as a versatile resource with diverse and promising anticancer effects that warrant further investigation in cancer therapy.

2.5 Conventional Methods for the Cultivation of *E. longifolia*

2.5.1 Seed germination in a nursery

Eurycoma longifolia is conventionally cultivated by germinating seeds in a nursery before transplanting them to the field. The germination process usually takes 30 to 100 days and is influenced by various factors, including seed treatment and soil type. The use of growing media such as Jiffy 7 and the removal of the hard seed coat have been shown to improve germination rates (Siregar et al., 2006; Fuad et al., 2015).

2.5.2 Soil and environmental conditions

The selection of a suitable soil is a crucial step in the mass cultivation of *E. longifolia*. The plant thrives in well-drained and fertile soils with a texture ranging from loam to sandy clay and a pH between 6.0 and 7.5. It has been observed that the

application of NPK fertiliser promotes growth (Wan et al., 2010). Optimal growing conditions are an average daily temperature of about 26°C, 73.6% humidity and 0.9 Klux light (Kartikawati et al., 2014).

2.5.3 Fertigation

Proper watering is crucial, especially in dry seasons, to provide the necessary moisture for the growth of *E. longifolia*. The amount of water required depends on the soil conditions and avoiding waterlogging is important to avoid stress and disease (Wan et al., 2010). Fertilisation, i.e. the application of a balanced fertiliser containing nitrogen, phosphorus and potassium, is used to supplement nutrients and increase the production of bioactive compounds (Wan et al., 2010).

2.5.4 Pest and Diseases

One of the major challenges in the large-scale cultivation of *E. longifolia* is the prevalence of pests and diseases (Wan-Muhammad-Azrul et al., 2018). Common pests affecting the plant include tiger moth larvae, scale insects, termites, and spider mites, while reported diseases encompass algal leaf spot, *Colletotrichum* leaf blight, sooty mold, and sudden death syndrome (Wan-Muhammad-Azrul et al., 2018). These issues underscore the importance of effective pest and disease management strategies for successful cultivation. According to Sajap et al. (2014), entomopathogenic fungi such as *Isaria fumosorosea* and *Metarhizium anisopliae* have shown potential in controlling tiger moths, a significant pest of *E. longifolia*.

2.5.5 Harvesting process

Eurycoma longifolia is usually harvested for medicinal purposes when the tree is 4 to 7 years old (Farag et al., 2022). Careful selection and cutting of mature stems, leaves and roots containing medicinal components are part of the harvesting process. The roots, especially the taproots, are the main focus of Tongkat Ali production and the often-destructive harvesting process is a critical step (Idris and Ghani, 2002). Given the long gestation periods and the potential impact of destructive harvests on the species, there is growing concern for the conservation of this important plant (Idris and Ghani, 2002). After harvesting, the refined product is obtained through various extraction processes (Mohamad et al., 2013).

2.6 Classification of Bioactive Compounds in *E. longifolia*

Hundreds of bioactive compounds have been isolated, identified and characterised in *E. longifolia*. Among the most abundant groups are quassinoids, triterpenes and alkaloids. Other compounds are claineanone derivatives and triterpene saponins. Figure 2.2 illustrates the classification of bioactive compounds in *E. longifolia* and highlights the groups of biochemicals studied in this research, namely eurycomanone, 9-methoxycanthin-6-one and 9-hydroxycanthin-6-one, as well as other examples (Figure 2.2).

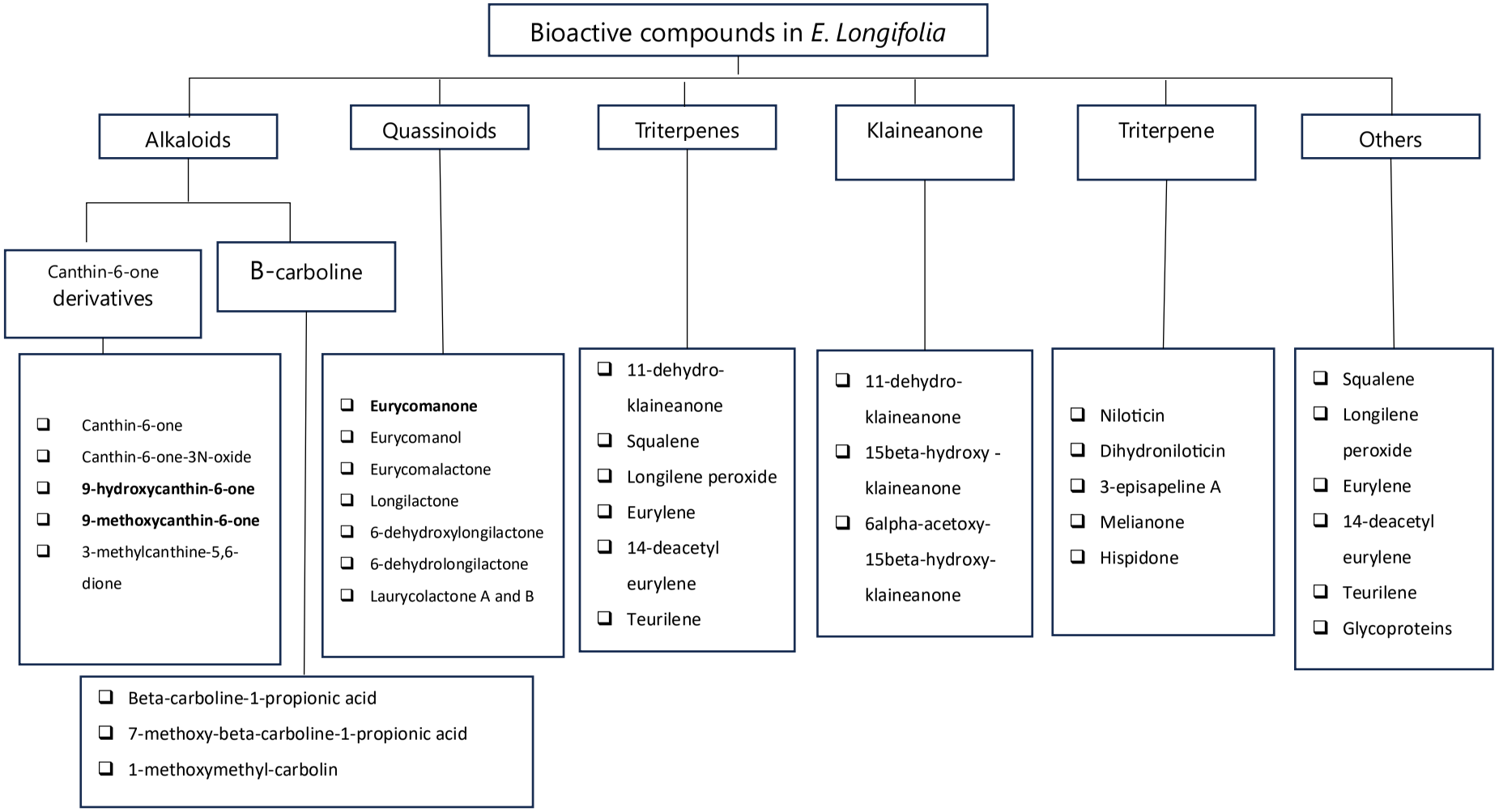

Figure 2.1: Classification of bioactive compounds in *E. longifolia*

2.7 Tissue Culture Techniques and Strategies for the Production of Bioactive Compounds in *E. longifolia*

2.7.1 Techniques for *in vitro* germination of *E. longifolia* seeds

The successful establishment of *in vitro* cultures for *E. longifolia* seeds depends on effective surface sterilisation techniques and optimised germination conditions. Various studies have used different methods to achieve these goals, presenting a range of surface sterilisation agents, culture media and light conditions (see Table 2.1).

2.7.1(a) Surface sterilization agents

Various agents have been used to remove contamination from the seeds of *E. longifolia* (Table 2.1). In general, the seeds are washed with detergent for 30 to 40 minutes and rinsed under tap water. They are then soaked in various concentrations of ethanol before being sterilised in a laminar flow hood (Hassan et al., 2012; Chee et al., 2015). Commonly used sterilising agents for *E. longifolia* include sodium hypochlorite (in Chlorox®) and mercuric chloride (Ngoc et al., 2015; Alttaher et al., 2020). For effective sterilisation, treatments are usually carried out with higher concentrations on the intact seeds and then repeated with lower concentrations after the seed coat has been removed (Sireagar et al., 2006; Danial et al., 2012; Chee et al., 2015). Table 2.1 provides a summary of the different protocols for successful surface sterilisation, listing the key steps.

2.7.1(b) Culture media and growth regulators

The choice of culture media plays a crucial role in supporting seed germination. Most studies opted for MS media without plant growth regulators (PGRs), while others used MS and WP media with PGRs such as BAP (benzylaminopurine) (Hassan, 2012), kinetin (Chee et al., 2015) and GA3 (gibberellic acid) (Ngoc et al., 2015).

2.7.1(c) Light conditions for *in vitro* germination

The light conditions have a considerable influence on the success of *in vitro* germination. Various photoperiods have been used in studies, with 16/8-hour light/darkness being a common choice. In addition, 24-hour continuous light and a one-week dark period followed by a light/dark cycle have also been used. The duration and quality of light exposure influence the physiological responses of the germinating seeds.

The various approaches described in the literature provide a valuable basis for the refinement and optimisation of protocols for the *in vitro* germination and micropropagation of *E. longifolia*.

Table 2.1 Techniques for surface sterilization and *in vitro* germination of *E. longifolia* seeds

Main sterilizing agents	Culture media	Light conditions	Reference
20% Clx + T20 for 15 min	MS without PGR	16/8 h light/dark of 150 µmol/m^2/s	(Hussein et al., 2005)
-96% EtOH -20% Clx + T20 for 15 min -5% Clx for 5 min (after removal of seed coat)	MS without PGR	24 h light of 30 µmol/m^2/s	(Siregar et al., 2006)
-70% EtOH + T20 for 3 min -50% Clx +T20 for 15 min	MS and WPM + BAP		(Hassan, 2012)
-20% Clx for 20min -12% Clx for 15 min and 5% for 5 min (after removal of seed coat)	MS without PGR	16/8 h light/dark of 150 µmol/m^2/s	(Danial et al., 2012)

-30% Clx + T20 for 15 min -15% and 5% Clx for 15 min (after removal of seed coat)	MS + 5mg/L KIN	16/8 h light/dark of 150 µmol/m^2/s	(Chee et al., 2015)
-70% EtOH for 1 min -0.1 $HgCl_2$ for 3 min	MS + 0.3 mg/L GA3		(Ngoc et al., 2015)
-70% EtOH for 5 min -20% Clx + T20 for 20 min	MS without PGR	dark for 1 week then 16/8 h light/dark of 35 µmol/m^2/s	(Alttaher et al., 2020)

Key: Clx= Chlorox®; T20= Tween 20, EtOH= ethanol; PGR= plant growth regulators; MS= Murashige and Skoog medium; WPM= woody plants medium; BAP= 6-Benzylaminopurine; KIN = Kinetin; GA3= gibberellic acid

2.7.2 Techniques for direct organogenesis in *E. longifolia*

The induction of organs, such as roots and shoots, directly on the explant is a micropropagation technique that enables rapid clonal mass propagation of plants. In *E. longifolia*, various plant growth regulators (PGRs) have been employed on different types of explants to stimulate direct organogenesis. (Table 1). Hussein et al. (2005), using shoot tips as explants, induced shoots on Murashige and Skoog (MS) with 5.0 mg/L kinetin and roots on 0.5 mg/L Indole-3-butyric acid (IBA), while Hussein et al. (2006) using *in vitro* roots as explants produced shoots on Driver and Kuniyuki Walnut (DKW) media with 1.0 mg/L zeatin and roots on MS with 0.5 mg/L IBA.

Similarly, using *in vitro* stems as explants, shoots were induced on Woody Plant Media (WPM), supplemented with 2.0 mg/L each of 6-Benzylaminopurine (BAP) and zeatin, and induced roots on MS, supplemented with 0.5 mg/L IBA. Using nodal segments, cotyledons and *in vitro* leaves as explants; shoots were induced on half-strength MS supplemented with 0.5 mg/L BAP and full-strength MS supplemented with 1.0 mg/L BAP, respectively. In addition, roots were induced on half-strength MS, supplemented with 10 mg/L IBA; full-strength MS supplemented with 0.5 mg/L IBA; and half-strength MS supplemented with 0.5 mg/L IBA (Hassan et al., 2012; Alttaher et al., 2020; Alttaher et al., 2021). The light conditions used in the above studies

include 16 h/day at 150 µmol/m^2/s (Hussen et al., 2005; Hussein et al., 2006) and 16 h/day at 35 µmol/m^2/s (Alttaher et al., 2020; Alttaher et al., 2021).

2.7.3 Techniques for induction and elicitation of callus

Callus induction is a crucial event in various tissue and cell culture systems, as it can serve as a transient tissue for organogenesis, somatic embryogenesis, cell culture (Fehér, 2019) and more. It can also serve as a latent tissue for the synthesis of bioactive compounds. Table 2.2 summarises the main techniques for callus induction, proliferation and initiation in *E. longifolia*. Siregar et al. (2004) tested the effects of different genotypes, media and naphthaleneacetic acid (NAA) concentrations on callus induction using leaves as explants. The result showed that MS modification and genotype have effects on callus induction.

Siregar et al. (2006) investigated the effect of BAP and NAA on callus formation with different explants. They found that MS supplemented with 8.0 mg/L NAA and 2.0 mg/L BAP produced the highest callus biomass on petioles. In contrast, Mahmood et al. (2010), using different plant parts as explants, discovered that different concentrations of 2,4-Dichlorophenoxyaceticdichlorophenoxyacetic acid (2,4-D) and picloram were effective in inducing callus formation. Similar results have shown that 1.0 mg/L 2,4-D is effective in callus induction (Shim et al., 2015). Studies on the effect of different concentrations of 2,4-D and NAA showed that 1.0 mg/L NAA plus 1.0 mg/L BAP induced callus on leaves, while 1.0 mg/L 2,4-D plus 1.0 mg/L BAP induced callus on petioles (Rosmaina et al., 2015).

The production of bioactive compounds in the callus of *E. longifolia* has been well documented. According to Rosli et al. (2009), higher levels of methoxycanthin-6-one were found in quarter-strength MS (¼ MS) with 2.0 % fructose and 2.0 mg/L

dicamba and the addition of 1.65 × 10−2 mg/L phenylalanine. In addition, gamma irradiation has been shown to reduce callus biomass, total phenolics and flavonoids (Hussein et al., 2012a). Interestingly, increasing the dose to about 60 Gy increased the synthesis of soluble protein. This suggests that gamma irradiation at a certain dose can stimulate changes in certain metabolic pathways. However, the available data are not sufficient to fully explain the mechanisms involved.

Different photoperiods were applied, including continuous darkness and different light intensities from 15 to 50 µmol/m^2/s. The variety of induction conditions shows that callus can be induced in *E. longifolia* from different explants using different types, combinations and concentrations of PGRs and under different light conditions. However, leaves appear to be the predominant explants, with 2,4-D and NAA emerging as the most commonly used PGRs.

Table 2.2 Summary of the techniques for callus induction and elicitation in *E. longifolia*

Explants	**Media + PGR + Additives**	**Other Culture Conditions**	**Morphogenic Response/Outcome**	**Refs.**
Leaf	MS + NAA and various macro nutrients	Various plant sources	Eu 9 plant, pH 5.75 and modified MS formed more callus	(Siregar et al., 2004)
Leaf, stem, and petiole	MS + BAP, NAA	24 h light of 30 µmol/m^2/s	8.0 mg/L NAA + 2.0 mg/L BAP formed higher callus on petiole while 10 mg/L NAA formed callus on leaves	(Siregar et al., 2006)
All plant parts	MS, SH, WH, and B5 + auxins, sugars and amino acids		¼ MS + 2% fructose + 2 mg/L dicamba; and 1.65 × 10^{-2} mg/L phenylalanine produced higher canthin-6-one	(Rosli et al., 2009)
All plant parts	MS + 2,4-D, dicamba, picloram, NAA and IAA	Continuous dark	1.0 – 4.0 mg/L 2,4-D produced callus on leaf, petioles, rachis, stem, roots, and cotyledons etc.	(Mahmood et al., 2010)
Callus	MS+ 1 mg/L 2,4-D	16/8 h light/dark of 15 µmol/m^2/s and gamma	Gamma radiation decrease biomass, total phenol and flavonoids but improve soluble protein at 60 Gy	(Hussein et al., 2012a)

Leaf and petioles	MS + 2,4-D, NAA, BAP and KIN		1.0 mg/L NAA + 1 mg/L BAP induced callus on leaf and 1.0 mg/L 2,4-D + 1 mg/L BAP induced callus on petioles	(Rosmaina et al., 2015)
Root segments	MS + 1 mg/L 2,4-D	16/8 h of 40 µmol/m^2/s	Treatment produced callus	(Shim et al., 2015)
Callus	MS + 2,4-D, NAA and KIN	8/16 h light/dark	1.5 mg/L NAA and 1.0 mg/L KIN gave better biomass	(Nhân et al., 2019)

Key: PGR = Plant growth regulators; BAP = 6-Benzylaminopurine; KIN = Kinetin; IBA = Indole-3-butyric acid; TDZ = thidiazuron; NAA = 1-Naphthaleneacetic acid; 2,4-D = 2,4-Dichlorophenoxyacetic acid; IAA = Indole-3-acetic acid; MS = Murashige and Skoog medium; SH = Schenk and Hildebrandt medium; WH = white's medium; and B5 = Gamborg (B5) Medium; Gy = Gray; W = watts; Eu 9 = code given by the author.

2.7.4 Establishment and production of bioactive compounds in cell suspension cultures

Plant cell suspension cultures are increasingly used for the synthesis of bioactive compounds. This approach, applied to *E. longifolia*, provides a convenient method for the production of compounds for agricultural, pharmaceutical and industrial applications. Various culture conditions and elicitation techniques were investigated, as summarised in Table 2.3. It should be noted that the initial steps of callus formation have already been presented in the previous sections (section 2.8.2). Therefore, only the steps associated with the production of cell suspensions from callus are discussed in this section.

One of the earliest reports is that of Siregar et al. (2003), in which the effects of manipulating MS nutrients and pH on growth were tested. The best results were obtained at a pH of 5.75 in the modified MS. Similarly, variations between different cell sources were tested under these conditions by Siregar et al. (2004). The results showed that different cell lines responded differently, with Eu9 producing the highest biomass and Eu8 the most alkaloids. Other studies found that MS supplemented with 0.5 mg/L NAA and 0.25 mg/L 2,4-D produced more canthin-6-on alkaloids, and MS