ESTABLISHMENT OF CALLUS AND CELL SUSPENSION CULTURES OF *Clitoria ternatea* FOR THE PRODUCTION OF PENTACYCLIC TRITERPENOIDS

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ESTABLISHMENT OF CALLUS AND CELL SUSPENSION CULTURES OF *Clitoria ternatea* FOR THE PRODUCTION OF PENTACYCLIC TRITERPENOIDS

by

TEOH SIEW CHIN

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LIST OF ABBREVIATIONS

%	Percentage
±	Plus-minus sign
°C	Degree celsius
2,4-D	2,4-Dichlorophenoxyacetic acid
ANOVA	Analysis of variance
BAP	6-Benzylaminopurine
CL	Cell line
CSL	Cell suspension line
DMRT	Duncan’s Multiple Range Test
g	Gram
GC-MS	Gas chromatography-Mass spectrometry
mg/L	Milligram per litre
mL	Millilitre
MS	Murashige and Skoog (1962)
NAA	α-Naphthalene acetic acid
PGR	Plant growth regulator
ppm	Parts per million
rpm	Rotation per minute
SE	Standard error
SEM	Scanning electron microscopy
SPSS	Statistical Package for the Social Sciences
TDZ	Thidiazuron
w/v	Weight per volume

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PENUBUHAN KALUS DAN KULTUR AMPAIAN SEL BAGI *Clitoria ternatea* UNTUK PENGHASILAN TRITERPENOID PENTACYCLIC

ABSTRAK

Clitoria ternatea, atau lebih dikenali sebagai bunga telang, adalah tumbuhan herba yang tergolong dalam keluarga Fabaceae. Dikenali oleh sifat perubatannya, bunga, daun, batang, dan akar tumbuhan ini mengandungi pelbagai sebatian fitokimia yang berkaitan dengan sifat perubatan dan farmakologi, termasuk triterpenoid pentacyclic, terutamanya taraxerol dan lupeol, yang berkait rapat dengan mekanisme neuroprotektif mamalia. Kajian ini bertujuan untuk mendorong pembentukan kalus rapuh daripada eksplan kotiledon *C. ternatea* bagi penubuhan kultur ampaian sel dan penghasilan metabolit yang mampu menambahbaikan ingatan. Eksplan kotiledon *in vitro* telah tertakluk kepada induksi dan proliferasi kalus dengan rawatan zat pengatur tumbuh (2,4-D, NAA, BAP, kinetin, dan TDZ) serta kepekatan sukrosa (15 g/L dan 30 g/L) dalam media Murashige dan Skoog (1962) kepekatan separuh (1/2 MS). Media proliferasi yang optimum kemudian digunakan untuk penubuhan kultur sel ampaian, diikuti dengan analisis GC-MS untuk mengenal pasti dan mengkuantifikasi triterpenoid pentasiklik. Hasil kajian ini menunjukkan bahawa 0.4 mg/L 2,4-D dengan 15 g/L sukrosa (CL1) telah menghasilkan kalus rapuh kekuningan dengan berat basah purata sebanyak 0.323 ± 0.0623 g, manakala 0.5 mg/L NAA bersama 0.5 mg/L BAP dan 15 g/L sukrosa (CL2) menghasilkan 0.3586 ± 0.0670 g kalus rapuh yang berwarna hijau muda dan kalus kompak yang berwarna hijau. Gabungan rawatan 0.5 mg/L NAA bersama 1.0 mg/L BAP adalah optimum dalam memproliferasi kalus CL1 manakala 0.8 mg/L 2,4-D adalah optimum dalam proliferasi kalus CL2. Dalam penubuhan kultur sel ampaian, CSL1 mencapai puncaknya pada hari ke-15 (berat basah 0.772 ± 0.013 g/20 mL media), manakala CL2 mencapai puncaknya pada hari ke-18 (0.788 ± 0.017 g/20 mL media). Selain itu, hari optimum untuk menuai sel adalah pada hari ke-13 bagi CSL1 dan hari ke-17 bagi CSL2. Analisis GC-MS telah mendedahkan kewujudan triterpenoid pentasiklik dan fitosterol dalam semua kultur *in vitro*, dengan kewujudan lupeol yang paling tinggi dalam CL2 (12.71-kali lebih daripada kawalan), manakala CL1 mempunyai kandungan taraxerol yang lebih tinggi berbanding dengan kultur *in vitro* lain walaupun rendah daripada kawalan. Kajian ini telah berjaya menubuhkan kultur kalus dan sel ampaian untuk *C. ternatea* dan mengenal pasti triterpenoid pentacyclic terutamanya lupeol dan taraxerol yang berkaitan dengan sifat penambah kognitif mamalia secara kualitatif.

ESTABLISHMENT OF CALLUS AND CELL SUSPENSION CULTURES OF *Clitoria ternatea* FOR THE PRODUCTION OF PENTACYCLIC TRITERPENOIDS

ABSTRACT

Clitoria ternatea, or commonly known as butterfly pea, is a perennial herbaceous plant belonging to the Fabaceae family. Renowned for its medicinal properties, the flowers, leaves, stems, and roots consist of a rich array of novel phytochemical compounds associated with medicinal and pharmacological properties, including pentacyclic triterpenoids particularly taraxerol and lupeol, known for their memory-enhancing properties. The current study aims to establish callus and cell suspension cultures from cotyledon explants of *C. ternatea* for production of novel plant secondary metabolites associated to the mammalian neuroprotective mechanism. The *in vitro* cotyledon explants were subjected to callus induction and proliferation in treatments of plant growth regulators (2,4-D, NAA, BAP, kinetin and TDZ) and sucrose concentrations (15 g/L and 30 g/L) in half-strength Murashige and Skoog (1962) media. The optimal proliferation media were then used for the establishment of cell suspension cultures, followed by GC-MS analysis to identify and quantify the pentacyclic triterpenoids. Results in this study indicated that 0.4 mg/L 2,4-D with 15 g/L sucrose (CL1) resulted in the induction of yellowish friable callus with the average fresh weight of 0.323 ± 0.0623 g, whereas combination of 0.5 mg/L NAA and 0.5 mg/L BAP with 15 g/L sucrose (CL 2) yielded 0.3586 ± 0.0670 g of light green friable callus and greenish compact callus. Combination treatment of 0.5 mg/L NAA with 1.0 mg/L BAP was optimum in proliferating the CL1 callus while single treatment of 0.8

mg/L 2,4-D was optimum in proliferating CL2 callus. In the establishment of growth curves for the suspension cultures, CSL1 reached its peak on day 15 (fresh weight of 0.772 ± 0.013 g/20 mL medium) whereas CSL2 on day 18 (0.788 ± 0.017 g/20 mL medium). In addition, the optimum day to harvest the cells were observed to be at day 13 and 17 for CSL1 and CSL2, respectively. GC-MS analysis revealed the presence of pentacyclic triterpenoids and phytosterols in all callus and cell suspension cultures with CL2 contained the highest amount of lupeol (12.71-folds higher than the control) whereas CL1 had higher amount of taraxerol in comparison to other *in vitro* cultures regardless being lower than the control. The current study has successfully established callus and cell suspension cultures of *C. ternatea* and qualitatively quantified the pentacyclic triterpenoids associated with the mammalian cognitive enhancing properties. This study has revealed the potential of callus and cell suspension cultures in producing pentacyclic triterpenoids particularly taraxerol and lupeol which could be a therapeutic alternative to neurodegenerative disorders.

CHAPTER 1

INTRODUCTION

1.1 Introduction

The butterfly pea plant (*Clitoria ternatea*) is a perennial leguminous twiner from the Fabaceae family. Despite being an ornamental plant and a natural dye, *C. ternatea* too, serves as a tropical medicinal plant as it possesses numerous pharmacological properties associated to the phytochemical compounds of the plant. The different parts of the *C. ternatea* plant contains a variety of phytochemical compounds such as pentacyclic triterpenoids, flavonoids, alkaloids, anthocyanins, steroids, phenols, saponins and tannins associated with pharmacological properties that are reported to be antioxidant, anti-diabetic, antipyretic, anti-inflammatory, anti-cancer and possesses promising neuroprotective properties (Kumar et al., 2007; Neda et al., 2013; Murugalakshmi et al., 2014; Al-Snafi, 2016; Damodaran et al., 2018; Adisakwattana et al., 2020; Lee et al., 2021; Swathi et al., 2021; Ginting et al., 2022; Ahad et al., 2023).

Nonetheless, the most essential secondary metabolites identified in *C. ternatea* are the pentacyclic triterpenoids such as taraxerol and lupeol, which are found mainly in the roots of the plant (Shahnas & Akhila, 2014; Lijon et al., 2017; Dash et al., 2023). These phytochemical compounds had been shown to exhibit potential neuroprotective properties, improving brain activities for the treatment of neurodegenerative diseases (Rai, 2002; Kumar et al., 2007; Kaundal et al., 2017; Sinha et al., 2019; Ahmad et al., 2020; Hashmi et al., 2020; Huang et al., 2021). For instance, a study by Kumar et al. (2007) demonstrated that the anti-acetylcholinesterase activity of *C. ternatea* extracts were compatible to that of the standard medication (physostigmine) used to reverse cholinesterase inhibitors, which further affirms the potential of these phytochemical compounds in treatments of Alzhemier's. In addition, *in vivo* studies had proven that lupeol is a bioactive compound that holds promising effect in addressing neurodegenerative diseases which attributed by its anti-inflammatory, antioxidant, and neuroprotective properties (Kaundal et al., 2017; Ahmad et al., 2020).

Generally, secondary metabolites obtained from the roots or aerial parts of the medicinal plants only accounted for 1% by weight, thus, requiring large amounts of raw plant material in satisfying the commercial needs, leading to overexploitation or even extinction of the specific species (Motolinía-Alcántara et al., 2021). Direct harvesting of the roots of this plant species for its valuable medicinal and pharmaceutical properties is irrational. Moreover, its cultivation in different geographical areas can result in the variations in the accumulation of the secondary metabolites, resulting in inconsistent production of those compounds for the pharmaceutical industry. Thus, other alternatives such as plant tissue culture techniques could be an alternative to produce and boost plant secondary metabolite production.

Plant cell cultures are known as promising alternatives in the production of novel secondary metabolites for medicinal purposes. In such, callus culture techniques had been widely utilized in the biotechnology field for the production of novel secondary metabolites (Efferth, 2019). Apart from callus culture, cell suspension culture too, serves as a significant approach in mass producing the cells and secondary metabolites. Hence, the incorporation of both these culture techniques is relatively crucial in the production of novel secondary metabolites for the fields of medicinal, pharmaceutical, cosmetic, or even dietary (Eibl et al., 2018; Motolinía-Alcántara et al., 2021). These alternatives have been successfully established for medicinal plants such as *Commiphora gileadensis* L. (Al-Abdallat et al., 2023), *Eurycoma longifolia*

(Tongkat Ali) (Nhan & Loc, 2017), *Panax quinquefolium* (American ginseng) (Kochan et al., 2019), *Sutherlandia frutescens* (cancer bush) (Nosov et al., 2023), to produce novel phytochemical compounds. Moreover, cell suspension cultures are capable of yielding high secondary metabolites *in vitro* and later to industrial scale (Yue et al., 2016). For instance, cajaninstilbene acid (phenolic compound) obtained from the cell suspension culture of *Cajanus cajan* (Linn.) were significantly higher than that of the field-grown *C. carjan* (Gai et al., 2020). Besides, the cell suspension cultures derived from the friable callus consisted of higher phenolic compounds and flavonoids (catechin, luteolin, quercetin, and kaempferol) as compared to the field-grown plant (Bong et al., 2021). Moreover, another study done by Nhan and Loc (2017) had successfully established a more comprehensive method in producing the eurycomanone from cell suspension culture of *Eurycoma longifolia (Tongkat Ali)* which resulted in higher secondary metabolites production in only 14 days as compared to the 5 years-old field-grown plant, further affirmed that this *in vitro* technique was an efficient, time-saving, and environmentally friendly approach in producing and harnessing the valuable secondary metabolites.

Notably, Lee et al. (2021) have reported the presence of pentacyclic triterpenes, phytosterols and fatty acids in the adventitious root cultures of *C. ternatea* that are linked to the mammalian neuroprotective properties. This further indicated that *in vitro* cultures of *C. ternatea* such as callus and cell suspension cultures could potentially be applied for studies on the accumulation of valuable secondary metabolites particularly pentacyclic triterpenoids. Moreover, callus and cell suspension cultures could be the ideal approach in the production of novel plant secondary metabolites with memory enhancing properties due to the totipotent nature that endeavours the full genetic information of the mother plant (Efferth, 2019).

Despite the wide spectrum medical uses and high potential value of *C. ternatea* in pharmaceutical field, information about callus and suspension cultures of this species for the secondary metabolites production is limited. Therefore, the focus of the current study is to induce friable callus and to establish cell suspension cultures of *C. ternatea* with the aim to assess the production of pentacyclic triterpenoids associated to the mammalian neuroprotective properties. This included growth kinetics and a preliminary analysis of the spectrum of secondary metabolites.

1.2 Objectives

1. To induce friable callus in different concentrations and combinations of plant growth regulators and sucrose from cotyledon explants of *Clitoria ternatea*.
2. To determine the optimum callus proliferation medium by investigating the effects of plant growth regulators and subculture frequency on callus proliferation.
3. To establish cell suspension cultures of *C. ternatea* and study the growth kinetics of the cell suspension cultures.
4. To identify and qualitatively quantify the pentacyclic triterpenoids and phytosterols between *in vitro* callus and cell suspension cultures via gas chromatography mass spectrometry (GC-MS) analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 Clitoria ternatea

2.1.1 Taxonomy, description and nutritional values

Clitoria ternatea, which is commonly known as butterfly pea plant is a leguminous twiner with elliptic leaves from Fabaceae family (Figure 2.1). It is a dicotyledon plant with a diploid chromosome number of 2n = 16 (Gandhi, 1993; Shahzad et al., 2007). This plant species consists of eye-catching vivid-blue flowers in which the blue pigments were attributed by the anthocyanins which derived from delphinidin (Adisakwattana et al., 2020; Jeyaraj et al., 2021; Thuy et al., 2021). This botanical marvel is renowned for its vibrant blue flowers, which not only captivate the eye but also hold significant value in traditional medicine and culinary practices. This plant species is recognized for its pea-sized blooms that resemble butterfly wings and is referred to as 'bunga telang' in Malaysia (Figure 2.2). Apart from its ornamental value due to its vibrant flower colour, *C. ternatea* holds significant medicinal value in Ayurvedic practice, boasting a rich array of phytochemicals renowned for therapeutic applications (Mukherjee et al., 2008).

This plant species is native to Asia but has been naturalized in regions like South and Central America, East and West Indies, China, and India (Barik et al., 2007; Gupta & Bhatia, 2010). Exhibiting longevity as a perennial herb, it adopts an erect growth habit (Kalamani & Gomez, 2001). *C. ternatea* thrives in various habitats, particularly in mesic forests or shrublands with ample moisture and temperature ranging from 24 °C to 32 °C (Collins & Grundy, 2005; Oguis et al., 2019).

Figure 2.1: *C. ternatea* plant grown at Herbarium unit of School of Biological Sciences, Universiti Sains Malaysia (USM).

Figure 2.2: The flower of *C. ternatea.*

According to Cook et al. (2005), *C. ternatea* exhibits self-pollination but also undergoes cross-pollination, which is influenced by genotype segregation. Despite its ability to thrive in various habitat, the seed germination rate of this plant species is relatively low (Mullick & Chatterji, 1967). Hence, *in vitro* propagation of this plant species could therefore combat this issue and at the same time, conserving and mass propagating *C. ternatea* lines (Oguis et al., 2019).

C. ternatea can grow up to 162 cm in size, with older stems appearing woody or grey, while new growth stems are greenish in colour (Kalamani & Gomez, 2001; Collins & Grundy, 2005). It bears vivid deep blue pentamerous zygomorphic pea-shaped flowers with a tubular calyx consisting of five free petals which bloom year-round. The petals can be divided into three categories which are standard, wing, and keel that ranged from 3.8 × 2.8 to 4.2 × 3.00 cm; 2.6 × 1.4 to 2.7 × 1.8 cm; 1.8 × 0.5 to 2.0 × 0.8 cm, respectively (Taur et al., 2010). The flowers have a simple stigma and long style that curved at the distal end. Its leaves are pinnatifid with 5 to 7 leaflets, 6 to 11 cm in length, 5 to 7 cm in width, and obovate with emarginate apex.

The flowers will then develop into seed pods (6 - 13 cm) that are linear-oblong in shape which each pod can bear around 10 blackish-oval seeds (Bishoyi & Geetha, 2012). The newly developed seedpods appear greenish and moist but will subsequently turn brown and dry upon maturation (Figure 2.3). These matured seedpods undergo explosive dehiscence, thereby dispersing the seeds within for propagation purposes. Trichomes and cuticles are present at both surfaces of the leaves within the epidermis (Collins & Grundy, 2005; Taur et al., 2010). The extensive deep-root system of *C. ternatea* comprises a fairly stout taproot with several branches of slender lateral roots that can grow up to 2 m long while bearing purple and greyish-green wiry stems (Gomez & Kalamani, 2003; Mukherjee et al., 2008) (Figure 2.4).

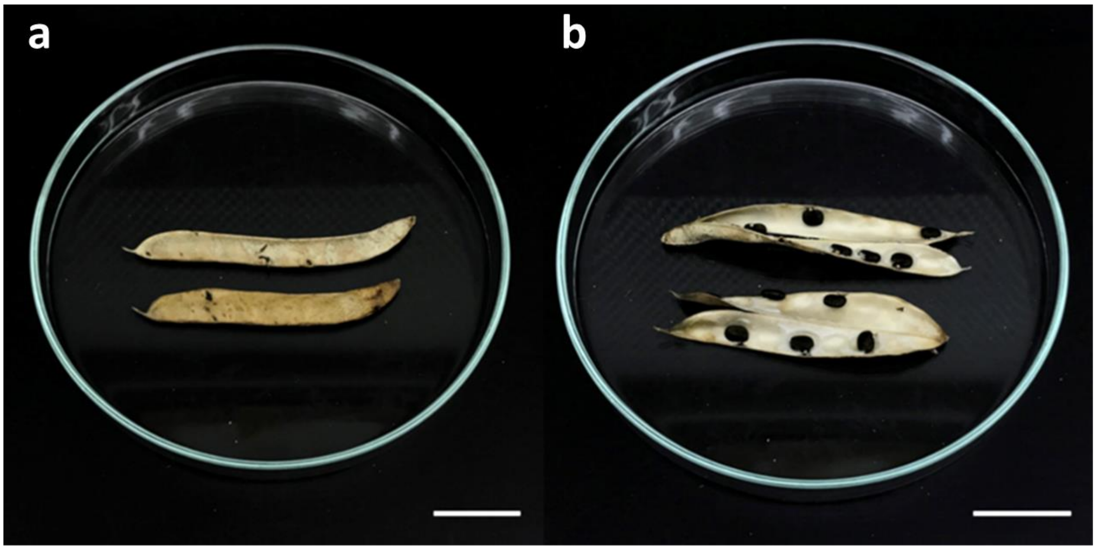

Figure 2.3: Mature seedpods of *C. ternatea*. a) Seedpods; b) Inner part of the seedpods with seeds. The scale bar represents 2 cm.

Figure 2.4: Roots of *C. ternatea* harvested from the mother plant planted at Herbarium unit of School of Biological Sciences, Universiti Sains Malaysia (USM).

The flowers of this plant are multifunctional not just because of their ornamental value but also their abundance of nutrients. A study on the assessment and characterization the nutraceutical values of *C. ternatea* had been performed by Morris (2009) and the results indicated that this medicinal plant is high in protein (20%). The seeds on the other hand, marked a protein percentage of 38%. Apart from protein and fibre, this plant too, contains oils (10%) and sugars (5%) (Morris, 2009). Besides, Neda et al. (2013) too, performed comprehensive research on the flowers of *C. ternatea* with proximate and mineral analysis. The analysis conducted on the flowers provides a strong basis for emphasizing the nutritional value of *C. ternatea* which are relatively high in calcium, magnesium, potassium, zinc, sodium, and iron (Neda et al., 2013). In such, calcium is an essential mineral for the body because it is required to develop strong teeth, skeletal, and blood clotting (Forbes, 2012). Furthermore, Neda et al. (2013) reproted that the heavy metal content of *C. ternatea* flower was relatively low, thereby indicating that the plants are safe to be consumed.

2.1.2 Conventional and traditional applications

Al-snafi (2016) reported that almost every part (flower, root, seed, and leaves) of the *C. ternatea* plant is nutritious and can serve as traditional medicine. According to Jain et al. (2003), the roots, seeds, and leaves of this plant are functional in treating dementia, inflammation, asthma, bronchitis, and fever. The study of Al-snafi (2016) had highlighted the beneficial effect of this plant in which the roots had been widely utilized in treating ascetics, enlargement of the abdominal viscera, sore throat, and skin diseases. Apart from that, the roots could also serve as a health tonic that is effective in improving muscular strength (Ngadni et al., 2021). Seeds of *C. ternatea* could also be used to treat swollen joints, dropsy, and enlargement of abdominal viscera due to the presence of glucoside, phenol glycoside, and anthoxanthin, and adenosine (Al-snafi, 2016). Furthermore, the seeds and leaves of *C. ternatea* were found to be functional in treating swollen joints, urinary issues, snakebite, and as well as in promoting intelligence and memory (Al-snafi, 2016). *C. ternatea* serves as traditional medicine, especially in India. It has been utilized for centuries as a memory enhancer, tranquillizing, and sedative agent (Mukherjee et al., 2008). The properties of nootropic, antidepressant, anxiolytic, anticonvulsant, and anti-stress in *C. ternatea* further make it a renowned conventional medicine in India (Gomez & Kalamani, 2003; Jain et al., 2003; Taranalli & Cheeramkuzhy, 2000).

Butterfly pea flower extract has been explored as a natural colourant due to its vibrant blue hue. Gamage et al. (2021) mentioned that natural colourants giving red, orange and yellow hues are widely available but not applicable to that of blue hues. Hence, *C. ternatea* is renowned for its blue flowers as a natural source of colourant. Notably, butterfly pea flowers are gaining popularity in beverages as they exhibit the ability to transform from blue to purple colour with the addition of acidic compounds such as lemon juice in which this ability is the result of a chemical change of anthocyanin in response to changes in pH (Abdullah et al., 2010). The blue flower extracts are widely utilized in the Thailand community as natural food colouring in the preparation of various delicacies (Adisakwattana et al., 2020).

2.1.3 Medicinal and pharmaceutical properties

2.1.3(a) Improvement in cognitive function

Numerous studies had proposed that *C. ternatea* serves as a medicinal plant that could aid in intelligence and as well as in memory enhancement. For instance, the root extracts were proposed to have the ability to boost the synthesis of neurotransmitters such as acetylcholine, and the effect of the root extracts was similar to that of the synthetic drugs, Nefiracetam (Van der Schyf et al., 2006). The root extracts too, had been proven to induce permanent changes in the brain by increasing acetylcholine content in the hippocampus of the animal model which could subsequently improve the learning abilities (Rai et al., 2002). Moreover, Scalbert et al. (2005) demonstrated that the leaves of *C. ternatea* consisted of a great number of alkaloids and flavonoids which could potentially help in preventing neurodegenerative diseases. Next, Kumar et al. (2007) had isolated triterpenoids which comprised mainly of taraxerol from *C. ternatea* and evaluated its ability in inhibiting acetylcholinesterase (AChE), an enzyme involves in breaking down the neurotransmitter acetylcholine. In their research which inclusive of *in vitro* and *in vivo* assays, the activity of taraxerol on AChE inhibition was compatible to that of the standard medication (physostigmine), indicating that this bioactive compound obtained from *Clitoria ternatea* could be a highly potential medicine for memory enhancement (Kumar et al., 2007).

On the other hand, Damodaran et al. (2018) demonstrated that *C. ternatea* root extract managed to improve the memory performance in chronic cerebral hypoperfusion rat model, further affirms the potential of *C. ternatea* root extract to be a potential therapeutic strategy to prevent the progression of cognitive deterioration in vascular dementia and Alzheimer's disease patients. Besides, synaptic function in rat model was significantly enhanced with the treatment using *C. ternatea* root extract, demonstrating that the root extract exhibited cognitive-enhancing properties (Damodaran et al. 2018). Another study by Damodaran et al. (2020) discovered that the root extract exhibited nootropic effects as the acetylcholinesterase activity in the chronic cerebral hypoperfusion rat model was suppressed. Furthermore, a recent study by Lee et al. (2021) documented the presence of secondary metabolites such as pentacyclic triterpenoids, flavonoids, phytosterol, and fatty acids in the *in vitro* adventitious root cultures of *C. ternatea* which associated with memory enhancing properties. These secondary metabolites possessed anti-acetylcholinesterase (AChE) activities which could potentially delay the occurrence of neurodegenerative diseases (Lee et al., 2021). Besides, a recent study by Ahad et al. (2023) demonstrated that the root extracts of *C. ternatea* could effectively inhibit the cholinesterase enzyme activities, suggesting that the phytochemical compounds present in this plant extracts could be a promising therapeutic approach for cognitive dysfunction, in which the pharmaceutical properties were attributed to the presence of clitorienolactones A (CLA).

2.1.3(b) Anti-diabetic and hypoglycemic

Diabetes mellitus is a chronic medical condition characterized by elevated blood glucose levels due to insulin deficits (Widowati et al., 2023). To date, the drugs used in addressing this disease are synthetic oral hypoglycemic drugs and insulin. However, these drugs might cause the patients to experience some side effects such as nausea, diarrhea, and stomachache (Ginting et al., 2022). According to Ginting et al. (2022), the flower and leaves extracts of *C. ternatea* exhibits the potential in managing diabetes by boosting insulin secretion, impeding the formation of advanced glucose end products, and suppressing the activity of enzymes responsible for glucose production in blood. Hence, indicating that medication or supplements derived from natural sources such as *C. ternatea* that possesses anti-diabetic properties and at the same time being safer with fewer side effects as compared to the synthetic drugs would be highly recommended. Apart from that, according to Scalbert et al. (2005), phytochemicals such as polyphenols that are widely present in the leaf of this medicinal plant could also help in preventing diabetes mellitus.

Numerous *in vivo* studies have revealed the potential of *C. ternatea* extract as an anti-diabetic and antihyperglycemic agent. For instance, Gunjan et al. (2010) demonstrated that the glucose level in diabetic rats had decreased dramatically after 14 days of treatment with *C. ternatea* flower extract, in which the effect was comparable to the conventional antidiabetic medicine (Glibenclamide), thereby revealed the potential of *C. ternatea* to be a natural source in reducing blood glucose levels (Gunjan et al., 2010). Furthermore, research conducted by Ginting et al. (2022) revealed that the antidiabetic effect of *C. ternatea* extracts was better than that of the conventional antidiabetic medicine (Glibenclamide). In such, the alloxan-induced rat treated with 400 mg/kg body weight of *C. ternatea* extracts managed to reduce the blood glucose level from 322.8 mg/dl to 31.2 mg/dl in 10 days whereas the alloxan-induced rat treated with 5 mg Glibenclamide resulted in a glucose level reduction from 253.2 mg/dl to 48.6 mg/dl, showcasing that the potential of *C. ternatea* extracts as a potential therapeutic approach in addressing diabetics (Ginting et al., 2022).

Moreover, the hypoglycaemic effects *C. ternatea* extracts at a 400 mg/kg diet dosage were observed in the diabetic mellitus rats. In such, the *C. ternatea* extract managed to significantly reduce the glucose level while elevate the insulin level of the diabetic mellitus rats (Widowati et al., 2023). Besides, it was also noteworthy that the anti-diabetic properties of *C. ternatea* extract were highly associated with the antioxidant and anti-inflammatory properties which play vital role in decreasing the damage of pancreatic β-cell, thereby normalized the production of insulin (Widowati et al., 2023).

2.1.3(c) Antipyretic

According to an antipyretic study conducted by Parimaladevi et al. (2004), the antipyretic effect of *C. ternatea* root extract was comparable to that of paracetamol, a standard medicine used to treat fever. For instance, the yeast-induced pyrexia albino rats showed a remarkable reduction in temperature after being treated with 400 mg/kg body weight methanolic root extract of *C. ternatea* in 2 hours from 39.9 ± 0.02 °C to 37.7 ± 0.03 °C, which was non-significantly different to the paracetamol-treated group which marked the temperature from 39.8 ± 0.02 °C to 38.2 ± 0.03 °C. The result, therefore, confirmed the antipyretic effect of *C. ternatea* extract, revealing its potential to be developed into a versatile drug or medication in treating fever (Parimaladevi et al., 2004). Furthermore, apart from root extracts, the leaves extracts of *C. ternatea* too, were reported to possess antipyretic properties (Murugalakshmi et al., 2014). The study demonstrated that the oral administration of *C. ternatea* ethanolic extract to the yeast-induced pyrexia albino rats resulted in a significant reduction in body temperature as compared to the control group. Besides, the antipyretic effect of the leaves extracts was better than that of the positive control group, which was treated with paracetamol, a standard drug used in fever treatment as the leaf extracts treated rats had greater reduction in temperature as compared to that of the positive control group (Murugalakshmi et al., 2014).

2.1.3(d) Antioxidant

According to Adisakwattana et al. (2020), the flowers of *C. ternatea* exhibit antioxidant properties which capable of protecting biological molecules against oxidative damage attributed by free radicals such as reactive oxygen species (ROS). With reference to Chauhan et al. (2012), terpenoid, flavonoid, tannin, and steroids act as antioxidants that could effectively reduce reactive oxygen species (ROS) activity and oxidative stress. Besides, Scalbert et al. (2005) claimed that polyphenols that act as antioxidants may protect cell constituents against oxidative damage and, therefore, limit the risk of various degenerative diseases associated to oxidative stress.

The antioxidant activities of *C. ternatea* flower extracts were evaluated by Jeyaraj et al. (2021) via DPPH ((2,2-diphenyl-1-picryl-hydrazyl)) assay and the result indicated that the radical scavenging activity of both the ethanolic and water flower extracts were almost similar, where the water extract was slightly greater than that of ethanolic extract. In such, the IC_{50} values for ethanolic extract and water extract were 1.24 ± 0.05 and 1.18 ± 0.07 mg/mL, respectively. Even the results revealed that the radical scavenging activity of both the ethanolic and water flower extracts were less potent than that of the positive control ascorbic acid (IC_{50} = 0.0039 mg/mL), it was still noteworthy that the natural sources exhibited approximately 30% antioxidant activity of the positive control, which could serve as an antioxidant agent as health supplements in pharmaceutical field (Jeyaraj et al., 2021).

Apart from that, the antioxidant properties of ethanolic extract of *C. ternatea* were further confirmed by Swathi et al. (2021) through *in vitro* assays and *in vivo* studies. For instance, the *in vitro* assays such as DPPH and ABTS assays had revealed the antioxidant properties of the ethanolic extract with the IC_{50} value of 34.71 µg/mL and 3.82 µg/mL, respectively. As for the *in vivo* antioxidant studies, treatment of 400 mg/kg/day of *C. ternatea* ethanolic extract to the arthritic rats was significantly different to that of the control group, further affirmed the antioxidant potential of *C. ternatea* (Swathi et al., 2021).

2.1.3(e) Anti-inflammatory

In vivo study was employed in evaluating the anti-inflammatory activity of the ethanolic extract of *C. ternatea* and the result showed the extract possessed remarkable anti-inflammatory properties (Swathi et al., 2021). In such, the extract exerted a significant inflammation inhibition on the histamine-induced rat paw edema model, revealing the anti-inflammatory effects of *C. ternatea* extract that were comparable to that of the standard medication used in treating inflammation which is diclofenac. Swathi et al. (2021) suggested that the presence of various phytochemical compounds such as triterpenoids, terpenoids, flavonoids, quercetin, delphidin, kaemferol and malvidin attributed to the anti-inflammatory properties of C. *ternatea*. Hence, the authors suggested that further research is needed to confirm, identify, and isolate the specific phytochemical compounds from *C. ternatea* that is responsible for the anti-inflammatory activity and healing the inflammation related diseases (Swathi et al., 2021).

The anti-inflammatory potential of *C. ternatea* was also confirmed by Yazhini et al. (2023) through an *in vitro* study which evaluated the anti-inflammatory property of mouthwash using ethanolic extract of *C. ternatea*. The study revealed that the ethanolic extract of *C. ternatea* showed a promising inhibition of 80% in BSA (Bovine Serum Albumin) and EA (Egg Albumin) assays in which the assays are commonly used in the research to study anti-inflammatory activity. The authors thereby suggested that the ethanolic extract of *C. ternatea* that possessed promising anti-inflammatory activity could therefore be utilized as anti-inflammatory agents for dental therapeutic purposes (Yazhini et al., 2023).

2.1.3(f) Anti-cancer

According to Jacob and Latha (2012), the seeds of *C. ternatea* comprised of anti-cancer activity in which their study revealed that the methanolic extract of *C. ternatea* managed to lower the tumour volume, packed cell volume and viable count against the Dalton's lymphoma tumor bearing mice by two-folds as compared to the control group. It was also noteworthy that the survival time of the animal models being fed with the methanolic extract of *C. ternatea* (100 mg/kg and 200 mg/kg) was elevated as compared to the control group which received no treatment. In such, the average lifespan of the control group was 18 days while the treatment groups managed to survive up to 34 days. The results thereby indicated that methanolic extract of *C. ternatea* exhibited promising capacity in the inhibition of cancerous cells (Jacob & Latha, 2012). Moreover, Neda et al. (2013) reported that the water extract of *C. ternatea* flowers possessed remarkable cytotoxic effects that were effective against breast cancer cell line (MCF-7) due to the presence of various bioactive compounds. In such, the researchers conducted MTT assay and the water extract of *C. ternatea* flowers showed promising anti-proliferation activity towards the MCF-7 cell line with the IC_{50} value of 175.3 µg/mL at 72 hours. Besides, GC-MS analysis revealed that mome inositol (38.7%) and pentanal (14.3%) were the main active compounds present in the water extract of *C. ternatea* flowers. Hence, the study therefore concluded that water extract of *C. ternatea* could serve as an anti-cancer agent which could be utilized in cancer treatment (Neda et al., 2013).

2.1.4 Other applications

C. ternatea is a type of legume that possesses nitrogen fixing capability due to the presence of rhizobia such as *Bradyrhizobium elkanii* and *Rhizobium grahamii* in its root nodules (Lopez-Lopez et al., 2012; Duangkhet et al., 2018). Its ability to fix nitrogen in the soil through a symbiotic relationship with rhizobia bacteria is particularly noteworthy as the soil fertility could be restored and hence benefits the neighbouring plants. In such, this nitrogen-fixation process allows *C. ternatea* to convert atmospheric nitrogen into soluble nitrogenous compounds such as ammonia which can be directly utilized by the plants nearby, therefore, enriching the soil fertility while being sustainable as the use of synthetic fertilizer can be reduced (Checcucci et al., 2017; Kumar et al., 2019). Field trials conducted in Mexico by Alderete-Chavez et al. (2011) revealed that planting of *C. ternatea* managed to improve the growth of other plants around this legume as the organic matters such as nitrogen, potassium and phosphorus contents of the soil were elevated in just 6 months. Thus, planting of *C. ternatea* is beneficial as this could improve soil quality by enriching it with nitrogen, making it a valuable companion plant for existing vegetation. The plant's multifaceted nature makes it a valuable resource not only in traditional medicine and culinary practice, but also in agriculture and environmental conservation efforts.

Other than being utilized as natural food colourant in dishes and beverages, natural dyes extracted from the blue flowers of *C. ternatea* could potentially serve as colorimetric pH sensor in wound dressing application (Kiti et al., 2022). In such, the flower extract consisted anthocyanins in which the pigments within are sensitive to pH and would result in differ colour with changing pH values. In the case of wound dressing applications, the extract changes colour based on the pH level of the wound area, allowing for visual indication of the wound status. The colour changes of the extract ranges from red in acidic conditions to blue in mildly basic conditions and green in basic conditions. This observation is due to the structural alteration occurring in anthocyanin molecules alongside with variation in hydrogen and hydroxide ions (Gamage et al., 2021). This colour variation would therefore serve as a visual indicator for evaluating wound infection, with specific colour changes indicating different pH ranges and infectious status (Kiti et al., 2022).

A recent study by Chaksupa et al. (2022) revealed the potential of *C. ternatea* extract as a hair growth enhancer. In such, the extract managed to stimulate human dermal papilla cells and promote initial hair growth in mice, in which its function was compatible to minoxidil, a synthetic drug used to increase new hair growth. Besides, the Thailand community tends to utilize the flower of *C. ternatea* as hair growth stimulator and as well as in hair dyeing (Adisakwattana et al., 2020). Therefore, incorporating *C. ternatea* extract in consumable product such as hair shampoo can potentially contribute to improving scalp health and stimulating hair growth.

Moreover, Sero-X®, the first plant extract bio-pesticide in the world, is the product from *C. ternatea*. The active ingredients in Sero-X® comprised of mainly cyclotides, a type of peptide found in *C. ternatea* which exhibit insecticidal properties. This bio-pesticide has been developed as a safe an effective approach to traditional pesticides, with a focus on protecting bees and other pollinators. It is designed to offer organic pest control solutions for various crops, such as cotton and macadamia. Notably, Sero-X® is deemed as non-hazardous pesticides according to the Globally Harmonized System of Classification and labelling of Chemicals as it showed no toxicity towards testes rodents or bee pollinators, making it a unique and environmentally friendly option for pest management in agriculture (Oguis et al., 2019).

2.1.5 Plant secondary metabolites

The term secondary metabolites refer to a diverse array of organic compounds synthesized by plants in response to stresses. According to Bhaskar et al. (2022), these compounds are synthesized in plants as a defense mechanism against pathogen invasion, environmental factors, and nutrient deficiencies. These compounds are not involved in the plant growth and development but instead, mediate plants survival under stress conditions. Generally, secondary metabolites obtained from the roots or aerial parts of the medicinal plants only accounted for 1% by weight, thus, requiring large amounts of raw plant material in satisfying the commercial needs, leading to overexploitation or even extinction of the plants (Motolinía-Alcántara et al., 2021). It is noteworthy that these secondary metabolites exert an effect on biological system which thereby considered as bioactive (Azmir et al., 2013).

According to Jamwal and Puri (2018), plant-derived secondary metabolites could be grouped into three categories: (i) terpenes that composed of 5-C isopentanoid units, (ii) phenolics that derived from the shikimic acid, and (iii) nitrogen and sulphur synthesized from common amino acids. Surprisingly, plant-derived secondary metabolites are synthesised specifically from certain plant species or taxonomically related groups only, which is differ from primary metabolites that are widespread across the entire plant kingdom (Jamwal & Puri, 2018; Jain & Vijayvergia, 2019).

2.1.5(a) Secondary metabolites of *C. ternatea*

With a rich phytochemical profile, *C. ternatea* offers a plethora of bioactive compounds such as polyphenols, flavonoids, alkaloids, glycosides, phytosterols, pentacyclic triterpenoids, showcasing potential therapeutic benefits ranging from antioxidant and anti-inflammatory properties to cognitive enhancement (Adisakwattana et al., 2020). This plant species contains unique phytochemicals with multipurpose properties in which the phytochemicals are biologically active compounds found in the plant. A qualitative analysis of *C. ternatea* performed by Manjula et al. (2013) had revealed the presence of bioactive compounds such as alkaloids, tannins, glycosides, resins, steroids, saponins, flavonoids, and phenols. According to Chauhan et al. (2012), terpenoid, flavonoid, tannin, and steroids act as antioxidants that could effectively reduce reactive oxygen species (ROS) activity and oxidative stress. Apart from that, according to Scalbert et al. (2005), phytochemicals such as alkaloids and flavonoids that are widely present in the leaf could help in preventing neurodegenerative diseases and as well as diabetes mellitus. Meanwhile, the flowers of this medicinal plant that are rich in saponin, alkaloids, and tannins could work against inflammation and diabetes (Widowati et al., 2023).

Notably, the different parts of the *C. ternatea* plant contains a variety of phytochemical compounds such as pentacyclic triterpenoids, flavonoids, alkaloids, anthocyanins, steroids, phenols, saponins and tannins associated with pharmacological properties that are reported to be antioxidant, anticancer, analgesic, antipyretic, anti-inflammatory, anti-diabetic, anti-cancer, and possesses potential memory enhancing properties (Swain et al., 2012; Al-Snafi, 2016; Adisakwattana et al., 2020; Lee et al., 2021; Ginting et al., 2022). Furthermore, clitorienolactone (CLA), a distinctive phenolic compound found exclusively in *C. ternatea*, features a C8-C7' linkage and an α, β-unsaturated-γ-lactone structure (Vasisht et al., 2016). This compound was first isolated and identified by Vasisht et al. (2016) from the roots of *C. ternatea* and was proven to exhibit cognitive-enhancing properties (Ahad et al., 2023). Nonetheless, the most essential secondary metabolites identified in *C. ternatea* are the pentacyclic triterpenoids of taraxerol and taraxerone, which are found mainly in the roots of the plant (Lijon et al., 2017). These phytochemical compounds had been shown to exhibit potential neuroprotective properties, improving brain activities for the treatment of neurodegenerative diseases (Rai, 2002; Kumar et al., 2007).

The presence of pentacyclic triterpenoids such as taraxerol and taraxerone in *C. ternatea* had been confirmed in numerous studies (Kumar et al., 2008; Chauhan et al., 2012; Lijon et al., 2017; Damodaran et al. 2018). These bioactive compounds demonstrated a broad spectrum of pharmacological activities, including anti-cancer, anti-inflammatory, antimicrobial, anti-carcinogenic, cytotoxic, and acetylcholinesterase inhibitory activities (Takasaki et al., 1999; Lin et al., 2001; Singh et al., 2002; Jang et al., 2004; Lee et al., 2004; Naik et al., 2004). Furthermore, a study by Lee et al. (2021) had documented the presence of secondary metabolites such as pentacyclic triterpenoids, flavonoids, phytosterol, and fatty acids in the *in vitro* adventitious root cultures of *C. ternatea* which associated with memory enhancing properties. These secondary metabolites possessed anti-acetylcholinesterase (AChE) activities which could potentially delay the occurrence of neurodegenerative diseases (Lee et al., 2021).

Numerous studies on root extract of *C. ternatea* had revealed its potential in improving central nervous system (CNS) activity as well as in treating neurodegenerative diseases (Rai, 2002; Kumar et al., 2007; Damodaran et al. 2018). Of these, the main phytochemical compounds that counteract with the CNS mechanisms are pentacyclic triterpenoids such as taraxerol and taraxerone (Rai, 2002; Kumar et al., 2007; Mukherjee et al., 2008; Damodaran et al. 2018). For instance, the taraxerol obtained from *C. ternatea* exhibit the property of anti-acetylcholinesterase on rats and its effect was compatible to that of the standard medication (physostigmine) which had been used in inhibiting acetylcholinesterase (Kumar et al., 2007), indicating that taraxerol could be a highly potent medicine for memory enhancement. Besides, Rai et al. (2002) discovered that these phytochemical compounds aided in neurogenesis stimulation which could significantly improve the brain performance while preventing cognitive deterioration. Hence, it is evident that production of these valuable phytochemical compounds particularly pentacyclic triterpenoids is relatively crucial.

Moreover, the presence of lupeol in *C. ternatea* is also worth to be highlighted (Shahnas & Akhila, 2014; Khatoon et al., 2015; Lee et al., 2021; Dash et al., 2023). In such, lupeol had been proven to exhibit various pharmaceutical properties such as neuroprotective, anti-inflammatory, antioxidant, hepatoprotective, renoprotective, anti-cancer and anti-diabetic properties (Kaundal et al., 2017; Sinha et al., 2019; Ahmad et al., 2020; Hashmi et al., 2020; Huang et al., 2021). In addition, lupeol is often employed to be the standard in quantifying or determining the presence of triterpenoids (Khatoon et al., 2015; Dash et al., 2023; Thatipelli et al., 2023). Nonetheless, the most outstanding function of lupeol was its neuroprotective properties. For instance, Kaundal et al. (2017) demonstrated that lupeol served as a potential therapeutic compound in treating Alzheimer's disease as their study revealed that the amyloid-beta induced rat model of Alzheimer's disease treated with lupeol (50 mg/kg/day) orally showed significant reduction in behavioral, biochemical, and neurochemical abnormalities in the brain. The study therefore suggested that lupeol holds promising effect in addressing neurodegenerative diseases attributed by its anti-inflammatory, antioxidant, and neuroprotective properties. Similarly, Ahmad et al. (2020) reported that oral administration of lupeol at 50 mg/kg for two weeks resulted in significant suppression on the elevated oxidative stress, neuroinflammation, memory and cognitive deficits against the amyloid-beta-induced mouse model of Alzheimer's disease (Ahmad et al., 2020).

2.2 Plant tissue culture

Plant tissue culture was first introduced by Gottlieb Haberlandt, a German scientist, who was revered as the father of plant tissue culture in the late 19th and early 20th centuries. Nowadays, plant tissue culture serves as a classic yet renowned approach in the field of plant biotechnology which allows the propagation of plants under aseptic *in vitro* condition over a short period of time. It is built on the fundamentals of plant's plasticity, totipotency and high regeneration capacity in which a small piece of tissues from the original plant is capable of generating large amounts of genetically identical plantlets, given that the growing condition is optima in terms of lights, temperature, humidity, and supplementation of appropriate plant growth regulators (Bhatia et al., 2015; Fehér, 2019; Sivagamasundari, 2022). This technique is relatively crucial and versatile across various domains as in micropropagation, genetic modification, secondary metabolite production, conservation, or even in research and experimentation. In addition, plant tissue culture techniques utilized in producing secondary metabolites involved root culture, shoot culture, hair root culture, callus culture, cell suspension culture, and many more which mostly studied in the laboratory scale (Motolinía-Alcántara et al., 2021).

Nutrient requirements vary among different species and parts of plants. Consequently, several media formulations have been developed. Culture media such as root culture medium of White (1943) and callus culture medium of Gautheret (1939) were created by modifying nutrient formulations originally used for whole plants (Bhojwani & Razdan, 1986). Saad and Elshahed (2012) noted that the media commonly used in plant tissue culture were Murashige and Skoog (MS) medium (1962), Linsmaier and Skoog (LS) medium (1965), Gamborg (B5) medium (1968), and Nitsch and Nitsch (NN) medium (1969). Moreover, according to a five-year citation analysis by Herman (2015) on plant tissue culture regeneration media, MS medium (1962) was the most used (82%) medium. This popularity was due to its high nitrogen content in both nitrate and ammonium forms, making it suitable for both dicots and monocots especially in callus proliferation and shoot differentiation (Phillips & Garda, 2019).

Plant growth regulators play an important role in plant tissue culture as they could affect the development pathway of plant cells and tissues (Sivagamasundari, 2022). For instance, addition of auxin such as 2,4-D, IAA, NAA, and picloram would favour root formation, presence of cytokinin such as BAP, TDZ, kinetin, and zeatin would promote shoot induction, while combination of both auxin and cytokinin