ESTABLISHMENT OF *IN VITRO* CULTURE TECHNIQUES FOR *Clinacanthus nutans* AND THE EVALUATION ON ITS ANTIOXIDANT POTENTIAL

BONG FUI JOO

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BONG FUI JOO

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LIST OF ABBREVIATIONS

µg/mL	Microgram per millilitre
2, 4-D	2, 4-dichlorophenoxyacetic acid
ANOVA	Analysis of variance
BAP	6-benzylaminopurine
CRD	Completely randomized design
DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	Dry weight
GC	Gas chromatography
HPLC	High-performance liquid chromatography
IC_{50}	Half maximal inhibitory concentration
KIN	Kinetin
mg/L	Milligram per litre
MS	Murashige and Skoog (1962)
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAA	1-naphthaleneacetic acid
PGR	Plant growth regulator
rpm	Rotation per minute
SE	Standard error
TDZ	Thidiazuron
TFC	Total flavonoid content
TPC	Total phenolic content
w/v	Weight per volume

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PENUBUHAN TEKNIK KULTUR *IN VITRO* UNTUK *Clinacanthus nutans* DAN PENILAIAN KE ATAS POTENSI ANTIOKSIDAN

ABSTRAK

Clinacanthus nutans (Burm.f.) Lindau merupakan sejenis tumbuhan ubatan tradisional yang bernilai dan telah mendapat perhatian sebagai rawatan sampingan untuk penyakit kanser terutamanya di Asia Tenggara. Fenolik dan flavonoid yang dikenalpasti dalam tanaman ini mempunyai aktiviti antioksidan dalam menangkap radikal bebas dikaitkan dengan sifat anti-kansernya. Kajian ini bertujuan untuk membangunkan kultur kalus dan ampaian sel *C. nutans* serta menilai kandungan fenolik, flavonoid dan aktiviti antioksidan dari ekstrak kultur *in vitro* (kalus dan ampaian sel) dan tumbuhan induk (daun dan batang). Hormon tumbuhan yang berbeza (2, 4-D dan BAP, picloram) dan kombinasi auksin (2, 4-D atau NAA) dengan sitokinin (BAP, TDZ atau KIN) telah digunakan untuk menginduksi kalus yang rapuh. Selain itu, medium ditambah dengan hormon tumbuhan yang berbeza dan kepekatan sukrosa yang sesuai telah diuji untuk proliferasi kalus. Kinetik pertumbuhan bagi dua jenis sel turunan dari kultur ampaian telah dikaji. Jumlah kandungan fenolik, flavonoid dan aktiviti antioksidan bagi ekstrak dari kultur *in vitro* dan tumbuhan induk telah dinilai dengan menggunakan analisis spektrofotometri dan analisis kromatografi cecair prestasi tinggi (HPLC) untuk mengenalpasti flavonoid terpilih di dalam ekstrak. Kalus rapuh berwarna kuning telah diinduksikan daripada eksplan daun muda *C. nutans* yang dikulturkan atas medium MS yang ditambah dengan 2, 4-D dan BAP manakala kalus padat yang berwarna perang telah diinduksikan atas medium ditambah dengan picloram. Kalus rapuh dengan biomas yang maksimum (0.623 ± 0.016 g) telah

diinduksikan atas medium MS ditambah dengan kombinasi 0.25 mg/L 2, 4-D dan 0.25 mg/L BAP. Medium ini didapati optimum untuk proliferasi kalus. Kajian ini juga menunjukkan sukrosa pada kepekatan 30g/L didapati optimum untuk proliferasi kalus. Kalus rapuh menunjukkan pertumbuhan yang stabil selepas pengkulturan keempat yang mana sel turunan CN2 menunjukkan kadar pertumbuhan yang lebih cepat berbanding dengan sel turunan CN1. Kultur ampaian sel *C. nutans* dikultur sehingga 10 subkultur dengan agregat kecil dan sel halus. Jumlah kandungan fenolik yang tertinggi telah didapati dalam sel turunan CN2 dengan 55.35 mg GAE/g DW manakala kandungan flavonoid yang tertinggi diperolehi dari daun dengan 25.13 mg QE/g DW. Daun menunjukkan aktiviti antioksidan terkuat dengan nilai IC_{50} terendah (117.42 µg/mL) diikuti oleh sel turunan CN2. Analisis HPLC menunjukkan bahawa adanya catechin, luteolin, quercetin dan kaempferol di sel turunan CN2 dan daun dengan jumlah yang berbeza yang merupakan sebatian bioaktif yang berkaitan dengan sifat anti-kanser. Kajian ini telah berjaya membangunkan ampaian sel kultur *C. nutans* dengan flavonoid yang serupa dengan tanaman induk dan dapat dimanipulasi lebih lanjut sebagai strategi alternatif untuk pengeluaran fenolik dan flavonoid yang bernilai dalam keadaan terkawal untuk kegunaan farmaseutikal.

ESTABLISHMENT OF *IN VITRO* CULTURE TECHNIQUES FOR *Clinacanthus nutans* AND THE EVALUATION ON ITS ANTIOXIDANT POTENTIAL

ABSTRACT

Clinacanthus nutans (Burm.f.) Lindau is a valuable traditional medicinal plant that has gained interest as an alternative side treatment for cancer, particularly in South East Asia. Phenolic and flavonoid compounds identified in this plant have been linked to its anti-cancer properties due to their antioxidant activity in scavenging radical species. The present study aims to establish callus and cell suspension cultures of *C. nutans* and to evaluate the accumulation of phenolics, flavonoids and antioxidant activity from extracts of *in vitro* cultures (callus and cell suspension cultures) and intact plants parts (leaf and stem). Different plant growth regulators (2, 4-D and BAP, picloram) and combinations of auxin (2, 4-D or NAA) and cytokinin (BAP, TDZ or KIN) were used for the induction of friable callus. Besides, different proliferation medium and sucrose concentrations were tested on callus proliferation. Growth kinetics of two suspension cell lines was studied. The total phenolic and total flavonoid contents as well as the antioxidant activity of the extracts from intact plants and *in vitro* cultures were evaluated using spectrophotometric analysis. High-performance liquid chromatography (HPLC) analysis was performed to detect the selected flavonoids. Friable and pale-yellow callus was induced from young leaf explants of *C. nutans* on MS medium augmented with 2, 4-D and BAP whereas compact and brownish callus was induced on medium supplemented with picloram. Friable callus with maximum biomass (0.623 ± 0.016 g) was produced on MS medium supplemented with a combination of 0.25 mg/L 2, 4-D and 0.25 mg/L BAP. This medium

was also found to be optimum for callus proliferation. Results indicated that sucrose at the concentration of 30 g/L was optimum for callus proliferation. The friable callus showed a stable growth pattern after four subculture cycles where cell line CN2 showed a faster growth rate than cell line CN1. The suspension culture of *C. nutans* was maintained up to 10 subcultures with small aggregates and fine cells. The highest total phenolic content was obtained in cell line CN2 with 55.35 mg GAE/g dry weight (DW) whereas the highest flavonoid content obtained in leaf with 25.13 mg QE/g DW. The leaf exhibited the highest antioxidant activity with the lowest IC_{50} value (117.42 µg/mL) followed by cell line CN2. HPLC analysis revealed the presence of catechin, luteolin, quercetin and kaempferol in the cell line CN2 and the leaf samples at varying amounts indicating the presence of bioactive compounds linked to anti-cancer properties. The present study successfully established cell suspension cultures of *C. nutans* with the present of selected flavonoid compounds as the intact plant which can be further manipulated as an alternative strategy in the production of valuable phenolics and flavonoids under controlled conditions for various pharmaceutical purposes.

CHAPTER 1

INTRODUCTION

Clinacanthus nutans (Burm.f.) Lindau, locally known as Sabah Snake Grass is a valuable medicinal herb and belongs to the Acanthaceae family. It has been used in traditional medicine to treat insect, snake bites and viral infection and now gained popularity in Asia due to its anticancer properties (Sakdarat *et al.*, 2008). The leaves of this plants have been widely used as a side treatment for cancers by infusing the dried leaves in water or boil in water and consume as a tea (Fong *et al.*, 2019). Cancer patients claimed that the use of *C. nutans* as complementary medicine therapies help to mitigate disease progression and reduce cancer symptoms (Ng *et al.*, 2017). Numerous studies have been reported on the anticancer properties of *C. nutans* in exhibiting antiproliferative against human cancer cell lines (Yong *et al.*, 2013; Arullappan *et al.*, 2014). Apart from that, Rosli *et al.* (2018) proved that the ethanol extract of *C. nutans* increased the potency of anticancer drugs, paclitaxel to inhibit the viability of MDA-MB-231 cells. These reports suggested that *C. nutans* possess natural biochemical compounds correlated to cancer prevention and could also be an alternative side treatment for cancer patients. Besides, this plant has been reported to possess significant pharmacological activities such as anti-inflammatory (Sriwanthana *et al.*, 1996), antiviral (Kunsorn *et al.*, 2013), immune and neuromodulating (Le *et al.*, 2017) and antiangiogenic activity (Ng *et al.*, 2018).

Many of the beneficial medicinal effects of *C. nutans* have been reported attributed to the polyphenol compounds. The study by Sarega *et al.* (2016) showed that *C. nutans* is abundant in phenolics and the synergistic effects of these phenolic compounds can be used

to cure or prevent oxidative stress-related illnesses. Phenolics are antioxidants with redox properties and scavenge the free radicals generated during biochemical processes in the human body (Matkowski, 2008). They play a significant role in protection against disorders caused by oxidative damage such as cancers, inflammation and cardiovascular diseases by delaying or inhibiting the oxidative capacity of reactive oxygen species (Bag *et al.*, 2015; Chong *et al.*, 2018). Besides, phenolics are potential sources of natural antioxidants functioning as chemopreventive agents and are present in abundance in certain plant species than in common vegetables or fruits (Cai *et al.*, 2004). Futhermore, the interest in natural antioxidant has grown rapidly due to their low side effect.

Previous studies revealed that the antioxidant activity of *C. nutans* strongly attributed to the presence of polyphenol compounds associated with anti-cancer properties (Tan *et al.*, 2010). Ghasemzadeh *et al.* (2014) reported that *C. nutans* is rich in flavonoids and demonstrated high free radical scavenging activity. Flavonoids linked to anti-cancer properties such as quercetin, catechin, kaempferol and luteolin have been previously detected in the aerial, leaf, stem and bud extracts of *C. nutans* (Ghasemzadeh *et al.*, 2014; Khoo *et al.*, 2015; Mustapa *et al.*, 2015). *C. nutans* usually propagated by stem cuttings. Field crop production of herbal plants is relative ease of cultivation but the production of bioactive compounds in intact plants is often dependent on the physiological and developmental stages of the plant itself (Ketaren *et al.*, 2015). Besides, the conventional propagation of *C. nutans* in the field is subjected to various ecological problems such as climate change, seasonal variation, soil suitability, epidemic diseases (Ismail *et al.*, 2017) and post-harvesting factors such as storage duration and conditions (Raya *et al.*, 2015) which result in inconsistent accumulation of phenolic and flavonoids.

Plant cell cultures provide an effective alternative source to produce valuable secondary metabolites at a consistent and efficient rate under controlled conditions without the interference of seasonal and geographical restrictions as compared to conventional cultivation methods (Yue *et al.*, 2016). Cell suspension culture is the preferred methods for large scale production of secondary metabolites due to the rapid growth cycles providing higher rate of metabolism coupled with shorter biosynthetic cycles in a system (Vanisree *et al.*, 2004; Tan *et al.*, 2010). In cell cultures, nutritional factors and environment can be easily controlled to produce useful compounds. Moreover, various culture parameters can be exploited to enhance the accumulation of secondary metabolites. Production of secondary metabolites from callus and cell suspension cultures have been successfully reported in many plants to harness bioactive compounds such as accumulation of taxol in *Taxus baccata* (Khosroushahi *et al.*, 2006), artemisinin in *Artemisia annua* (Chan *et al.*, 2010) and ginsenoside in *Panax ginseng* (Kim *et al.*, 2018).

To date, studies on the production of secondary metabolites from *in vitro* cultures of *C. nutans* is limited. Haida *et al.* (2020) revealed that the tissue-cultured leaves of *C. nutans* contained higher total phenolic content, total flavonoid content and antioxidant activity than conventionally propagated leaves. However, the phenolic content and antioxidant properties of callus and cell suspension culture of *C. nutans* have not been determined. Phua *et al.* (2018) has reported the presence of quercetin, catechin and luteolin in *C. nutans* cell suspension cultures but no quantification of flavonoids in *in vitro* cultures has been conducted. In addition, there is lack of study on the optimization of culture media and conditions for *in vitro* culture of *C. nutans*.

The present study aims to develop an effective protocol for establishment of callus and cell suspension culture of *C. nutans* and to evaluate their potential antioxidant activity in comparison with intact plants and followed by quantification of their phenolic and flavonoid contents. The objectives of this study are:

1. To optimize the culture medium for *C. nutans* callus induction and proliferation,
2. To initiate cell suspension culture of *C. nutans* and study the effect of light and subculture frequency on cell proliferation,
3. To analyze the phenolic content and antioxidant activities between *in vitro* cultures and intact plant parts of *C. nutans* through *in vitro* assays,
4. To quantify and compare the amounts of selected falvonoids (catechin, luteolin, quercetin and kaempferol) between *in vitro* cultures and intact plant parts of *C. nutans* using high-performance liquid chromatography (HPLC) analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 *Clinacanthus nutans*

2.1.1 History and distribution

Medicinal plants are widely used in folklore medicine due to ancient belief in their protective effects and this has been increasingly recognized as a useful alternate and complementary regime due to their multitargeted characteristics. They are rich sources of medicinal phytochemicals and received well by the public due to their lower side effects as compared to allopathic medicines (Roslan *et al.*, 2018). *Clinacanthus nutans* (Burm. f.) Lindau belonging to the family of Acanthaceae, is a well-known medicinal herb in folk medicine and has gained much attention due to its multiple medicinal properties. It was originally found in tropical Asia and is highly reputable in Thailand commonly used to treat insect bites and various viral infections. Since 1992, the *C. nutans* leaf extracts have been reported exhibited antiviral activity against herpes simplex virus for both *in vitro* and *in vivo* samples (Jayavasu *et al.*, 1992). Besides, this plant has been shortlisted as one of the essential medicinal plants for primary healthcare in Thailand (Arullappan *et al.* 2014).

C. nutans gained its popularity in Malaysia due to its anticancer properties as claimed by a patient who recovered from terminal stage of lymph node cancer after consuming this plant daily (Tan *et al.*, 2020). In recent years, the use of *C. nutans* as side treatment for cancer increases and many cancer patients claimed that they have a better recovery after consuming *C. nutans* alongside chemotherapy prolonging their lifespan and promoting cancer remission (Hii *et al.*, 2019). It also ranked top 5 commonly used

medicinal plant in Singapore and is also widely used in China for general detoxification, boost immunity and to prevent cancer (Siew *et al.*, 2014; Li *et al.*, 2019).

C. nutans has long been cultivated in Thailand, Indonesia, Malaysia and China and is now widely distributed throughout the tropical countries of South East Asia (Ayudhya *et al.*, 2001). It has a wide range of geographical distribution and can be found in the wild, cultivated habitat such as grasslands, shrubs, hillsides and open forest (Ismail *et al.*, 2016). It is identified with multiple vernacular names based on different regions in Asia. In Malaysia, this plant is locally known as "Sabah Snake Grass" since it was found in Sabah of East Malaysia. It is also known as “Daun Belalai Gajah” (elephant's trunk) due to its curved stem that seems like the curve of elephant's trunk (Ismail *et al.*, 2017). In Thailand, this plant is popularly known as “phaya yo” or “phaya plongtong” while in China it is named “you dun cao” or “e zui hua” (Aslam *et al.*, 2015).

C. nutans has been commercialized throughout the country especially in South East Asia. In Malaysia, there are various types of *C. nutans* products being sold as herbal-based products in the form of powder, tablets, capsules and herbal tea (Fong *et al.*, 2014; Roslan *et al.*, 2018). These products are generally marketed as a health supplement in herbal therapies for illnesses and for general health. The unfermented *C. nutans* were found to exhibited stronger antioxidant activity compared to the fermented one due to the degradation of phenolics during fermentation (Lusia Barek *et al.*, 2015).

2.1.2 Botany

Acanthaceae is one of the family of dicotyledonous flowering plants and comprises of about 346 genera and 4300 species (Khan *et al.*, 2017). They are mainly distributed in tropical, subtropical while some in temperate regions and consist of mainly tropical herbs and shrubs. They are one of the largest sources of medicinal plants that possess numerous pharmacological activities. *Clinacanthus nutans* is one of the well-known species from this family and widely used in traditional medicines in tropical regions. The genus *Clinacanthus* consists of two species, namely *Clinacanthus nutans* and *Clinacanthus siamensis*. These two species are often misidentified as they have relatively similar growth habit and leaves appearance. Both species showed similar major components of cross section of midrib and stem but different in leaves measurement index based on macroscopic and microscopic analysis (Kunsorn *et al.*, 2013). In general, they can be distinguished by their flowers and pharmacological properties (Fong *et al.*, 2014). Besides, molecular identification using *trnH-psbA* has been proved can be used to distinguish *C. nutans* from *C. siamensis* (Ismail *et al.*, 2018).

The taxonomic classification of *C. nutans* is as follow:

Kingdom : Plantae

Phylum : Magnoliophyta

Class : Magnoliopsida

Order : Lamiales

Family : Acanthaceae

Genus : *Clinacanthus*

Species : *nutans*

C. nutans is a tall herbaceous perennial shrub, generally can grow up to 3 m. The leaves are pale green, opposite and narrowly elliptic-oblong or lanceolate in shape with apex acute with obtuse rounded or truncate leaf base (Plate 2.1). The leaves have four to seven pairs of side veins (Plate 2.2). The stomata are classified as diacytic type and only present at the lower epidermis part of the leaf (Kunsorn *et al.*, 2013). The petiole is sulcate, bifariously pubescent and 0.2 - 1.5 cm long. The stems are green, pubescent branches and cylindrical, glabrescent and with white internodes (Kosai *et al.*, 2016). The flowers are dull red with yellow streaks on lower lips and present at the top of branches. The capsule is oblong and basally contracted into a solid stalk 4-seeded whereas the ovary is compressed into two cells (Hu *et al.*, 2011; Yahaya *et al.*, 2015).

Generally, *C. nutans* is vegetatively propagated by stem cutting, preferably from the mature stem which is able to produce a high rate of multiplication. They are able to grow well under moderate temperature with relatively high humid conditions, full sunlight or shaded region. Flowers are rarely formed on this plant. Fong *et al.* (2014) explained this could be due to the long terms of vegetative propagation and the frequent harvesting before maturity to meet the high demand of the leaves from the consumers resulting in the inability to mature for sexual reproduction. Ismail *et al.* (2016) reported that in the long term, these practices would reduce the genetic diversity and impact on long-term survival and evolution of the plant.

Plate 2.1: **The *Clinacanthus nutans* plant grown in the Herbarium Unit, School of Biological Sciences, Universiti Sains Malaysia.**

Plate 2.2: **Leaves of *Clinacanthus nutans*.** (A) Front view and (B) Back view. Scale bar represents 1 cm.

2.1.3 Ethnomedicinal uses

In Thailand, the fresh leaves of *C. nutans* are widely used as folklore medicine, particularly for the treatment of skin rashes, snake and insect bites, herpes infection and inflammation (Ayudhya *et al.*, 2001; Sakdarat *et al.*, 2009). The leaves have been topically used as anti-venom for scorpion and snake bites (Vajrabhaya and Korsuwannawong, 2016). Uawonggul *et al.* (2006) suggested that the anti-snake venom activity of *C. nutans* attributed to anti-cell lysis activity instead of anti-neuromuscular blockage. The leaves are also soaked in hot water and consumed as tea to treat diabetes mellitus, diarrhea, fever and dysuria (P'ng *et al.*, 2012). Besides, the leaf extracts also provided as an alternative therapeutic use in the form of topical cream to cure lesions caused by herpes zoster and herpes genitalis (Kongkaew and Chaiyakunapruk, 2011).

In Malaysia, the usage of *C. nutans* for the treatment of various cancers has been spread throughout the country. The leaves of *C. nutans* are widely used as complementary and alternative medicine (CAM) to slow down cancer progression or to prevent cancer (Quah *et al.*, 2017). The utilization of CAM has been shown to enhance health and physical well-being, wound healing and reduce side-effects from chemotherapy among cancer patients (Dhanoa *et al.*, 2014). The fresh leaves highly demanded by patients with cancer, diabetes and general ailments (Raya *et al.*, 2015). The fresh leaves are freshly eaten or blended and serve as a fresh drink or boil with water and serve as herbal tea (Alam *et al.*, 2017; Kong and Abdullah, 2017). They also blended with juices such as apple juice and sugarcane to lessen the bitterness of the decoction. The leaves also have been used for detoxification and to promote health such as to lower blood cholesterol, blood pressure, glucose and uric acid.

In China, *C. nutans* have been broadly used in traditional Chinese medicine to treat various inflammatory illnesses such as contusion, haematoma, bruises on eye, anxieties, rheumatism and to relieve pain (Fong *et al.*, 2014). They also combined *C. nutans* with other herbs to treat various medical conditions including clearing away heat, nourishing the spleen and detoxification (Farsi *et al.*, 2016). In Indonesia, *C. nutans* fresh leaves have been used to cure diabetes, dysuria, hyperglycemia, dysentery and gastrointestinal problems (Arullappan *et al.*, 2014). They consume the decoction or boiled with hot water.

2.1.4 Phytochemical activities

Previous studies reported the presence of saponins, alkaloids, phenolics, flavonoids, diterpenes, triterpenes, steroids, phytosterols, protein and polysaccharide in *C. nutans* leaves and stem extracts (Chithra *et al.*, 2016; Rahim *et al.*, 2016; Esmailli *et al.*, 2019; Kong *et al.*, 2019). Khoo *et al.* (2015) reported that the leaf extracts contain higher amounts of terpenoids and phenolic compounds than the stem extracts. Based on the study performed by Mustapa *et al.* (2015), *C. nutans* was found rich in chlorophyll and mainly comprised of polyphenols, flavonoids, phytosterols, diterpene, triterpene, fatty acids and palmitic acid.

Lupeol and β-sitosterol have been previously isolated from the stems of *C. nutans* (Dampawan *et al.*, 1977). Six C-glycosyl flavones namely isovitexin, isomolrupentin 7-O-β-glucopyranoside, shaftoside, isoorientin, vitexin and orientin and five sulfur-containing glucosides have been separated from the butanol fraction of methanolic extract of *C. nutans* (Teshima *et al.*, 1997; Teshima *et al.*, 1998). Chelyn *et al.* (2014) reported that the C-glycosidic flavones are the major flavonoids in the leaf of this plant. Tu *et al.* (2014)

also isolated four sulfur-containing compounds from the ethanol extract of the aerial parts of this plant and showed anti-dengue virus, anti-inflammatory and immune-modulating activity.

C. nutans extracts have been reported to be rich in chlorophyll. Three chlorophyll a and chlorophyll b namely purpurin 18 phytylester, 13^2-hydroxy-(13^2-S)-phaephytin b and phaeophorbide a were isolated from the chloroform extract of *C. nutans* and three chlorophyll derivatives compounds namely 13^2-hydroxy-(13^2-S)-phaeophytin a, 13^2-hydroxy-(13^2-R)-phaeophytin a and 13^2-hydroxy-(13^2-R)-phaeophytin b have also been separated from the same extract and exhibited anti-herpes simplex virus activity (Ayudhya *et al.*, 2001; Sakdarat *et al.*, 2009).

A study by Yong *et al.* (2013), they found that 1, 2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester, was the most abundant in the chloroform extract of *C. nutans* among 14 phytochemicals through GC-MS analysis and the extract demonstrated antioxidant and anticancer activities against cancer cell lines such as K-562 and Raji cell lines. Ghasmzadeh *et al.* (2014) reported *C. nutans* is a good natural antioxidant with high accumulation of phenolics and flavonoids and found that catechin is the most abundant in *C. nutans* compared to quercetin, kaempferol and luteolin.

Huang *et al.* (2015) detected seven known flavones namely 6,8-apigenin-C-α-L-pyranarabinoside, isoorientin, isovitexin, shaftoside, orientin and vitexin from the 30% ethanol extract of the aerial parts of *C. nutans* based on HPLC and LC-MS analysis. Besides, four compounds namely shaftoside, stigmasterol, β-sitosterol and lupeol have been identified from *C. nutans* leaves extract and the extracts showed anti-proliferative effects when tested on breast, ovarian and colorectal cancer cell lines (Murni *et al.*, 2015). Sulaiman *et al.* (2015) found that alpha tocopherol was the most abundant compound in

ethyl acetate and ethanol extracts of *C. nutans* among 23 tested phenolic and fatty acid compounds and the extracts exhibited cytotoxicity activity on tumorigenic cell MCF-7.

A novel polysaccharide-peptide complex which make up of about 87.25% of carbohydrate and 9.37% of protein has been extracted and purified from *C. nutans* ethanol leaf extract and inhibited the growth on human gastric cancer cells at 200 µg/mL with 92.34% of inhibition ratio (Huang *et al.*, 2016). Besides, a bioactive compound, purpurin-18 phytyl ester has been isolated from *C. nutans* which exhibited *in vitro* wound healing, anti-inflammatory and anti-biofilm activities (Roeslan *et al.*, 2019).

Rahim *et al.* (2016) reported that the main flavonoid compounds present in the *C. nutans* methanolic extract belongs to the flavone C-glycoside family. They identified the presence of 4-hydroxybenzoic acid, apigenin, caffeic acid, ferulic acid, coumaric acid, gallic acid, isoorientin, luteolin, isovitexin, schaftoside, vitexin and orientin in methanolic extract of *C. nutans* using UHPLC-ESI analysis. They also suggested these compounds exert antinociceptive activity synergistically. Besides, five phenolic acids namely caffeic acid, cinnamic acid, proto-catechuic acid, chlorogenic acid and ferulic acid have been detected in the most potent antioxidant capacities extracts of *C. nutans* by HPLC-DAD and PCA was identified as the major phenolic acid in this extract (Sarega *et al.*, 2016).

In another study, Ismail *et al.* (2017) revealed the presence of 20 phytochemicals constituents in *C. nutans* through GC-MS analysis and many of the bioactive compounds can be used to treat cancer such as methyl ester, octadecanoic acid, oleic acid and vitamin E. Additionally, Ng *et al.* (2017) identified 31 known compounds from hexane extract of *C. nutans* using GC-MS analysis and the extracts demonstrated antiproliferative activity against three cancer cell lines namely nasopharygeal cancer (CNE1), non-small cell lung cancer (A549) and liver cancer (HepG2) cells. Out of the 31 identified constituents,

vanillin, phytol, squalene, tetracosane, vitamin E, stigmasterol and beta-sitosterol have been reported exhibited anticancer properties. On the other hand, Teoh *et al.* (2017) reported that the root extracts of *C. nutans* are rich in phytosterols and terpenoids while the most abundant compounds in root extracts were lupeol and found inhibited the proliferation of MCF-7 and HeLa cells. They suggested that lupeol might work solely or cooperatively with other compounds in modulating apoptosis process.

Besides, GC-MS analysis showed the presence of 28 compounds in the dichloromethane fraction of *C. nutans*, most of them were fatty acids and the most abundant compound was N-(4-methoxyphenyl)-2-hydroxyimino-acetamide (Haron *et al.*, 2019). Another GC-MS analysis study conducted by Murugesu *et al.* (2019) revealed the presence of stigmasterol, phytol, hexadecenoic acid, 1-monopalmitin, heptadecanoic acid, palmitic acid, 1-linolenoylglycerol, pentadecanoic acid and stigmast-5-ene in the n-hexane fraction of *C. nutans*. Furthermore, Esmailli *et al.* (2019) reported that methanolic extract of *C. nutans* contained the highest concentration of flavonoids such as vitexin, orientin and isovitexin and showed strongest cytotoxicity activity against human colorectal carcinoma cell line (HCT-116) among other extracts.

2.1.5 Pharmacological activities

The pharmacological activities of *C. nutans* extracts and their fractions have been examined and found exhibited a wide range of biological activities including antioxidant (Arullappan *et al.*, 2014; Sarega *et al.*, 2016), anticancer (Yong *et al.*, 2013; Zakaria *et al.*, 2019), antiviral (Thongchai *et al.*, 2008; Kunsorn *et al.*, 2013), antimicrobial (Chithra *et al.*, 2016; Hamid and Mutazah, 2019), anti-inflammatory (Wanikiat *et al.*, 2008; Mai *et*

al., 2016), analgesic (Satatyavivad *et al.*, 1996; Hao *et al.*, 2020), antinociceptive (Rahim *et al.*, 2016; Zakaria *et al.*, 2018), neuroprotective (Huang *et al.*, 2015; Azam *et al.*, 2020) and immunomodulatory effects (Le *et al.*, 2017), anti-diabetic (Abdullah and Kasim, 2017; Imam *et al.*, 2019), anti-obesity (Sarega *et al.*, 2016; Abdulwahid *et al.*, 2018); anticoagulant (Rahman *et al.*, 2020) and wound healing effect (Roeslan *et al.*, 2019).

Toxicological studies showed that the extracts of *C. nutans* did not show toxicity on human gingival fibroblast cell line (Vajrabhaya and Korsuwannawong, 2016). P'ng *et al.* (2012) reported oral administration of 1.8 g/kg of *C. nutans* showed no adverse effects in male mice after 24 hours or 14 days. Khoo *et al.* (2018) revealed no abnormal toxicity symptoms or mortalities in all treated rats with oral administration of 5000 mg/kg. Besides, the toxicity of the n-hexane fraction of *C. nutans* extract has been tested on zebrafish embryos (Murugesu *et al.*, 2019). The toxic effects of the *C. nutans* on developing zebrafish embryos was found time and dose dependent. They reported that deformities in cardiological functions in zebrafish embryos may attributed to the present of cardiac glycosides in the extract and exclaimed that these compounds should be further identified.

Clinical trials have also been conducted to determine the antiviral effect of *C. nutans*. Studies revealed that *C. nutans* cream is efficient to treat herpes genitalis (Jayavasu *et al.*, 1992; Kongkaew and Chaiyakunapruk, 2011) and herpes zoster patients (Sangkitporn *et al.*, 1995). They also claimed that the pain was reduced and the lesions crusted and healed more rapidly in extract-treated group as compared to placebo group. These studies suggested that *C. nutans* is a potential therapeutic agent to treat various illnesses. Further studies on its phytochemical profile and their mode of action are required to confirm its safety for clinical applications.

2.1.5(a) Antioxidant activity

C. nutans is a potential source of antioxidant and therapeutic agent as it contains high amount of phenolic compounds (Khoo *et al.*, 2015). *C. nutans* extracts with strong antioxidant activity exhibited cytotoxic effect on cancer cell lines. Antioxidants present in the extracts scavenge reactive oxygen species that are potentially to be used as a source of antioxidant and anticancer (Sulaiman *et al.*, 2015). Sarega *et al.* (2016) also claimed that *C. nutans* is potential to be used to treat and prevent oxidative stress-related illnesses.

Pannangpetch *et al.* (2007) demonstrated that the extract of *C. nutans* possessed antioxidant properties and protective effect against free radical-induced hemolysis. The ethanolic extracts inhibited the production of peroxide in rat macrophages and reduced the red blood cell lysis. Their findings revealed that *C. nutans* extract is potential to be used as an antioxidant agent to ameliorate the oxidative damage. Yuann *et al.* (2012) evaluated the antioxidant activity and protective effects of *C. nutans* on plasmid DNA integrity in *E. coli*. DNA integrity assay revealed that *C. nutans* leaf extracts reduced the levels of DNA cleavages and retained the high levels of super-coiled plasmid DNA under riboflavin photochemical treatment and the results were better as compared to green tea extract.

Tiew *et al.* (2014) examined the antioxidant activity of methanolic extracts of *C. nutans* through DPPH assay and the extract exhibited radical scavenging activity in dose-dependent manner with IC_{50} value of 1.33 mg/ml. They suggested that the antioxidant activity of the extract contributed by the presence of phenolic and flavonoid compounds present in the extract. Sarega *et al.* (2016) reported that *C. nutans* extracts upregulated the expression of antioxidant genes (SOD 1, SOD 2, CAT, GPx, and GSR) and increased the activities of antioxidant enzyme which are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GSR) which attenuating

oxidative stress in rats. They suggested that the overall activities contributed by the synergistic effects of different phenolics present in the *C. nutans* extracts.

Ghasemzadeh *et al.* (2014) reported that the accumulation of bioactive compounds and antioxidant activity of *C. nutans* is organ-specific where the extract of buds contained higher amounts of phenolic acids and flavonoids than the leaf. Kong *et al.* (2016) found that the leaves of *C. nutans* have greater antioxidant potential than the stems. Kong *et al.* (2019) further investigated the antioxidant properties on leaves of *C. nutans* from different location and revealed that un-shaded leaves of *C. nutans* exhibited significantly higher antioxidant properties than shaded leaves. Besides, a study by Haida *et al.* (2020) demonstrated that the leaves of *C. nutans* from tissue-cultured plant produced higher total phenolic content and showed the higher antioxidant activities as compared to the leaves from field-grown plant.

Different solvents have been used for extraction of *C. nutans* and the extracts were found displayed different antioxidant activity. Yu *et al.* (2017) reported that 70% ethanol extracts achieved stronger antioxidant activities in 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and reducing capacity compared to distilled water extract. Similar finding was reported by Chong *et al.* (2018) where ethanolic extract of *C. nutans* leaves had the highest antioxidant capacity. Yong *et al.* (2013) proved a good antioxidant in *C. nutans* chloroform extract against galvinoxyl radicals and DPPH. Other study conducted by Sulaiman *et al.* (2015) showed that ethyl acetate and ethanol extracts showed the stronger antioxidant activity and cytotoxicity against cancer cell (MCF-7) than dichloromethane and n-hexane extracts while Kong *et al.* (2016) reported *C. nutans* extracted with 70% acetone showed the highest antioxidant power.

Alam *et al.* (2017) reported methanolic extract and ethyl acetate fraction of *C. nutans* showed significant DPPH free radical scavenging activity among all the fractions evaluated. They found that the total phenolic and flavonoid content of the extract showed a significant impact on antioxidant and α-glucosidase inhibitory activity. In addition, a positive correlation between antioxidant activity and the α-glucosidase inhibitory potency of different extracts was noticed. Besides, Jusoh *et al.* (2019) found that insoluble phenolic acids abundant in *C. nutans* extracts and exhibited highest antioxidant activity compared to free and soluble phenolic in *C. nutans* extracts. The results indicated that different forms of phenolic acid contributed to distinct total phenolic content and antioxidant capacity.

2.1.5(b) Anticancer activity

C. nutans leaves have been widely used for cancer treatment and prevention. The anticancer effect of *C. nutans* has been reported exhibited antiproliferative and cytotoxic effects on human cancer cell lines such as skin, breast, cervical, lung, liver, gastric, colon cancer and lymphatic disorder which suggested that this plant can be used as a complementary approach to cure and prevent cancer. With reference to the National Cancer Institute, a crude extract can be considered as an active anti-cancer agent when it possesses with less than 20 μg/mL of IC_{50} value (Ghasemzadeh *et al.*, 2014).

Previous studies showed that non-polar extracts of *C. nutans* generally exhibited stronger anti-proliferative effect than polar extracts. Yong *et al.* (2013) reported that chloroform extract of *C. nutans* at 100 μg/ml exhibited the highest antiproliferative activity on K-562 (91.28%) and Raji cell lines (88.97%) in a concentration-dependent manner. Arullappan *et al.* (2014) found that petroleum ether extracts exhibited the

strongest cytotoxic activity against K-562 and HeLa cells with IC_{50} of 20 and 18 µg/mL, respectively. In addition, Ng *et al.* (2017) found that hexane and chloroform extracts of *C. nutans* showed antiproliferative against nasopharygeal cancer (CNE1), non-small cell lung cancer (A549) and liver cancer cell lines (HepG2) cells and induced apoptosis which modulated by oxidative stress and through extrinsic and intrinsic caspase pathways.

However, Hamid and Yahaya (2016) reported the methanol extract from *C. nutans* exhibited the highest cytotoxicity effect with lowest IC_{50} values of 43.94 µg/ml compared to hexane, chloroform and ethyl acetate extracts against HepG2 cell line which might be due to the total flavonoid content which was noticed highest in methanol extract. Quah *et al.* (2017) also revealed that the methanolic extract of *C. nutans* showed the highest cytotoxic effect against liver cancer cells (HepG2) with IC_{50} value of 13.33 µg/mL and breast cancer cell lines (MDA-MB-231) with IC_{50} value of 18.67 µg/mL. Similar results also reported by Esmailli *et al.* (2019) that methanolic extract exhibited the highest activity on human colorectal carcinoma cell line (HCT-116) using MTT assay. Teoh *et al.* (2017) revealed that ethyl acetate and methanolic root extract of *C. nutans* induced apoptosis by suppressing the anti-apoptotic gene through mitochondria-independent or dependent way.

On the other hand, Zakaria *et al.* (2017) observed that aqueous extract of *C. nutans* showed a significant cytotoxic effect on HeLa cells with IC_{50} value of 13 µg/mL through apoptosis. Fazil *et al.* (2016) also demonstrated that the *C. nutans* water extract showed antiproliferative properties against human lung cancer cells (A549). Interestingly, Fong *et al.* (2019) reported that cold aqueous extract of *C. nutans* exhibited higher cytotoxic effect against D24 melanoma cells than the hot aqueous extract which induced apoptosis in the D24 cells in a dose and time-dependent manner.

Besides, Murni *et al.* (2015) showed that one of the fractions of *C. nutans* hexane extract exhibited higher anti-proliferative effect against ovarian (SKO-3), breast (MCF-7) and colorectal (HT-29) cancer cell lines than hexane extract. On the other hand, Zakaria *et al.* (2019) investigated the fractions from methanol extract of *C. nutans* on the anti-proliferative activity against liver cancer cells (Hep-G2) using MTT assay and reported that one of the fractions that contained triterpenes exhibited the strongest cytotoxicity against Hep-G2 cancer cells with lowest IC_{50} value of 1.73 µg/mL. Besides, Roslan *et al.* (2018) revealed that one of the fractions of *C. nutans* from the elution of ethyl acetate: hexane showed the lowest IC_{50} value with 27 µg/mL on human cervical cacer (HeLa cell).

Haron *et al.* (2019) found that the semi-polar and non-polar fractions demonstrated stronger cytotoxic effect than the polar extracts. They revealed that dichloromethane fraction of *C. nutans* displayed the lowest IC_{50} value of 70 µg/mL at 48 hour of treatment and exhibited marked morphological features of apoptosis on human cervical cancer (HeLa) cells with cell cycle arrested at the S phase. Ismail *et al.* (2020) also reported that dichloromethane extract of *C. nutans* inhibited MCF7 cell growth. Molecular docking studies revealed that linolenyl alcohol and palmitic acid from the extract could bind to the apoptosis-related proteins. Mutazah *et al.* (2020) examined the cytotoxic activity of methanol extract of *C. nutans* and its fractions on two breast cancer cell lines using MTT assay. They found that one of the fractions showed the highest activity with IC_{50} values of 5.68 µg/mL and 5.05 µg/mL against MDA-MB-231 and MCF-7 cell line, respectively. They revealed that the entadamide C and clinamide D that presence in the leaves of *C. nutans* bind to the caspase-3 binding site of cancer cell line and this binding affinity interaction activated the caspase-3 and induced apoptosis.

Wang *et al.* (2019) reported that *C. nutans* ethyl acetate fraction exert the strongest cytotoxic effect against human colorectal cancer cells (HCT116) with IC_{50} value of 48.81 µg/mL and revealed the apoptosis activated via intrinsic and extrinsic pathways. They suggested that this fraction induced ROS-dependent apoptosis and autophagy on HCT116 cells. Besides, Zainuddin *et al.* (2019) showed that the antiproliferative activity of a semi-purified fraction of *C. nutans* associated with induction of apoptosis in human cervical cancer cells (SiHa) with IC_{50} value of 9.60 µg/mL. The flowcytometric analysis revealed that this fraction induced apoptosis in SiHa cells via mitochondrial-dependent pathway.

The apoptotic effect of silver nanoparticle *C. nutans* (AgNps-CN) against oral squamous cell carcinoma cell lines have been studied by Yakop *et al.* (2018). MTT assay revealed AgNps-CN showed cytotoxic effects against HSC-4 cell lines with IC_{50} value of 1.61 µg/mL and no cytotoxic activity has been observed on normal cells. The combination of morphological cells analysis, flow cytometry and cell cycle analysis and western blot showed that AgNps-CN induced apoptosis against HSC-4 cell lines via intrinsic pathway.

Besides, the combination effect of *C. nutans* with anticancer drugs has been carried out by Rosli *et al.* (2018). The SRB assay revealed that the combination of ethanol extract of *C. nutans* (CNE) and paclitaxel (PTX) significant enhanced PTX-induced cytotoxicity on breast cancer cells (MDA-MB-231). The findings revealed that CNE increased the inhibitory effect of PTX and potentially to be used as new combination chemotherapy. In addition, Hii *et al.* (2019) also reported that the combination of *C. nutans* extracts and gemcitabine enhanced the sensitivity and anticancer effects of gemcitabine in squamous pancreatic cancer cells (SW1990 and BxPC3). They also found that the effective dose of gemcitabine could be reduced by 2.38- 5.28 folds when combined with *C. nutans* extracts.

2.2 Plant tissue culture

Plant tissue culture is the cultivation of plant cells, tissues or organs on medium supplemented with nutrients under aseptic and controlled environmental conditions (Mukta *et al.*, 2017). It relies on the totipotency of plant cells. Each cell in the cultures retains full genome of the parent plant and able to express it to develop into complete whole plant and able to produce the bioactive compounds found in the intact plant under appropriate chemical and physical environment (Rao and Ravishankar, 2002). Besides, the cultured plant cells also exhibit a high degree of plasticity which allow them to alter their metabolism, growth and development for plant regeneration. The cultures can be initiated from any parts of the plant and their grow varied based on the nutrient composition of the culture medium and physical conditions such as pH, illumination and temperature (Wang *et al.*, 2017; Jan *et al.*, 2019). Plant tissue culture has been used for research and application on clonal propagation, plant modification, production of pathogen-free plants and production of valuable secondary metabolites (Thorpe, 1990; Suwanseree *et al.*, 2019). In addition, *in vitro* culture systems have been used for genetic manipulation to facilitate the biosynthesis of desired plants and plant products (Wang *et al.*, 2017). Furthermore, it also used to study plant cell behaviour, genetics, biochemistry, physiology and pathology (El-Mawla, 2014).

Plant tissue culture approaches provide an alternative source for mass production of plants and bioactive compounds such as pharmaceuticals, pigments, flavours, fragrances and biopesticides (Murthy *et al.*, 2014). In recent years, medicinal plants have been widely studied due to their valuable medicinal values. However, the instabilities of the environmental and geographical factors increase the difficultly to acquire plant-derived compounds in a consistent manner (Jain *et al.*, 2019). Cell cultures have been

received great attention for the accumulation of valuable plant-derived secondary metabolites such as ginsenosides from *Panax notoginseng* (Zhong and Zhu, 1995), paclitaxel from *Taxus baccata* (Tabata, 2004), camptothecin from *Camptotheca acuminate* (Pi *et al.*, 2010) and shikonin from *Arnebia euchroma* (Hao *et al.*, 2014). Moreover, the compounds such as berberine, shikonin and sanguinarine have been successfully produced at industrial levels by using cell culture systems (Rao and Ravishankar, 2002).

In vitro culture system provides advantages over conventional methods as it is independent of seasonal variations, geographical and environmental restrictions. It allows better control of the developmental processes and the accumulation of bioactive compounds as the cultures maintained in sterile and controlled environmental conditions (Cardoso *et al.*, 2019). In addition, it also provides reliable and continuous supply of plant secondary metabolites for industrial processing and avoids the extinction of plants in the future (Vanisree *et al.*, 2004). Furthermore, it is efficient for downstream recovery as the structure of cells are simpler as compared to the intact plants. Moreover, novel compounds can be produced through cell cultures (Rao and Ravishankar, 2002).

The main target in the plant cell cultures is to produce maximum biomass and desired metabolite. However, these two processes usually do not occur at the same time (Georgieva *et al.*, 2015). With the goal to enhance the production of cell biomass and bioactive compounds, there are several strategies have been used such as the selection of high-yield cell cultures, optimization of culture medium and physical factors to improve the growth of cell biomass and to stimulate the biosynthesis of secondary metabolites using precursor feeding or elicitation (Murthy *et al.*, 2014).

2.2.1 Callus culture

Callus culture consists of unorganized dividing cell mass that form in response to mechanical injury or pathogen infection (Bekheet *et al.*, 2018). These undifferentiated cells mainly composed of parenchyma cells (Mastuti *et al.*, 2017). In general, callus is initiated by culture the small wounded pieces of plant tissues such as leaves, stems, roots, seeds and flowers of the plants on a solidified medium under aseptic conditions (Cardoso *et al.*, 2019). The length of the induction phase depends on the physiological status of the explants cell as well as the cultural conditions (Bhatia, 2015). Callus consists of morphogenic cells that cover the injured area of the explants and then initiate a complex developmental process such as induction of cell division and de-differentiation (Fehér, 2019). It can be homogenous or heterogenous as it is usually composed of non-differentiated and differentiated cells. Callus cultures can be grouped into non-embryogenic callus and embryogenic callus (Mukta *et al.*, 2017). In compact callus, the cells are aggregated while friable callus cells are loosely connected.

A successful callus formation largely relies on an appropriate culture medium. The selection of medium generally depends on the purpose of the tissue culture. Plant tissue culture medium provides mineral ions, vitamins, carbon sources, growth regulators, organic supplements and other nutrients for the growth of cells or tissue (Bhatia, 2015). The nature of callus involves a complex relationship between the plant species, explant selection, medium composition and culture conditions (Butnariu and Coradini, 2012; Bakar *et al.*, 2019). For example, callus derived from leaf, petiole and internode explants of *Gynura procumbens* was friable and compact in texture while node explant derived callus was compact in texture (Nurokhman *et al.*, 2019).

Plant growth regulators (PGRs) are synthetic molecules that used in plant tissue culture and usually supplemented in culture medium at low concentrations for plant growth and development (Bhatia, 2015). Callus actively divide with the stimulation of endogenous hormones and exogenous PGRs that supplemented in the medium. Endogenous hormones regulate gene expression and metabolic processes of the plant (Peng *et al.*, 2015). Gaspar *et al.* (1996) revealed that successful callus induction is the result of positive interaction between endogenous and exogenous PGRs. Exogenous supply of PGRs is often needed to initiate the callus formation. However, this requirement is strongly dependent on the genotype of the plant and endogenous hormone content.

Auxin and cytokinin are the most extensively used PGRs in culture medium (Ikeuchi *et al.*, 2013). The control of the process of differentiation was found to be mainly dependent on the presence of auxin and cytokinin. Auxins promote cell growth, cell elongation, adventitious root formation and somatic embryogenesis whereas cytokinins stimulate cell division and shoot formation (Sauer *et al.*, 2013; Nielsen *et al.*, 2019). The types and concentrations of auxin or cytokinin and the auxin/cytokinin ratio are important factors to be responsible for callus formation and secondary metabolites accumulation (Bhatia, 2015; Daffalla *et al.*, 2019). An intermediate ratio of auxin and cytokinin is needed for the callus formation (Murthy *et al.*, 2014). Besides, different PGRs also affect the biosynthesis pathway in callus and lead to the production of different kinds and amounts of secondary metabolites (Karakas, 2020).

Callus culture can be maintained for long period of time by successive subculture the callus to a fresh medium at intervals. The period of subculturing and the conditions to maintain callus cultures differ from species to species and depends on their regeneration potential and genotype variation (Bhatia, 2015). Subculture periodically is needed to