THE ESTABLISHMENT OF CALLUS AND CELL SUSPENSION CULTURE OF SABAH SNAKE GRASS (*Clinacanthus nutans* (Burm. f.) Lindau)

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by

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LIST OF ABBREVIATIONS

MS	Murashige and Skoog
GI	Growth Index
NAA	1-Naphthaleneacetic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
rpm	Rotation per minute
IC_{50}	50% growth cell proliferation inhibitory concentration
ATCC	American Type Culture Collection
MTT	30(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ANOVA	Analysis of variance
BAP	6-Benzylaminopurine
TDZ	Thidiazuron
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
CRD	Complete randomized design
IAA	Indole-3-acetic acid
TLC	Thin layer chromatography
v/v	Volume per volume
w/v	Weight per volume
R_f	Retention factor
NCED	9-cis epoxycarotenoid dioxygenase
ABA	Abscisic acid

PEMBANGUNAN KULTUR KALUS DAN AMPAIAN SEL BELALAI GAJAH (*Clinacanthus nutans* (Burm. f.) Lindau)

ABSTRAK

Clinacanthus nutans (Burm. f.) Lindau ialah sejenis tumbuhan ubatan dari famili Acanthaceae. Ia digunakan secara meluas untuk merawat radang kulit, luka kulit, jangkitan virus dan gigitan ular. Baru-baru ini, pesakit-pesakit kanser melaporkan penggunaan ekstrak daun *C. nutans* dapat menyekat kemajuan kanser dan memanjangkan hayat pesakit kanser pada peringkat kritikal. Pengenalpastian catechin, luteolin, kaempferol dan quercetin dalam *Clinacanthus nutans* ekstrak dikaitkan dengan kecekapan tumbuhan ini sebagai rawatan sampingan untuk rawatan dan pencegahan kanser. Banyak kajian telah membuktikan keupayaan biokompaun ini untuk menyekat pertumbuhan sel kanser. Kajian ini dijalankan bertujuan untuk menginduksi penghasilan biojisim kalus yang rapuh bagi penubuhan kultur ampaian sel dengan tujuan untuk memperoleh catechin, luteolin, kaempferol and quercetin daripada metabolit sekunder *Clinacanthus nutans*. Eksplan daun muda telah dikumpulkan dari sumber-sumber yang berbeza (pokok luar dan pokok "*in vitro*") untuk menentukan eksplan yang paling sesuai untuk penginduksian kalus. Eksplan daun kemudiannya dikulturkan di atas medium MS dengan kombinasi auksin (2,4-D dan NAA) dan sitokinin (Kinetin) yang berbeza untuk menentukan medium induksi kalus. Dua parameter (orientasi eksplan dan pencahayaan) telah dikaji untuk menentukan keadaan kultur yang sesuai bagi penghasilan kalus rapuh. Medium proliferasi kalus telah diubah untuk meningkatkan biojisim kalus rapuh dengan menggunakan pelbagai jenis ejen jel (agarose, gelrite dan agar tumbuhan). Hormon tumbuhan (2,4-D, NAA, Picloram, Kinetin dan BAP) yang berbeza telah ditambah ke

dalam medium MS cecair bagi penubuhan kultur ampaian sel. Tiga jenis sel turunan dari kultur ampaian, kalus dan pokok luar digunakan dalam pengekstrakan etil asetat dan analisis melalui kajian kromatografi lapisan nipis (TLC) untuk menilai kewujudan sebatian bioaktif yang disasarkan. Eksplan daun diperolehi dari pokok luar yang dikulturkan di atas medium MS yang diperkaya dengan 0.25mg/L 2,4-D dan 0.75mg/L Kinetin memperolehi biojisim kalus rapuh yang tertinggi pada nilai 0.896g selepas 8 minggu. Daripada semua parameter yang diuji, penghasilan biojisim kalus telah meningkat dengan ketara apabila eksplan daun diletakkan di permukaan adaksial pada media dan dikulturkan dalam keadaan bercahaya. Kajian ini menunjukkan bahawa medium proliferasi yang menggunakan agarose telah meningkatkan penghasilan kalus dengan ketara. Medium cecair diperkaya dengan 0.25mg/L 2,4-D dan 0.50mg/L NAA memperolehi biojisim kalus yang tinggi pada nilai 1.760g. Kajian kromatografi lapisan nipis dikenalpasti quercetin, catechin dan luteolin dalam kalus dan tiga sel turunan ampaian. Walau bagaimanapun, hanya quercetin telah dikenal pasti dalam daun dan batang *Clinacanthus nutans*. Kajian ini telah berjaya menubuhkan kultur ampaian sel dengan menggunakan eksplan daun dan juga memperolehi sebatian bioaktif disasarkan yang berkaitan dengan rawatan kanser. Kuantifikasi quercetin, catechin dan luteolin dan ujian ke atas sel turunan kanser akan dijalankan pada masa akan datang.

THE ESTABLISHMENT OF CALLUS AND CELL SUSPENSION CULTURE OF SABAH SNAKE GRASS (*Clinacanthus nutans* (Burm. f.) Lindau)

ABSTRACT

Clinacanthus nutans (Burm. f.) Lindau is a well-known medicinal plant belonging to the family Acanthaceae. It is widely used as a traditional medicine for skin inflammation, skin lesions, viral infection and snake bites. The consumption of fresh leaves of *C. nutans* was recently reported by cancer patients having the ability to suppress the advancement of cancer and able to prolong life of cancer patients at critical stages. The identification of catechin, luteolin, kaempferol and quercetin in the *Clinacanthus nutans* extracts was linked to the efficiency of this plant as side treatment for cancer treatment and prevention. Various studies have indicated the ability of these compounds in suppressing the cancer cell growth. The current study was carried out to induce the production of friable callus biomass for the establishment of cell suspension culture with the purpose to harness catechin, luteolin, kaempferol and quercetin in the secondary metabolites of *Clinacanthus nutans*. Young leaf explants were collected from different sources (outdoor plant and *in vitro* plantlets) to determine a suitable explant for callus induction. The leaf explants were then cultured on MS medium with different combinations of auxins (2,4-D and NAA) and cytokinin (Kinetin) to determine a suitable callus induction medium. Two parameters (orientation of explants and light illumination) were investigated to identify culture conditions in friable callus induction. Callus proliferation medium was also formulated to enhance friable callus biomass using different types of gelling agents (agarose, gelrite and plant agar). Different types of plant growth regulators (2,4-D, NAA, Picloram, Kinetin and BAP) with different concentrations and combinations were

supplemented into the liquid MS medium for the establishment of the cell suspension culture. Three cell lines of the suspension culture, callus and outdoor explants were subjected to ethyl acetate extraction and analysed through thin layer chromatography (TLC) to evaluate the presence of the targeted bioactive compounds. In this study, the leaf explants obtained from outdoor plant cultured on MS medium supplemented with 0.25mg/L 2,4-D and 0.75mg/L Kinetin induced the highest friable callus biomass at the value of 0.896g after 8 weeks of culture. Of all the parameters tested, callus production was significantly increased when leaf explants were placed on the adaxial surface on the medium and cultured under light condition. Results also indicated that solid proliferation medium with agarose significantly increased the callus formation. Liquid medium supplemented with 0.25mg/L 2,4-D and 0.50mg/L NAA recorded a significantly higher callus biomass at the value of 1.760g. TLC analysis identified quercetin, catechin and luteolin in the callus and the three cell lines of the suspension culture. However, only quercetin was identified in the leaves and stem of *Clinacanthus nutans*. The current study has successfully established the cell suspension culture of *Clinacanthus nutans* using leaf explants and also identified the targeted bioactive compounds linked to cancer treatment. Future work will involve further quantification of quercetin, catechin and luteolin and tests on cancer cell lines.

CHAPTER 1

INTRODUCTION

Cancer is defined as the advancement and continuous uncontrolled growth of cells in the mammalian body. Malignant tumor is able to spread and invade the surrounding cells resulting in death. According to the National Cancer Registry of Malaysia (NCR), one out of four Malaysian citizens is threated with cancer during advanced age and the number of cancer patients increases from year to year in Malaysia (Ariffin and Saleha, 2011). There are many methods to treat cancer which includes surgery, radiation therapy, chemotherapy and immunotherpy. In recent years, the use of herbal medicine is increasing in popularity around the world for health maintenance, disease prevention and treatment. Medicinal plants have long been used in traditional medicine particularly in developing countries to meet their health care needs. Plants are rich in a variety of compounds. Plants secondary metabolites containing terpenoids, alkaloids and flavonoids have been proven efficient in preventing occurrence of oxidative stress associated diseases such as cancer due to its potent antioxidant properties (Reddy *et al*., 2003; Lam, 2007). Some plants were found to have novel chemical constituent such as topotecan, irinotecan (CPT-11) and belotecan from *Camptotheca acuminata*, dronabinol and cannabidiol from *Cannabis sativa* and nitisinone from *Callistemon citrinus*.

Clinacanthus nutans (Burm.f.) Lindau, also known as the Sabah Snake Grass, is a herbal plant belonging to the Acanthaceae family. This plant is a well-known medicinal plant in Asia and is being utilized for traditional treatments of snake bites, rashes and inflammation (Yoosook *et al*., 1999; Wanikiat *et al*., 2008; Sakdarat *et al*., 2009; Vachirayonstien *et al*., 2010; Kongkaew and Chaiyakunapruk, 2011;

Maneenoon *et al*., 2015). Recently, the Malaysia government identified eleven nationally important herbs and *C. nutans* is listed in the National Key Economic Areas (NKEAs) proprietary list due to its medicinal properties (Narayanaswamy and Ismail, 2015). *C. nutans* leaves have long been used in herpes simplex treatment in Thailand and cream made from *C. nutans* extracts was shown to be as efficacious as Acyclovir in healing herpes simplex and herpex zoster (Jayavasu *et al*., 1992; Sangkitporn *et al*., 1995; Charuwichitratana *et al*., 1996; Vachirayonstien *et al*., 2010; Kunsorn *et al*., 2013). Reports from Gan *et al.* (2015) and Farooqui *et al.* (2015) showed that *C. nutans* is the most common herb being used in complementary and alternative medicine for cancer patients in Malaysia. In Malaysia and Singapore, leaves of *C. nutans* are usually juiced with a green apple to mask the unpleasant grassy smell and consumed by cancer patients as a side treatment (Siew *et al*., 2014). Many testimonials from cancer patients have reported the efficiency of *C. nutans* extracts in suppressing advancement of cancer and prolonging life of cancer patients at critical stages. Various reports have proved the efficiency *C. nutans* extracts in suppressing growth of selected cancer cell lines (Yong *et al*., 2013; Arullappan *et al*., 2014; Ghani *et al*., 2015; Sulaiman *et al*., 2015). These reports indicated that the leaf extracts of *C. nutans* could potentially be used as an alternative side treatment for cancer and inhibit the recurrence of cancer.

Cell suspension culture is a potential alternative method for the mass industrial production of secondary metabolites in plants (Dicosmo and Misawa, 1995). In recent years, biotechnology companies incorporated cell suspension culture techniques for the production of pharmaceutical, cosmetic and food related secondary products as a cheaper alternative in extracting metabolites rather than chemical synthesis. Wilson and Roberts (2012) reported that the plant cell culture system is more beneficial because it is not limited by the low production yield from natural harvest or the high

cost in complex chemical synthesis. The production of plant secondary metabolites via natural harvesting may pose some difficulties due to many biological and non-biological limitations such as seasonality, geographical, diseases issue and soil issue (Mulabagal and Tsay, 2004; Wilson and Roberts, 2012). Cell suspension culture could provide an alternative approach to produce useful secondary metabolites under controlled conditions, independent of climate and geographical factors. The totipotent nature of plant cells enables rapid multiplication of true-to-type clones which have similar genetic information from the mother cells (Verdeil *et al*., 2007). Different strategies such as manipulation of nutrient content and culture conditions can be used to enhance the synthesis of secondary metabolites enabling continuous and reliable production of plant pharmaceuticals at large-scale (Karwasara and Dixit, 2013).

The current study aims to establish cell suspension culture of *C. nutans* for the purpose of harnessing essential secondary metabolites related to cancer prevention and treatment. This investigation is also an attempt to evaluate if the cell suspension culture system is a more efficient alternative in the production of metabolites utilized in treatment of cancer. The objectives of this study are:

1. To determine a suitable callus induction and proliferation medium for *C. nutans*
2. To establish cell suspension culture of *C. nutans*
3. To identify targeted compounds (kaempferol, quercetin, catechin and luteolin) using Thin Layer Chromatography (TLC)

CHAPTER 2

LITERATURE REVIEW

2.1 *Clinacanthus nutans*

2.1.1 Taxonomy and morphology

C. nutans belongs to the family of Acanthaceae. There are 250 genera and 2500 species in the family Acanthaceae, which are mostly herbs or shrub. *C. nutans* is a shrub that can grow up to 1 to 3 m high and is widely distributed throughout the tropical regions including China and Southeast Asia. The leaves are simple, opposite and narrowly elliptic-oblong or lanceolate, while the flowers are dull red in colour and has green base on lower lip with yellow streaks that located on the top of branches with two stamens on the flower inserted in the throat (Kunsorn *et al*., 2013; Fong *et al*., 2014). Each flower has 1-centimeter long of calyx glandular-pubescent and 3.5 centimeter corolla glandular-pubescent. The acute apex green leaves have a cuneate-rounded base and dentate margin ("tooth-like" edges) (Figure 2.1). There are 6-7 pairs of side veins on each side of midvein and convex on both abaxial and adaxial surface of a leaf when dry. The capsule of *C. nutans* is oblong, basally contracted into a short, solid stalk with four seeds.

According to Kunsorn *et al.* (2013), the stomata of *C. nutans* is known as diacytic type (two subsidiary cells accompany the stomata of the leaves at the right angle) and these stomata is located at the lower epidermis of leaves together with lithocysts and glandular trichomes. The cross section of *C. nutans* stems indicated that xylem vessels in the group of xylem fibers interposed longitudinally with parenchyma ray in the central portion of parenchyma, while the phloem tissue is laid semi circularly in the parenchyma central portion (Kunsorn *et al*., 2013).

Figure 2.1: An image of the *C. nutans* leaves

2.1.2 Origin and traditional uses

C. nutans of family Acanthaceae is an important medicinal herb in Malaysia, China and Thailand. This plant is locally known as "sabah snake grass" and "belalai gajah" in Malaysia because this plant is found in Sabah of East Malaysia and the slightly curved stem looks like the curve of an elephant's trunk (Yahaya *et al*., 2015). Fresh leaves of *C. nutans* have long been used for traditional treatments in Asia. The leaves are usually juiced or consumed as raw vegetable in Thailand and the pounded leaves have been applied on the affected site to treat skin rashes and insect bites (Makhija and Khamar, 2010). *C. nutans* is also traditionally used for viral treatment of herpes simplex (HSV) and herpes zoster. The decoction of leaves is applied on the affected part to treat herpes infection (Kongkaew and Chaiyakunpruk, 2011). Various reports had proven the efficiency of *C. nutans* leaves extract in suppressing HSV-1 and HSV-2 infection (Yoosook *et al*., 1999; Vachirayonstien *et al*., 2010; Kunsorn *et al*., 2013; Pongmuangmul *et al*., 2016).

There are many herbal-based products in the form of tablets, capsules, herbal tea and concentrated plant extracts which are available in the market (Shim *et al*., 2013). *C. nutans* in the form of herbal tea have been used in Thailand and Indonesia to treat dysuria, dysentery, fever, diabetes mellitus and diarrhea (Uawonggul *et al*., 2011). Recently, *C. nutans* has gained high popularity and have widely been used as remedies for various health ailments due to its high medicinal value in suppressing the advancement of cancer. In addition, various scientific reports had also proven the extract of *C. nutans* contain antioxidant and antiproliferative properties when tested with cancer cell lines (Yong *et al*., 2013; Arullappan *et al*., 2014; Ghani *et al*., 2015; Sulaiman *et al*., 2015). This data suggested that the *C. nutans* leaf extract could be applied as an complementary treatment for cancer patients.

2.1.3 Composition and uses

C. nutans contains stigmasterol, botulin, lupeol and β-sitosterol. These chemical compounds were isolated from light petroleum extracts of the stems, roots and leaves of *C. nutans* (Dampawan, 1976; Dampawan *et al*., 1977; Lin *et al*., 1983). Lupeol, β-sitosterol and terpenoids flavonoids present in *C. nutans* possess anti-bacterial activity (Yang *et al*., 2013).

Six known C-glucosylfavones (shaftoside, orientin, isoorientin, vitexin, isovitexin and isomollupentin-7-O-β-glucopyranoside) were successfully extracted using advance chemical techniques and instruments from methanol extract of leaves and stems of *C. nutans* (Teshima *et al*., 1997). In a current study, high performance thin-layer chromatography (HPTLC) and high performance liquid chromatography with photodiode array detector (HPLC-UV/DAD) have been used to determine and quantify flavones C-glycosides in the *C. nutans* leaves (Chelyn *et al*., 2014). Teshima *et al*. (1998) has also successfully isolated five sulfur-containing glucosides, which are cycloclinacoside A1, cycloclinacoside A2, clinacoside A, clinacoside B and clinacoside C from butanol- and water- soluble portions of methanol extract of leaves and stems of *C. nutans*. Glucosides present in *C. nutans* was found to contributed to its anti-inflammatory properties (Melzig *et al*., 2001).

Satakhun (2001) discovered that two glycoglycerolipids (1-O-palmitoyl-2-O-linolenoyl-3-O-[α – D – galactopyranosyl – (1’ → 6’) – O – β – D – galactopyranosyl] glycerol and 1,2 – O- dilinolenoyl – 3 – O – β – D – galactopyranosylglycerol) have been isolated from leaves of *C. nutans*. These two compounds were found to contain anti-herpes simplex activity against HSV-1 and HSV-2 infection (Suwanborirux *et al*., 2003; Sakdarat *et al*., 2006; Yang *et al*., 2013). Dechatiwongse na Ayudhya and his

co-workers (2001) has successfully isolated 13^2 – hydroxyl – (13^2 – S) – phaeophytin b, purpurin 18 phytylester and phaeophorbide from chloroform extract of *C. nutans* leaves. A mixture of nine cerebrosides and monoacylmonogalactosylglycerol has also been isolated from *C. nutans* leaves (Tuntiwachwuttikul *et al*., 2004). Study from Ohnishi and Kinoshita (2004) indicated that plant cerebroside-derived compounds have the ability to induce apoptosis against human colon cancer cell line.

On the other hand, Sakdarat *et al.* (2006) discovered compounds that were related to chlorophyll a and chlorophyll b; 13^2-hydroxy (13^2-R)-chlorophyll b, 13^2-hydroxy-(132-S)-chlorophyll b, 13^2-hydroxy-(13^2-R)-phaeophytin b, 13^2-hydroxy-(13^2-S)-phaeophytin b, 13^2-hydroxy-(13^2-R)-phaeophytin a, 132-hydroxy-(132-S)-phaeophytin a, phaeophorbide a and purpurin 18 phytyl ester. Compounds such as 13^2–hydroxyl–(13^2–R)–phaeophytin b, 13^2–hydroxyl (13^2–S) – phaeophytin a and 13^2–hydroxyl–(13^2–R)–phaeophytin from chloroform extraction were reported to possess antiherpes simplex activity through pre-infection inhibition and herpes virus inactivation (Sakdarat *et al*., 2009; Vachirayonstien *et al*., 2010). Moreover, diglyceride and digalactosyldiglyceride from *C. nutans* were also found to exhibit significant anti-herpes simplex activity without cytotoxic effect (Pongmuangmul *et al*., 2016).

Ghasemzadeh *et al*. (2014) had successfully isolated catechin, quercetin, kaempferol and luteolin from methanolic extract of *C. nutans* leaves. Catechin is a natural antioxidant that has been reported to be protective against coronary heart disease, stroke and neurodegenerative diseases (Alzheimer's disease and Parkinson's disease) (Arts *et al*., 2001; Velayutham *et al*., 2008; Mak, 2012). This compound was also found to modulate the immune system through activation of signal transduction pathway involved in metastasis, cell proliferation, inflammation and transformation,

which promote angiogenesis (Na and Surh, 2006; Huang *et al*., 2008; Hu *et al*., 2010). Many studies concluded that catechin is a chemopreventive agent that have the ability to inhibit cancer cell growth and induce cancer cell apoptosis (Park *et al*., 2009; Lee *et al*., 2010; Roy *et al*., 2009; Connors *et al*., 2012; Hessien *et al*., 2012). Report from Butt *et al.* (2015) had also proven the efficiency of catechin in suppressing the advancement of various cancer cells including breast, colon, skin, prostate and lung cancer cell lines.

Kaempferol is a flavonoid compound found abundantly in a variety of vegetables and fruits. This compound was found to have the ability to modulate the key elements in cellular signal transduction pathways linked to metastasis, inflammation, apoptosis and angiogenesis at the molecular level (Ramos, 2007). Various studies suggested the consumption of kaempferol may suppress the growth of cancer cells by inducing cancer apoptosis, cell cycle arrest and inhibition of tyrosine phosphorylation (Lee *et al*., 1998; Sharma *et al*., 2007; Cho *et al.*, 2013). This compound has been proven successful in the studies on the suppression of non-small cell lung cancer cells (Hang *et al*., 2015; Jo *et al*., 2015; Kuo *et al*., 2015).

Luteolin is a flavonoid compound that can be found in many plants including fruits, vegetables, and medicinal herbs. Plants rich in luteolin are traditionally used to treat hypertension, inflammatory disorders and cancer (Harborne and Williams, 2000). Luteolin have been proven to exert its anticancer properties by inducing cancer cell apoptosis and inhibit cancer cell proliferation (Cai *et al*., 2012). Report from Lin *et al*. (2009) indicated that luteolin also has the potential in sensitizing cancer cells to therapeutic-induced cytotoxicity by stimulating the apoptosis pathway, indicating its potential use as an anticancer agent.

Quercetin is a common flavonoid in plants, mostly are in fruits and vegetables. Investigation by Lee *et al.* (2015) suggested that quercetin could be used as a chemosensitizer in cancer treatment to increase the sensitivity of cancer cells to chemotherapy. Many studies had also proven the efficiency of quercetin in inhibiting cancer cell growth and promoting angiogenesis in various cancer cells including pancreatic tumor, prostate cancer, breast cancer, leukemic cells, hepatoma cells and gastric carcinoma cells (Seufi *et al*., 2009; Vidya *et al*., 2010; Gibellini *et al*., 2011). These four compounds (catechin, kaempferol, luteolin and quercetin) isolated from methanolic extract of *C. nutans* leaves may have the potential to exhibit anti-cancer properties that has links to cancer prevention and treatment.

Khoo *et al.* (2015) carried out a metabolite profile of *C. nutans* leaves and stems using Nuclear Magnetic Resonance (NMR). They successfully isolated a few new compounds, namely gendarucin A, a gendarucin A isomer, ascorbic acid, 3,3-di-O-methylellagic acid and two isomeric oxoprolinates. These compounds were found to contribute to the anti-inflammatory effects of *C. nutans* (Mai *et al*., 2016). Another experiment performed by Huang *et al.* (2016) had successfully isolated polysaccharide–peptide complex CNP-1-2 from *C. nutans* using hot water extraction. This compound was found to exhibit anti-proliferative effects against human gastric cancer cells with the inhibition ratio of 92.34% (Huang *et al*., 2016).

2.1.4 Medicinal properties

2.1.4 (a) Antiviral activity

2.1.4 (a)(i) Anti-Herpes Simplex Virus (HSV)

C. nutans extracts were found to exhibit anti-viral activity against HSV-2 (Yoosook *et al*., 1999). From this study, HSV-2 yield drastically reduced with time when treated with *C. nutans* extracts. Acyclovir is a drug that is normally used to treat HSV infection, being used as positive control in this experiment. It was found that the plaque size formed in the culture well was smaller when treated with *C. nutans* extract in comparison to the control (Yoosook *et al.*, 1999). This study indicated that *C. nutans* extracts can potentially reduce virus replication within original infected cell and prevent the infected cell from continuously spreading.

The virucidal activities of *C. nutans* extracts have the ability to inactivate intracellular and extracellualar activity of HSV-2 and inhibit pre-infection (Yoosook *et al*., 1999; Sakdarat *et al*., 2009; Vachirayonstien *et al*., 2010). Galactosyl diglycerides, synthetic monoglycosyl diglycerides, glycoside, polyphenolic and terpenes were the important constituents which were reported effective in the inhibition of HSV virus (Satakhun, 2001; Janwitayanuchit *et al*., 2003; Lertsupwichit, 2003; Tuntiwachwuttikul *et al*., 2004). Janwitayanuchit *et al*. (2003) performed a study to examine 19 different types of monoglycosyldiglycerides from *C. nutans* leaves for its inhibitory effect on HSV virus. They identified 1,2-O-dilinolenoyl-3-O-b-d-glucopyranosyl-sn-glycerol having the highest inhibitory activity against HSV-1 and HSV-2.

On the other hand, Vachirayonstien *et al*. (2010) carried out a study to examine the inhibitory effect of *C. nutans* extracts on HSV-2 on the molecular level. The viral

protein and DNA quantities were determined after treatment with *C. nutans* extracts to examine extracellular inhibitory effect of the *C. nutans* extracts against HSV-2. From this study, late structural HSV-2 antigens were almost depleted. This indicated that *C. nutans* extract had successfully inactivated HSV-2 extracellularly in virucidal assay (Vachirayonstien *et al*., 2010).

Kunsorn *et al.* (2013) carried out an experiment to determine the efficiency of *C. nutans* extracts against HSV-1 and HSV-2 using plaque reduction assay. This study identified n-hexane, dichloromethane and methanol extracts isolated from the dried leaves of *C. nutans* exhibited inhibitory effects on HSV-1 and HSV-2 plaque formation. Pongmuangmul *et al.* (2016) also recorded 100% inhibition of HSV-1 replication against post-treatment of monogalactosyl diglyceride and digalactosyl diglyceride from *C. nutans* leaves. This study reported the similar anti-herpes simplex activity as standard anti-herpes synthetic compounds with IC_{50} value of 36.00 and 40.00 mg/mL and 41.00 and 43.20 mg/mL against HSV-1 and HSV-2 respectively. Monogalactosyl diglyceride and digalactosyl diglyceride from *C. nutans* successfully inhibited the viral multiplication at the late stages without causing significant cytototic effects. This investigation reported the efficiency of the extract of *C. nutans* being used as a treatment of HSV infections.

2.1.4 (a)(ii) Anti Varicella-Zoster Virus

Varicella-zoster virus (VZV) causes two types of diseases, known as shingles (herpes zoster) and chickenpox (varicella) that infect humans. Thawaranantha *et al*. (1992) carried out an experiment to determine *C. nutans* extracts against varicella-zoster virus (VZV) at three different stages: pre-treatment (applied on the cells before infection

with VZV), post-treatment (applied on the virus infected cells) and inactivation assay (applied directly on the VZV). This study reported that *C. nutans* extracts were effective in pre-treatment, post-treatment and inactivation assay, differing in the degrees of inhibition. Results showed that antiviral activities were more effective in the inactivation assay in comparison to pre-treatment and post-treatment. This study indicated the direct interaction of the *C. nutans* extract on varicella-zoster virus having significant inhibition on virus replication in the host cells (Thawaranantha *et al*., 1992).

2.1.4 (b) Antioxidant activity

Reactivated oxygen or nitrogen species (RONS) could lead to cancer initiation and progression (Wiseman and Halliwell, 1996; Kamiya, 2003; Shi *et al*., 2012). Halliwell (2012) discovered that cancer initiation and progression are normally caused by imbalances in cellular redox homeostasis with elevated free radical production. In fact, normal cells generate RONs as metabolic by-products. However, it is necessary for the cells to maintain RON levels below the cytotoxic level. Uncontrolled endogenous antioxidant capacity can cause damage to the lipid, DNA and cellular protein, leading to genomic instability. This leads from rapid progression into tumor development and promotion of metastasis (Halliwell, 1999; Xu *et al*., 2002; Klaunig and Kamendulis, 2004).

Reactivated RONs were found to cause oncogene activation and mitochondrial malfunction when at excessive cellular RONS levels (Valko *et al*., 2006). Antioxidants have the ability to salvage cells from oxidative stresses, preventing cancer initiation and progression (Inci *et al*., 2003; Storz, 2005). Arullappan *et al.* (2014) carried out an experiment to determine antioxidant activity of *C. nutans* extracts using 1, 1-diphenyl-

2-picrylhydrazyl (DPPH) assay. Petroleum ether extract of *C. nutans* leaves were found to exhibit the highest radical scavenging activity of 82.0% at the concentration of 4.0 mg/ml in comparison to α-tocopherol and ascorbic acid with the scavenging activities of 86.6% and 88.7% respectively.

Pannangpetch *et al*. (2007) also performed an experiment to determine ferric reducing antioxidant power (FRAP), free radical (1,1-diphenyl-2-picrylhydrazyl; DPPH), intracellularly antioxidant activity as well as the protective effect of the *C. nutans* extracts against 2,2'-azobis (2-amidinopropane) hydrochloride (AAPH)-induced rat red blood cell lysis. They reported that the ethanolic extracts of *C. nutans* have protective effects against oxidative hemolysis. The results obtained proved that *C. nutans* extracts have the potential to scavenge DPPH with a maximum scavange activity of 67.65± 6.59% and protect red blood cells from being haemolysed (Pannangpetch *et al*., 2007).

Ghasemzadeh *et al*. (2014) reported on the free radicals (1,1-diphenyl-2-picrylhydrazyl; DPPH) scavenging activity of *C. nutans* extract at different plant growth stages. This study reported that 1-year-old buds showed the highest scavenging value of 64.6µg/mL indicating that the extracts of *C. nutans* can be used as an antioxidant substance to salvage cells from oxidative stress. Wong *et al.* (2014) investigated the antioxidant properties of six tropical medicinal plants, namely *Clinacanthus nutans, Callicarpa formosana, Hedyotis diffusa, Vernonia amygdalina, Leonurus cardiac* and *Pereskia bleo* and indicated *C. nutans* as having the strongest antioxidation potential with high metal chelating and radical scavenging activities compared to other medicinal plants at the concentration of 10mg/ml.

Sulaiman *et al.* (2015) reported the efficiency of *C. nutans* extracts against oxygen radical absorbance capacity, 1,1-diphenyl-picrylhydrazyl (DPPH) radical scavenging activity and ß-carotene bleaching activity assays using different types of solvents. Results from this study proved that ethanol and ethyl acetate extracts of *C.s nutans* had strong antioxidant activity and cytotoxicity against breast cancer cells due to the high amount of phenolic compounds in the extracts. Sarega *et al.* (2016) performed an experiment to determine antioxidant activity of *C. nutans* extracts and its efficiency against hypercholesterolemia-induced oxidative stress using an animal model. They discovered that the aqueous extract and aqueous methanol extract of *C. nutans* leaves significantly reduced the hyperlipidemia-induced oxidative stress in rats by activating the hepatic antioxidant genes expression and increased the antioxidant enzymes activity.

2.1.4 (c) Anti-inflammatory activity

Neutrophils appear most abundantly in white blood cells of mammals which are responsible to the innate immune system. Neutrophils will attach to trauma sites when injury occurs to trigger inflammatory response releasing superoxide anions and tissue destructive enzymes (myeloperoxidase and elastase) during these processes (Fujie *et al*., 1999). Neutrophil elastase induces inflammation in chronic inflammatory processes and inhibit healing. Neutrophil extracellular trap (NET) which are nets of fibers will also be released to capture and kill microbe outside the cells.

Wanikiat *et al*. (2008) carried out an experiment to investigate the anti-inflammatory activities of *C. nutans* extracts against the models of carrageenan-induced paw oedema and EPP-induced ear oedema in rats. Results obtained indicated

that the *C. nutans* leaf extracts (0.1–100 μg/ml) had successfully inhibit the human neutrophil elastase release induced by fMLP (N-formyl-methionyl-leucyl-phenylalanine). This study reported the efficiency of the *C.nutans* extracts being used as a treatment for inflammation (Wanikiat *et al.*, 2008). On the other hand, Mai *et al.* (2016) also performed an experiment to study mechanisms underlying the anti-inflammatory effects of *C. nutans* using lipopolysaccharide-induced inflammation macrophage model. *C. nutans* extract successfully inhibit Toll Like Receptor-4 (TLR-4) activation as well as suppressing the production of nitric oxide (NO) and inflammatory cytokine TNF-α, IFN-γ, IL-1β, IL-1, IL-6 and IL-12p40. The extract was also found to reduce the phosphorylation of p38, p65, ERK1/2, JNK1/2 and IRF3, leading to TLR-4 inflammatory protein inhibition, preventing the NF-κB activation, which is a hallmark for inflammation, contributing to many inflammation-related chronic diseases, including systemic inflammatory response syndrome and inflammatory bowel disease.

2.1.4 (d) Anti-venom activity

C. nutans fresh leaves have been used traditionally in Thailand, Vietnam and Malaysia to treat scorpion and snake bite. The *C. nutans* leaves extracts being applied externally on the wound for one week to neutralize snake venoms (Makhija and Khamar, 2010). Regardless the extract of *C. nutans* being so commonly used by traditional healers to treat snake bites, research has proven that the extract was not able to inhibit neuromuscular transmission when tested on the *Naja naja siamensis* neurotoxin (Cherdchu *et al.*, 1977). This study has indicated that the *C. nutans* extracts cannot being used as a treatment to antagonize the cobra venom.

On the other hand, Uawonggul *et al*. (2006) studied the anti-venom activities of 64 plant extracts against scorpion venom activity on fibroblast cell lysis. The results obtained indicated that *C. nutans* extracts have the ability to neutralize scorpion venom with an efficiency of 46.51%, indicating that the extracts can be used as an anti-scorpion venom to prevent the cells from lysis.

2.1.4 (e) Anti-dengue Virus Type 2 infection

The *Aedes aegypti* mosquito is the primary vector for dengue virus. There are four antigenically distinct serotypes namely DENV-1, DENV-2, DENV-3 and DENV-4 where multiple genotypes are enclosed in each serotype (Holmes and Burch, 2000; Holmes and Twiddy, 2003; Salazar *et al.*, 2007). Sittiso *et al*. (2010) reported that hexane and chloroform extracts of *C. nutans* leaves significantly inhibited DENV-2 from spreading by preventing the synthesis of viral protein and RNA. Tu *et al.* (2014) also performed an experiment to investigate the efficiency of ethanolic extract of *C. nutans* against dengue virus by treated DENV-2 infected cell with *C. nutans* extracts for 72 hours. They discovered that the extract of *C. nutans* exhibit significant anti-dengue virus activity with IC_{50} value of 31.04 μg/mL, indicating its potential use in the dengue treatment.

2.1.4 (f) Anti-proliferative activity

Yong *et al.* (2013) performed an experiment to investigate the anti-proliferative properties of *C. nutans* extracts against selected cancer cell lines. They reported that chloroform extracts of *C. nutans* leaves can significantly inhibit cell proliferation of

cancer cells such as SNU-1, LS-174T, IMR32, HepG2, NCL-H23, HeLa, K562 and Raji cell lines. In this study, the cytotoxicity of *C. nutans* extracts was also tested on normal human umbilical vein (HUVECs). From the results obtained, chloroform extracts of *C. nutans* do not exert significant cytotoxic effects as anti-proliferative assays were performed on cancer cell lines.

Arullappan *et al.* (2014) performed a study to determine the cytotoxic effects of *C. nutans* extract agaist K-562 and HeLa cancer cells and discovered that non-polar solvent (petroleum ether) showed the greatest cytotoxic effect compared to methanol and ethyl acetate extracts. Petroleum ether extracts of *C. nutans* leaves exhibited the strongest cytotoxic activity against K-562 and HeLa cells with the values of IC_{50} of 20.0 and 18.0µg/mL respectively after 72-hours incubation. However, from the results obtained by Sulaiman *et al.* (2015), IC_{50} of 24.04 and 28.90 were recorded when ethyl acetate and ethanol extracts of *C. nutans* were treated on breast cancer estrogen positive (MCF-7) cell. Ghasemzadeh *et al*. (2014) also showed that *C. nutans* from 6-months-old buds had effectively inhibit HeLa cell proliferation with IC_{50} value of 56.8 µg/mL. On the other hand, *C. nutans* extracts was also found to possess strong cytotoxic activity against human lung adenocarcinoma cell line compared to *Piper sarmentosum* and *Ficus auriculata* with an IC_{50} value of 20 µg/mL (Ghani *et al*., 2015).

Huang *et al.* (2015) carried out studies on the antitumor and immunomodulatory activity of *C. nutans*. They indicated that the ethanolic extracts of *C. nutans* had potential antitumor activity on HepA tumor cells through up-regulation of the immune response efficacy of *C. nutans*. The extracts of *C. nutans* had successfully suppress the proliferation of cell nuclear antigen (PCNA) expression while increasing the immune cytokine levels and infiltration of $CD8^+$ T cells to HepA tumor cells. The increase of immune cytokine (IFN-γ and interleukin-2) levels in the

serum boost the immune response which successfully inhibited the proliferation of tumor cells. Vajrabhaya and Korsuwannawong (2016) studied the cytotoxicity of *C. nutans* using dimethylthiazol diphenyltetrazolium bromide (MTT) and neutral red uptake (NRU) assays and discovered that the extracts of *C. nutans* were not cytotoxic against mouse fibroblast L929 cells compared to polyvinyl chloride (positive control) which exhibited highest toxicity. These studies indicated that *C. nutans* extract were not cytotoxic and is a potential yet suitable plant to be used as a side treatment for cancer.

2.1.4 (g) Anti-microbial activity

The extract of *C. nutans* was also found to possess anti-microbial activities. Goonasakaran (2013) reported that chloroform extracts of *C. nutans* exhibited a larger inhibition zone against *Salmonella enterica* serovar Paratyphi B compared to absolute ethanol, 70% ethanol and aqueous extractions. This study indicated that the *Clincanthus nutans* extracts were also effective in forming the inhibition zone against all the five strains of Salmonella, which are *Salmonella enterica* serovar Paratyphi B, *Salmonella enterica* serovar Paratyphi C, *Salmonella enterica* serovar Typhi ATCC, *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Weltevreden. Moreover, Ho *et al*. (2013) carried out an experiment to investigate the antibacterial activities of methanolic extract of *C. nutans* leaves against five selected bacteria, namely *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Propionibacterium acnes*. They discovered that *C. nutans* extracts exerted significant antibacterial effect against *E. coli* and *S. aureus* with minimum inhibitory concentrations (MIC) of 12.5 mg/ml (Ho *et al*., 2013).

C. nutans has also been reported to have antimicrobial activity against *Bacillus cereus* (ATCC11778), *Candida albicans* (ATCC10231), *Escherichia coli* (ATCC25922) and *Salmonella enterica* Typhimurium (ATCC14028) (Arullappan *et al*., 2014). They reported that the microbial growth decreased as the treatment concentrations of fraction increased when tested on these microbial strains. In this study, anti-microbial activities were tested using ampicillin and amphotericin B as a positive control for antibacterial and antifungal assay respectively. *C. nutans* extract (Fraction 7) showed the strongest antimicrobial activity against *B. cereus* and *C. albicans* with the MIC value of 1.39 mg/ml. Similarly, petroleum ether and acetone extracts of *C. nutans* exhibited antimicrobial activity against *E. coli*, *Salmonella* sp., *Bacillus* sp. and *Streptococcus* sp. with zone of inhibition from 7.00 - 15.66 mm (Chithra *et al*., 2016). These results indicated that the *C. nutans* extracts can inhibit growth of certain microbes and is a potential anti-microbial agent.

2.1.4 (h) Antinociceptive effect

Opioids refers to the mediation used to relieve pain by reducing the pain signals being delivered to the brain. Rahim *et al.* (2016) performed an experiment to determine the antinociceptive effect mechanism of *C. nutans* against animal nociceptive models and reported that the methanolic extract of *C. nutans* leaves were found to exhibit peripheral and central mediated antinociceptive effect via the opioid and nitric oxide-mediated pathways. The extracts of *C. nutans* have the ability to attenuate acetic acid- and formalin- induced nociception preventing peripheral nociceptive neurons from being entering the dorsal horn of central nervous system. The extract was also found to attenuate thermal-induced nociception by suppressing the painful thermal stimulus