CYTOTOXIC, MUTAGENIC AND ORAL PATHOGEN RELATED ANTIMICROBIAL PROPERTIES OF *CLINACANTHUS NUTANS*

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by

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3T3	3-day transfer, inoculum 3×10^5 cells
AMR	Antimicrobial resistance
AST	Antimicrobial susceptibility testing
ATCC	American type culture collection
ATP	Adenosine triphosphate
BA	Blood agar
BHI	Brain heart infusion
CLSI	Clinical and laboratory standards institute
DDMP	2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	diphenyl-1-picrylhydrazyl
DTT	Dithiothreitol
EPP	Entry point projects
EtOAc	Ethyl acetate
ETP	Economic transformation programme
EUCAST	European committee for antimicrobial susceptibility Testing
FRIM	Forest research institute of Malaysia
GC	Gas chromatography
GCMS	Gas chromatography mass spectrometry
Glc-His MRPs	Glucose-histidine Maillard reaction products
GM	Glucose minimal

HEK-Blue^{TM}hTLR-4	Human embryonic kidney cells
HeLa	Henrietta Lacks
HGF	Human gingival fibroblast cell
his	Histidine
HPLC	High performance liquid chromatography
HSV-1	*Herpes simplex* virus-1
HSV-2	*Herpes simplex* virus-2
HUVECs	Human umbilical vein endothelial cells
IC	Inhibitory concentration
IMR-32	Human neuroblastoma cell line
IUPAC	International union of pure and applied chemistry
K-562	Human erythroleukemia line
LC	Liquid chromatography
LS-174T	Human colon adenocarcinoma cell line
MBC	Minimum bactericidal concentration
MCF-7	Michigan cancer foundation-7
MHA	Mueller-Hinton agar
MIC	Minimal inhibitory concentration
MPO	Myeloperoxidase
MS	Mass spectrometry
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium
MTT	3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide

NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide plus hydrogen
NCI-H23	National Cancer Institute, human lung adenocarcinoma
NIH 3T3	National Institutes of Health, 3-day transfer, inoculum 3×10^5 cells
NKEAs	National key economic areas
PMS	Phenazine methosulphate
Raji	Human Burkitt's lymphoma cell line
RAW2647	Macrophage; Abelson murine leukemia virus transformed
R*t*	Retention time
R*v*	Retention volume
SDA	Sabouraud dextrose agar
SNU-1	Human gastric cancer cell line
SOD	Superoxide dismutase
VZV	Varicella-zoster virus
WHO	World health organization
WST-1	Water soluble tetrazolium salt-1
XTT	2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-carboxanilide-2H-tetrazolium

SIFAT-SIFAT SITOTOKSIK, MUTAGENIK DAN PATOGEN ORAL YANG BERKAITAN SIFAT-SIFAT ANTIMIKROB *CLINACANTHUS NUTANS*

ABSTRAK

Clinacanthus nutans (*C. nutans*) atau nama tempatan dikenali sebagai rumput Belalai gajah adalah pokok herba saka yang telah digunakan secara tradisional untuk rawatan pelbagai penyakit di Malaysia. Objektif kajian ini adalah untuk menentukan sebatian fitokimia dalam ekstrak etanol dan ekstrak akueus daun *C. nutans* menggunakan Kromatografi Gas Spektrometer Jisim (GCMS) dan menilai kesan sitotoksik menggunakan ujian MTT pada sel-sel fibroblas gingiva manusia dan untuk menilai kesan mutagen menggunakan ujian Ames serta menentukan sifat-sifat antimikrob dengan menggunakan ujian resapan agar dan ujian kepekatan perencat minima (MIC). Jumlah keseluruhan, 53 sebatian fitokimia telah dikenal pasti dari ekstrak daun *C. nutans*. Sebatian ini diklasifikasikan kepada beberapa kelas sebatian seperti fenol, flavonoid, terpenoid, asid lemak, alkana dan hidrokarbon, ester, keton, furan, sulfur dan sebatian nitrogen. Kajian sitotoksik *in vitro* ekstrak etanol dan ekstrak akueus daun C. nutans dalam pelbagai kepekatan (6,400; 3,200; 1,600; 800; 400; 200; 100; 50 dan 25 μg / ml) pada sel-sel fibroblas gingiva manusia (HGF-1) menggunakan ujian MTT tidak menunjukkan sebarang kesan sitotoksik. Ujian Ames terhadap empat jenis *S.* Typhimurium, TA98, TA100, TA1535 dan TA1537 dirawat dengan ekstrak etanol dan ekstrak akueus daun *C. nutans* tidak menunjukkan sebarang kesan mutagen dalam kehadiran dan tanpa kehadiran campuran sistem pengaktifan metabolik (S9) berasaskan pada bilangan koloni. Kajian antimikrob dengan menggunakan ujian resapan agar menunjukkan bahawa pertumbuhan *Staphylococcus aureus* (*S. aureus*)

dan *Actinomyces viscosus* (*A. viscosus*) direncatkan oleh ekstrak etanol dan ekstrak akueus daun *C. nutans*. Walau bagaimanapun, ekstrak-ekstrak ini tidak menunjukkan sebarang aktiviti antimikrob terhadap *Enterococcus faecalis* (*E. faecalis*), *Porhyromonas gingivalis* (*P. gingivalis*) dan *Candida albicans* (*C. albicans*). Ekstrak etanol daun *C. nutans* menunjukkan aktiviti antimikrob yang lebih baik berbanding ekstrak akueus daun *C. nutans* terhadap *A. viscosus*. Ekstrak etanol daun *C. nutans* menunjukkan nilai MIC sebanyak 102,400 µg/ml terhadap A. viscosus dan 204,800 µg/ml terhadap *S. aureus*. Ekstrak akueus daun *C. nutans* menunjukkan nilai MIC sebanyak 204,800 µg/ml terhadap *A. viscosus* dan *S. aureus*. Hasil kajian semasa menunjukkan bahawa daun *C. nutans* boleh bertindak sebagai sumber berpotensi ejen antimikrob dalam memerangi patogen oral.

CYTOTOXIC, MUTAGENIC AND ORAL PATHOGEN RELATED ANTIMICROBIAL PROPERTIES OF *CLINACANTHUS NUTANS*

ABSTRACT

Clinacanthus nutans (*C. nutans*) locally known as Sabah snake grass is a perennial herb that has been used traditionally in the treatment of various diseases in Malaysia. The objectives of this study were to determine the phytochemical compounds in the ethanol and aqueous extracts of *C. nutans* leaves using Gas Chromatography Mass Spectrometry (GCMS) and assessing their cytotoxic effect of using MTT assay on human gingival fibroblast cell line and then to evaluate their mutagenic effect using Ames test as well as to determine their antimicrobial properties using agar diffusion test and Minimal Inhibitory Concentration (MIC) assays. A total of 53 phytochemical compounds were identified from the extracts of *C. nutans* leaves. The compounds were classified into several classes: phenols, flavonoids, terpenoids, fatty acids, alkanes and hydrocarbons, esters, ketones, furans, sulfur and nitrogen compounds. *In vitro* cytotoxic study of ethanol and aqueous extracts of *C. nutans* leaves in various concentrations (6,400; 3,200; 1,600; 800; 400; 200; 100; 50 and 25 µg/ml) on human gingival fibroblast cell line (HGF-1) using MTT assay did not exhibit any cytotoxic effect. Ames test on four types of *S.* Typhimurium strains, TA98, TA100, TA1535 and TA1537 treated with ethanol and aqueous extracts of *C. nutans* leaves did not show any mutagenic effect both in the presence and absence of metabolic activation system (S9) mix based on the number of revertant colonies. Antimicrobial study using agar well diffusion assay showed that the growth of *Staphylococcus aureus* (*S. aureus*) and *Actinomyces viscosus* (*A. viscosus*) were

inhibited by the ethanol and aqueous extracts of *C. nutans* leaves. However, the extracts did not exhibit any antimicrobial activity towards *Enterococcus faecalis* (*E. faecalis*), *Porhyromonas gingivalis* (*P. gingivalis*) and *Candida albicans* (*C. albicans*). Ethanol extract of *C. nutans* leaves showed better antimicrobial activity compared to the aqueous extract of *C. nutans* leaves against *A. viscosus*. Ethanol extract of *C. nutans* leaves showed MIC value of 102,400 µg/ml against *A. viscosus* and 204,800 µg/ml against *S. aureus*. Aqueous extract of *C. nutans* leaves showed MIC values of 204,800 µg/ml against *A. viscosus* and *S. aureus*. The results of the current study show that *C. nutans* leaves could act as a potential source of antimicrobial agent in combating oral pathogens.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Clinacanthus nutans (Burm.f.) Lindau (*C. nutans*) known as Sabah snake grass in Malaysia belongs to Acanthaceae family. *C. nutans* is known as Sabah snake grass because this plant can be found mostly in Sabah. However, due to its beneficial value, *C. nutans* has been cultivated all around Malaysia (Sekar and Rashid, 2016). It has been used traditionally in Southeast Asia as a medicine to treat various ailments including lesions caused by *Herpes simplex,* diabetes mellitus and also as diuretics (Lau *et al.*, 2014). *C. nutans* has been reported as an important traditional medicine that is widely used in China, Thailand and Malaysia that possesses various properties such as analgesic and anti-inflammatory activities (Satayavivad *et al.*, 1996), antiviral activities against varicella-zoster virus (Thawaranantha *et al.*, 1992) and *Herpes simplex* virus-2 (HSV-2) (Jayavasu *et al.*, 1992). Research has shown that *C. nutans* also possesses antioxidant and anti-proliferative activity against cultured cell line, which suggests the potential of this plant to be a candidate for cancer therapy (Yong *et al.*, 2013).

A study revealed some bioactive compounds detected in *C. nutans* such as chlorophyll phaeophytin derivatives isolated from chloroform extract of *C. nutans* leaves and a mixture of cerebrosides and monoacylmonogalactosylglycerol isolated from the ethanol extracts of *C. nutans* leaves (Chelyn *et al.*, 2014). These bioactive compounds

also have valuable pharmacological properties to prevent the *Herpes simplex* virus-1 (HSV-1) activity (Chelyn *et al.*, 2014).

1.2 Research problem

According to World Health Organization (WHO) in 2013, oral health is important to human health and determination of human quality of life. Oral health can be defined as being free from mouth and facial pain, oral and throat cancer, oral infection and sores, periodontal (gum) disease, tooth decay, tooth loss, and other diseases and disorders that limit an individual's capacity in biting, chewing, smiling, speaking, and psychosocial wellbeing. The most common oral diseases that affect humans worldwide are dental cavities, oral cancer, periodontitis, oral infectious disease, trauma due to injuries and hereditary lesions (WHO, 2013).

Some of the preventions and treatments of oral diseases include lowering the sugar intake and maintaining a well-balanced nutritional intake to avoid tooth decay and premature tooth loss. Also, maintaining a constant low level of fluoride in the oral cavity also plays a major role in the prevention of dental cavities. Some of the clinically established sources of fluoride are antimicrobial mouth rinse and toothpaste. According to Shekar *et al.* in 2015, chlorhexidine gluconate is commonly used as mouth rinse. However, frequent use of chlorhexidine alters the taste sensation, staining of teeth and develops resistance of bacteria that incapacitate its application on long term basis (Shekar *et al.*, 2015). Therefore, there is a need of finding alternative prevention and treatment options for oral diseases that are safe, effective and economical.

Discovery or development of new compounds from the medicinal plants with novel mode of action may also be a solution for other health complications which are antibiotic resistant bacteria outbreaks and diseases related to infection by bacteria (Nascimento *et al.*, 2000; Dewanjee *et al.*, 2007). Shekar and colleagues also suggested the use of medicinal plants for the control of oral or dental related diseases due to their lower negative impact and used to overcome an intrinsic (primary) resistance or secondary resistance to the drug during treatments. Plant extracts, essential oils and purified phytochemicals can be used as potential agents to treat and prevent oral diseases (Palombo, 2011).

C. nutans is one of the medicinal plants that has been proved to have antimicrobial properties towards bacteria. However, the complete potential of *C. nutans* has not been explored yet in the treatment and prevention of oral related diseases. The discovery of *C. nutans* as a treatment is highly important as bacterial infections are one of the major health problems all over the world (Adwan and Mhanna, 2008). The findings of new antimicrobial agents are very crucial since some of the bacterial strains are resistant against most of the clinical antibiotics (Adwan and Mhanna, 2008). In other words, *C. nutans* might be suitable for dental caries treatment and further studies must hence be conducted.

1.3 Justification of the study

Even though medicinal plants have been used traditionally by folklores to treat various diseases internally and externally, the possibility of these plants to become cytotoxic or mutagenic towards consumers should not be neglected. The fact that medicinal

plants contain advantageous bioactive compounds are known widely; however, the fact that these bioactive compounds might be poisonous and contain toxic compounds are not really exposed to the consumers. This study focuses on determining the possible phytochemical compounds present in *C. nutans* leaves and then to study its cytotoxicity, mutagenicity and antimicrobial properties. This will eventually enable in commercialization and prove its safety to be used in the treatment of dental caries or any oral related diseases.

1.4 Objectives

1.4.1 General objective

The general objective of the study is to investigate the potential beneficial properties of *Clinacanthus nutans* for use in commercialization.

1.4.2 Specific objectives

1. To analyze the phytochemical compounds of *Clinacanthus nutans* using Gas Chromatography Mass Spectrometry.
2. To determine the cytotoxic effect of *Clinacanthus nutans* using MTT assay on human gingival fibroblast cell line.
3. To evaluate the mutagenic effect of *Clinacanthus nutans* using Ames test.
4. To study the antimicrobial properties of *Clinacanthus nutans* using agar diffusion test and Minimal Inhibitory Concentration assays.

1.5 Research hypothesis

Clinacanthus nutans leaves have non-cytotoxic, non-mutagenic and antimicrobial properties against oral pathogens.

CHAPTER 2

LITERATURE REVIEW

2.1 *Clinacanthus nutans* (Burm. f.) Lindau

2.1.1 Botanical description

Clinacanthus nutans (*C. nutans*) is a shrub that grows up to 1 to 3 meters in height. The leaf of this plant is simple, oppositely arranged, narrowly elliptic oblong or lanceolate and usually is 2.5-13 cm in length and 0.5-1.5 cm in width (Figure 2.1) (Ali *et al.*, 2010). The leaf of *C. nutans* has a pointed or acuminates (tapering to a point) apex and the leaf margin is either exculpate-dentate or subentire. The leaf base of *C. nutans* is rounded, cuneate, obtuse and pubescent on the nerves and the petiole is around 3-15 mm in length (Ali *et al.*, 2010). The leaf blade is lanceolate-ovate, lanceolate or linear-lanceolate, while, the surface is pubescent when young then glabrescent. This plant has pubescent branches with cylindrical, striate and glabrescent stems (Perry and Metzger, 1980). The stem of *C. nutans* is small, soft, thin and slightly curved that looks like the curve of an elephant's trunk (Shim *et al.*, 2013). Therefore, in Malay *C. nutans* was known as Belalai gajah (elephant's trunk). Generally, *C. nutans* plant reproduces using vegetative propagation by stem cuttings (Panyakom, 2006; Ali *et al.*, 2010). The synonyms of *C. nutans* are *Justicia nutans*, *C. agustus*, *C. burmanni*, *C. spirei* and *C. siamensis* (Hu *et al.*, 2011; Ng, 2013). Common vernacular names for *C. nutans* are listed in Table 2.2. Taxonomically, *C. nutans* is classified by kingdom, phylum, class, order, family, genus and species (Table 2.1) as per Hassler, (2000).

Figure 2.1: a: whole plant; b: leaves with stem of *Clinacanthus nutans*

Table 2.1: Taxonomy of *C. nutans*

Kingdom:	Plantae
Phylum:	Tracheophyta
Class:	Magnoliopsida
Order:	Lamiales
Family:	Acanthaceae
Genus:	Clinacanthus
Species:	*Clinacanthus nutans* (Burm. f.) Lindau

Table 2.2: Vernacular names of *C. nutans*

Vernacular name	Country/Language	References
Belalai gajah	Peninsular Malaysia, Brunei	Alam *et al.*, (2016)
Sabah snake grass	East Malaysia, Brunei	Alam *et al.*, (2016)
Dandang gendis, Ki tajam	Indonesia	Ng, (2013)
Sha Be She Cao, Qing jian	China	Teshima *et al.*, (1998)
Phaya yo, Phak man kai	Thailand	Teshima *et al.*, (1998); Ali *et al.*, (2010)

2.1.2 Origin and distribution

C. nutans is native throughout Southeast Asia, particularly in Thailand, Malaysia and China (Tuntiwachwuttikul *et al.*, 2004). *C. nutans* is also commonly found in tropical Asian regions such as Vietnam, Indonesia, Brunei and some temperate regions (Le *et al*., 2017). *C. nutans* belongs to Acanthacea family, which is a large flowering plant family that comprises around 220 genera and 4000 species (Hu *et al*., 2011). Most of the species are pantropical and subtropical family and mainly distributed in Indonesia, Malaysia, Africa, Madagascar, Brazil and Central America with a few of them distributed in Mediterranean, Australia, United States and China (Meyer and Lavergne, 2004; Alam *et al*., 2016).

2.1.3 Phytochemical compounds in *Clinacanthus nutans*

Phytochemicals are bioactive chemical compounds that are present naturally in medicinal plants (Wadood *et al.*, 2013) and possess defence mechanisms or disease preventive properties (Afolabi and Afolabi, 2013). Phytochemicals are composed of two categories which are primary and secondary constituents (Wadood *et al*., 2013) based on their function in the plant metabolism (Afolabi and Afolabi, 2013). Primary constituents contain amino acids including chlorophyll, proteins and sugar (Wadood *et al*., 2013). Meanwhile, secondary constituents consist of terpenoids, flavonoids, phenolics, saponins and alkaloids (Afolabi and Afolabi, 2013). Compounds produced by plants having pharmacological or toxicological effects in humans and animals when ingested are known as bioactive compounds (Bernhoft *et al*., 2010). Basically, bioactive compounds such as n-hexadecanoic acid, 1, 2-benzenedicarboxylic acid, diisooctyl ester and tetradecanoic acid present in the plants are produced as secondary constituents.

As part of medicinal plants, *C. nutans* also possesses a wide range of phytochemicals such as terpenoids, flavonoids, glycosides, steroids, phenolic compounds, saponins and tannins. Previous study on preliminary screening of phytochemical compounds reported the presence of secondary constituents in methanol extract of *C. nutans* leaves including saponins, phenolic compounds, flavonoids, diterpenes and phytosterols (Ho *et al*., 2013). Recent study reported the presence of flavonoid compounds such as catechin, quercetin, kaempferol and luteolin in methanol extract of *C. nutans* leaves and buds (Ghasemzadeh *et al*., 2014).

An earlier study by Dampawan *et al*. (1977) reported the isolation of compounds known as stigmasterol, lupeol and β-sitosterol from light petroleum extract of *C. nutans* stems (Dampawan *et al*., 1977). In 1997, Teshima and colleagues successfully isolated six known C-glycosyl flavones; vitexin, isovitexin, shaftoside, isomollupentin 7-O-β-glucopyranoside, orientin and isoorientin from the butanol and water-soluble portion of the methanol extract of *C. nutans* stems and leaves (Teshima *et al*., 1997). In a further study on *C. nutans* extract, Teshima *et al*. (1998) isolated and identified five new sulfur-containing glucoside compounds known as clinacoside A, clinacoside B, clinacoside C, cycloclinacoside A1 and cycloclinacoside A2 using column chromatography (Teshima *et al*., 1998).

Study on leaf extract of *C. nutans* resulted in identification of two glycoglycerolipids; 1,2-Odilinolenoyl-3-O-β-D-galactopyranosyl-glycerol and 1-O-palmitoyl-2-Olinolenoyl-3-O-[α-D-galactopyranosyl-(1"→6')-O-β-D-galacctopyranosyl] glycerol (Satakhun, 2001). Another study on separation of compounds of chloroform extract of *C. nutans* leaves resulted in identification of three new compounds related to chlorophyll a and b which were 13^2-hydroxy-(13^2-S)-phaeophytin b (chlorophyll b), purpurin 18 phytyl ester and phaeophorbide a (chlorophyll a) (Dechatiwongse na Ayudhya *et al*., 2001). Previous study has reported the isolation of a mixture of nine cerebrosides and a monoacylmonogalactosylglycerol from ethyl acetate (EtOAc) soluble fraction of ethanol extract of *C. nutans* (Tuntiwachwuttikul *et al*., 2004).

Sakradat and team discovered five new compounds related to chlorophyll a and b; (i) 13^2-hydroxy-(13^2-*S*)-chlorophyll b, (ii) 13^2-hydroxy-(13^2-*R*)-chlorophyll b, (iii) 13^2-hydroxy-(13^2-*R*)-phaeophytin b, (iv) 13^2-hydroxy-(13^2-*S*)-phaeophytin a and (v) 13^2-

hydroxy-(13^2-*R*)-phaeophytin a from *C. nutans* extract (Sakdarat *et al*., 2006). Recent study on ethanol extract of *C. nutans* aerial parts identified four new sulphur-containing compounds which are clinamide A, clinamide B, clinamide C and 2-cis-entadamide A (Tu *et al*., 2014). Huang and team identified flavonol glycoside compounds which were shaftoside, orientin, vitexin, isoorientin, isovitexin and 6,8-apigenin-*C*-α-L-pyranarabinoside in ethanol extract of *C. nutans* aerial parts (Huang *et al*., 2016). They also reported that the ethanol extract of *C. nutans* aerial parts possessed antitumor and immunomodulatory. Recently, some studies and investigations on *C. nutans* extract have identified some phytochemical compounds as listed in Table 2.3. Lupeol has been identified by most of the researchers in their study.

Table 2.3: Phytochemical compounds present in *C. nutans* plants

Compound	Class	Reference
alpha-tocopherol	Terpenoids	Alam *et al*., (2017)
beta-amyrin	Triterpenoids	Teoh *et al*., (2017)
beta-sitosterol	Phytosterols	Teoh *et al*., (2017)
beta-tocopherol	Terpenoids	Alam *et al*., (2017)
Betulin	Triterpenoids	Teoh *et al*., (2017)
Campesterol	Phytosterols	Teoh *et al*., (2017)
Dimethyl trisulfide	Organosulfur	Mustapa *et al*., (2015)
gamma-sitosterol	Phytosterols	Teoh *et al*., (2017)
gamma-tocopherol	Terpenoids	Alam *et al*., (2017)
Hexadecanoic acid/palmitic acid	Fatty acid	Alam *et al*., (2017)
Isoorientin	Flavones	Mustapa *et al*., (2015)
Isoschaftoside	C-Glycosylated flavone	Mustapa *et al*., (2015)
Kaempferol	Flavones	Mustapa *et al*., (2015)
Kaempferol-7-neohesperidoside	Flavones	Mustapa *et al*., (2015)
Linoleic acid, ethyl ester	Fatty acid	Mustapa *et al*., (2015)
Linoleic acid, methyl ester	Fatty acid	Mustapa *et al*., (2015)
Lup-20(29)-en-3-one	Triterpenoids	Teoh *et al*., (2017)
Lupeol	Triterpenoids	Teoh *et al*., (2017)
Myristic acid	Fatty acid	Alam *et al*., (2017)
Neophytadiene	Diterpene	Mustapa et al., (2015)
Oleic acid	Fatty acid	Teoh *et al*., (2017)
Palmitic acid, methyl ester	Fatty acid	Mustapa et al., (2015)
Phenol,2,6-dimethoxy	Phenol	Mustapa *et al*., (2015)
Phytol	Diterpene	Mustapa et al., (2015)
Quercetin	Flavonoids	Alam *et al*., (2017)
Squalene	Triterpenoids	Teoh *et al*., (2017); Mustapa et al., (2015)
Stearic acid	Fatty acid	Alam *et al*., (2017); Mustapa et al., (2015)
Stearic acid, methyl ester	Fatty acid	Mustapa et al., (2015)
Stigmasterol	Phytosterols	Teoh *et al*., (2017)
Vitamin E	Terpenoids	Teoh *et al*., (2017)
Vitexin	C-Glycosylated flavone	Mustapa *et al*., (2015)
2-cyclopenten-1-one, 2-hydroxy	Cyclic ketone	Mustapa *et al*., (2015)
4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl	Flavonoid fraction	Mustapa *et al*., (2015)
4-vinyl-2-methoxy-phenol	Phenol	Mustapa *et al*., (2015)
4-vinylphenol	Phenol	Mustapa *et al*., (2015)
7,9-Dodecadien-1-ol	Alcohol	Mustapa *et al*., (2015)

2.1.4 Medicinal uses of *Clinacanthus nutans*

In Thailand, fresh leaves of *C. nutans* have been used traditionally to treat ailments like skin rashes, pruritic rashes, insect and snake bites as well as lesions caused by *Herpes simplex* virus (HSV) and Varicella-zoster virus (VZV) (Sakdarat *et al*., 2006; Chotchoungchatchai *et al.*, 2012). The fresh leaves of *C. nutans* are used as anti-snake venom among traditional practitioners in Thailand (Sakdarat *et al.*, 2009). Apart from that, *C. nutans* has been used as lotion or cream for the treatment of genital Herpes and Varicella-zoster lesions as well as to relieve skin inflammation and insect bites (Sakdarat *et al.*, 2009). Chotchoungchatchai *et al.* (2012) suggested that the use of ethanol extract of *C. nutans* dissolved in glycerine solution to treat aphthous ulcer and *Herpes simplex* and the extraction of *C. nutans* plant with palm oil to treat burns (Chotchoungchatchai *et al.*, 2012). In Indonesia, decoction of fresh leaves of *C. nutans* is used to treat dysentery and diabetes (Roeslan *et al.*, 2012; Arullappan *et al.*, 2014).

In China, the whole plant of *C. nutans* is used to treat inflammatory conditions such as haematoma, contusion, strains and sprains of injuries and rheumatism (Ghasemzadeh *et al.*, 2014). In Malaysia and Singapore, most of the traditional practitioners prefer to consume the decoction of fresh leaves of *C. nutans* with water as herbal tea to boost immunity and in the treatment for benign growth on pituitary glands (Siew *et al.*, 2014). In instances, raw stems and leaves of *C. nutans* are eaten for detoxification purposes and for benign growth on thyroid glands. Apart from that, the stems of *C. nutans* are consumed as juice and tea for health promotion, breast cancer treatment, bowel movement promotion, cold and flu treatment as well as bath to promote healthier skin (Siew *et al.*, 2014).

2.2 Reported pharmacological studies of *Clinacanthus nutans*

C. nutans possesses variety of pharmacological properties including antimicrobial, anti-venom, anti-inflammatory, anti-rheumatism, antiviral and antioxidant properties.

2.2.1 Antimicrobial properties

Chomnawang and colleagues reported the antimicrobial activity of *C. nutans* on bacteria triggering inflammation in acne, namely, *Propionibacterium acnes* (*P. acnes*) and *Staphylococcus epidermidis* (*S. epidermidis*) (Chomnawang *et al.*, 2005). Ho and colleagues also reported that the methanol extract of *C. nutans* possessed antibacterial effect against skin pathogens, *P. acnes* and *S. epidermidis* as well as *Staphylococcus aureus* (*S. aureus*), *Bacillus cereus* (*B. cereus*) and *Escherichia coli* (*E. coli*) (Ho *et al.*, 2013). Study on the antibacterial activities of medicinal plants against *S. aureus*, *Micrococcus luteus* (*M. luteus*), *E. coli* and *Pseudomonas aeruginosa* (*P. aeruginosa*) showed that *C. nutans* extract exhibited antibacterial activity against these bacteria (Wong *et al.*, 2013).

Ethyl acetate crude extract of *C. nutans* leaves and its seven fractions (F1, F2, F3, F4, F5, F6 and F7) exhibited antimicrobial activity against *B. cereus*, *Candida albicans* (*C. albicans*), *E. coli* and *Salmonella enterica* Typhimurium with F7 showed the strongest antimicrobial activity (Arullappan *et al.*, 2014). Recent study on the antimicrobial activity of aqueous and methanol extracts of *C. nutans* against three aquatic pathogenic bacteria, *Streptococcus agalactiae* (*S. agalactiae*), *Aeromonas hydrophila* (*A. hydrophila*) and *Enterobacter cloacae* (*E. cloacae*) showed that the

aqueous extract of *C. nutans* exhibited moderate antimicrobial activity against *S. agalactiae* and *E. cloacae*, while, methanol extract inhibited the growth of *A. hydrophila* (Raina and Hassan, 2016; Sekar and Rashid, 2016).

2.2.2 Anti-inflammatory properties

Traditionally, *C. nutans* has been used as an anti-inflammatory agent in the treatment of insect bites and allergic responses, although there is a limited study on the investigation of anti-inflammatory activity of *C. nutans*. A study by Wanikiat *et al.* proposed the anti-inflammatory properties of *C. nutans* extract on human neutrophils using two neutrophil-dependent acute inflammatory models (Wanikiat *et al.*, 2008). The results suggested that there was significant inhibition of myeloperoxidase (MPO) activity in the inflamed tissue showing that the anti-inflammatory effect of the *C. nutans* extracts was related to reduced neutrophil migration (Wanikiat *et al.*, 2008).

2.2.3 Antiviral properties

Kunsorn *et al.* suggested *C. nutans* to be an anti-HSV agent and their results revealed that n-hexane, dichloromethane and methanol extracts of *C. nutans* can inhibit the plaque formation of HSV-1 and HSV-2. Phytochemical compounds from *C. nutans* leaves such as polyphenolic, glycosides and terpenes were found to possess antiviral properties against HSV (Kunsorn *et al.*, 2013). Sakdarat *et al.* (2009) discovered the inhibitory activities of *C. nutans* as an antiviral agent against HSV-1F in pre-viral entry step (Sakdarat *et al.*, 2009). The meta-analysis study by Kongkaew and Chaiyakunapruk (2011) reported that *C. nutans* extract possessed potential beneficial

antiviral properties in the treatment of *Herpes genitalis* and *Herpes zoster* (Kongkaew and Chaiyakunapruk, 2011).

2.2.4 Antioxidant properties

Investigation of *C. nutans* extracts (chloroform, methanol and water) on diphenyl-1-picrylhydrazyl (DPPH), galvinoxyl radical, nitric oxide, and hydrogen peroxide scavenging assays revealed that *C. nutans* extracts contained antioxidant agents that were capable of negating free radicals (Yong *et al.*, 2013). However, the antioxidant activities of the extract varied for each test due to the solubility of the extracts in different testing systems and the stereo selectivity of the radicals (Yong *et al.*, 2013). A study by Yuann *et al.* inspected the antioxidant activity of *C. nutans* extracts on plasmid DNA integrity in *E. coli* (Yuann *et al.*, 2012). The antioxidant activities, DPPH radical scavenging activity, reducing power activity, SOD activity, and total phenolic contents of *C. nutans* were lowered when compared with green tea. However, *C. nutans* leaf extract showed better retention of the integrity of super-coiled plasmid DNA under riboflavin photochemical treatment compared to extracts of green tea (Yuann *et al.*, 2012).

2.2.5 Cytotoxic properties

Chloroform and water extracts of *C. nutans* leaf extracts did not exhibit cytotoxic effect on human umbilical veins endothelial cells (HUVECs) and only showed significantly lower per cent of inhibition with IC_{50} below 20 µg/ml compared to other cancer cells (Yong *et al.*, 2013). *In vitro* cytotoxicity study on petroleum ether, ethyl

acetate and methanol extracts of *C. nutans* leaves using MTT assay after incubation for 72 hours revealed that petroleum ether extract showed strongest inhibition on human cervical cancer cell line, Henrietta Lacks (HeLa) and human erythroleukemia cell line (K-562) with IC_{50} values with 18 and 20 µg/ml, respectively (Arullappan *et al.*, 2014). Recent study by Teoh *et al.* (2017) demonstrated that the methanol and ethyl acetate extracts of *C. nutans* roots promoted apoptosis on human breast cancer cell line, Michigan cancer foundation-7 (MCF-7) and HeLa but only showed no or little inhibition towards cells mouse embryonic fibroblast cell line, National Institutes of Health, 3-day transfer, inoculum 3×10^5 (NIH 3T3) (Teoh *et al.*, 2017). Several researchers claimed IC_{50} value for *in vitro* screening of cytotoxicity activity of crude extract, which was less than 20 µg/ml for incubation between 48-72 hours (Yong *et al.*, 2013; Kuete and Efferth, 2014; Graidist *et al.*, 2015).

2.3 Determination of phytochemical compounds by chromatography analysis

According to International Union of Pure and Applied Chemistry (IUPAC), the term chromatography can be defined as physical method of separation between two phases of compounds which are stationary phase and mobile phase (moves in a definite direction). Chromatography analysis has been used widely in the separation and analyses of complex mixtures or compounds (Christian, 2004). Basically, chromatography analysis can be divided into two principles of analysis including gas chromatography (GC) and liquid chromatography (LC) (Christian, 2004).

Theoretically, chromatography analysis is based on the solubility of the components in a mixture to the stationary and mobile phases. The components in a mixture will be separated according to their mobility differences (Sliepcevich and Gelosa, 2009).

2.3.1 Gas chromatography separation

Main components required in GC separations include (i) column (packed with stationary phase), (ii) heater (to heat the column), (iii) gas flow (inert carrier gas) and (iv) detection device (detection of compounds either by flame ionization or electron capture) (Harborne, 1973). According to Christian, the test sample injected to the heated port is converted to the vapour state. Then, the results are presented in terms of retention volume, R*v*, or retention time, R*t* (Christian, 2004). R*v* can be defined as volume of carrier gas needed for elution of components from the column, while, for R*t* specific time required for sample to be eluted (Harborne, 1973).

The chromatographic peaks are identified by measuring the peaks at each R*t* area and compare their height with standard of pure substance. Detection system device can read the readout of the peak area automatically together with the R*t*. However, the identification of the peaks in more complex mixtures or samples are not easy to be identified, thus, a more sensitive and selective detector can be used which is mass spectrometry (MS) instrument. MS is known based on the detection of mass of the compound (formula weight and fragmentation pattern) (Christian, 2004). Combination of GC and MS technique is known as gas chromatography mass spectrometry (GCMS) (Christian, 2004).

Phytochemical compounds analysis of the plant extracts is very important commercially to produce new drugs by identifying specific compounds that possess pharmacological properties to cure various diseases (Wadood et al., 2013). Several techniques are used by several researchers to determine the phytochemical compounds present in plant extracts and one of the techniques is GCMS.

2.3.1(a) Gas chromatography mass spectrometry analysis

GCMS technique is the combination of gas chromatography technique with mass spectrometry technique and established mainly for the study of volatile, active and stable compounds. Scientifically, GC is used widely for the determination of organic compounds whereas, MS is a very sensitive and selective detector to identify various components based on their mass spectra. The universal detection technique, high selectivity and very high sensitivity have made GCMS suitable for qualitative and quantitative analyses for volatile and semi-volatile compounds (Hubschmann, 2008; Kumar *et al.*, 2011).

2.3.2 Liquid chromatography separations

LC separation requires a sample or mixture to be dissolved in solvent before separating the ions or components (Sliepcevich and Gelosa, 2009). Conventional LC used large column and large particles resulted in slow diffusion rates compared to GC (Christian, 2004). High performance liquid chromatography (HPLC) has been developed to improve the classical LC and to minimize diffusion as well as the time required for the movement of sample components (Christian, 2004).

2.3.2(a) High performance liquid chromatography analysis

HPLC is a technique for the separation and determination of organic and inorganic compounds in test samples from biologicals, pharmaceuticals, foods, industrials and environment (Ingle *et al.*, 2017). HPLC analysis uses a specific pump to force the mobile phase through, thus, giving higher resolution in a short time of analysis (Sliepcevich and Gelosa, 2009). Usually, HPLC analysis is used to detect non-volatile compounds such as lipids, sugars, higher terpenoids and alkaloids (Harborne, 1973).

GCMS and HPLC have been used widely to analyse phytochemical compounds in plant extracts qualitatively and quantitatively (Proestos *et al.*, 2006). However, comparing HPLC with GCMS, HPLC has limitation in analysing and identifying the compounds, thus, GCMS analysis can provide more accurate results with better analysis (Proestos *et al.*, 2006). In this study, the phytochemical compounds of the extracts of *C. nutans* leaves were determined by GCMS analysis.

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2.4 Cytotoxicity

Cytotoxicity is a broad term and has ambiguous meaning (Niles *et al.*, 2008). Cytotoxicity is defined as quality of being toxic to cells; either the cells are killed or have their metabolism being altered (Freshney, 2010). The term cytotoxicity can also be described as the cascade of molecular events that interrupt with macromolecular synthesis, resulting in unequivocal cellular, functional, and structural damage (Aldridge, 1993). In other words, cytotoxicity indicates which test substances have specific destructive action on certain cells (Freshney, 2010). Therefore, cytotoxicity

assays are widely performed to identify the test substances or compounds, including plant-derived extracts and purified compounds that are intended for use as pharmaceutical or cosmetic products are non-toxic (McGaw *et al.*, 2014).

Plant extracts contain various compounds that attribute to biological activities and properties. Based on Paracelsus' recognition, all compounds including compounds from plant extracts, have the capacity to be poisonous depending on the dosage or concentration. Cytotoxicity study is considered as a first step used in evaluating and screening the toxicity of test substances including biological activity of plant extracts and active compounds isolated from plants (McGaw *et al.*, 2014). Niles and team suggested that cytotoxicity testing is important to identify and define safety thresholds for all new potential chemotherapeutics (Niles *et al.*, 2009). Besides, cytotoxicity testing also allows researchers to evaluate new antimicrobial or other bioactivities of plant extracts or compounds since these compounds or plant extracts are identified as non-toxic (McGaw *et al.*, 2014).

In vitro cytotoxicity tests on cell line serve as useful method to define basal cytotoxicity such as intrinsic ability of a certain test substances to cause cell death. In conjunction with *in vitro* cell culture systems, test substances are considered being cytotoxic if the test substances interfere with the cell attachments, significantly alters cell morphology, affects the rate of cell growth and causes the cells to die (Horvath, 1980). Apart from that, cytotoxicity is also very important in determining the concentration range for further and more detailed *in vitro* testing by providing useful and meaningful information on parameters such as genotoxicity, induction of

mutations or programmed cell death. Cytotoxicity data also can be applied as a predictor of acute systemic toxicity (Eisenbrand *et al.*, 2002).

There are different types of cytotoxicity assays used to evaluate cell viability and the choice of suitable viability assay to use for cytotoxicity test depends on the nature of the test substance, the expected response, and the target cell (Freshney, 2010; McGaw *et al.*, 2014). Common cytotoxicity or cell viability assays are identified based on metabolic activity measurements for detection of cytotoxic effects of plant extracts and purified plant compounds (Bunel *et al.*, 2014; McGaw *et al.*, 2014). Metabolism reductase viability assays can be classified into two classes; tetrazolium and resazurin assays (Bunel *et al.*, 2014; McGaw *et al.*, 2014).

2.4.1 Tetrazolium-based assays

Tetrazolium assay is the most widely used assay to screen the viability of cells. Tetrazolium assays are known as quantitative colorimetric assays for mammalian cell survival and these assays detect living cells and not the dead cells (Mosmann, 1983). Tetrazolium assays can be differentiated by the different salts used in the assays. Common salts used in the tetrazolium assays include MTT, XTT, MTS and WST-1 (Bunel *et al.*, 2014; McGaw *et al.*, 2014). Tetrazolium salts act as redox sensors that can be reduced into coloured formazan products by metabolically active cells (Bunel *et al.*, 2014). According to Riss *et al.* (2013), MTT salt is positively charged, while, XTT, MTS and WST-1 salts are negatively charged (Riss *et al.*, 2013).

2.4.1(a) MTT assay

In this study, MTT assay was applied to determine the cytotoxicity effect of *C. nutans*. According to Akhir *et al.* (2011), MTT assay is a rapid and high accuracy colorimetric assay that has been used widely to determine cell growth and cell cytotoxicity (Akhir *et al.*, 2011). The first tetrazolium salt, MTT or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay introduced by Mosmann in 1983 is a technique that determines the cell survival using different end points (Mosmann, 1983). MTT assay is a quantitative and reliable colorimetric assay that measures viability, proliferation and activation of cells in a multi-well.

The principle of this assay is based on the reduction of yellow water soluble 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Figure 2.2) by metabolically active viable cells (via mitochondrial and cytosolic enzymes) to a blue-purple formazan derivative after treating with test chemicals or substances (Mosmann, 1983; McGaw *et al.*, 2014). The mitochondrial dehydrogenase at the cytochrome b and cytochrome c sites of living cells alter the formazan salt from soluble to an insoluble, purple formazan derivative (Li *et al.*, 2015). The insoluble formazan derivative accumulates within cells as it cannot pass through cell membranes (McGaw *et al.*, 2014). Dimethyl sulfoxide (DMSO) is used to solubilize the insoluble formazan derivative and then, the coloured product can be measured quantitatively by spectrophotometer where the results are directly proportional to the number of viable cells (Bean *et al.*, 1995; Ruben and Neubauer, 1987; Riss *et al.*, 2013). The formation of purple coloured product shows the presence of viable cells since when the cells die, they lose the ability to convert MTT into formazan. Based on McGaw *et al.* (2014),