CARDIO-PROTECTIVE EFFECT OF STANDARDIZED EXTRACT OF CLINACANTHUS NUTANS LINDAU AGAINST DOXORUBICIN-INDUCED TOXICITY

by

KAMEH ESMAILLI

Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

December 2017

ACKNOWLEDGEMENT

Firstly, I would like to express my sincere gratitude to my advisor Professor Dr. Zhari Ismail for the continuous support of my Ph.D study and related research, for his patience, motivation, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my Ph.D study.

Besides my advisor, I would like to thank my co-supervisors, Associate Professor Dr. Amin Malik Shah Amdul Majid for his insightful comments and encouragement, but also for the hard question which incented me to widen my research from various perspectives. My sincere thanks also goes to Prof. Munavvar Zubaid Bin Abdul Sattar for providing finicianal support. I am grateful to my angel friend and fellow labmate Dr. Armaghan Shafaie for the stimulating discussions, for the sleepless nights we were working together before deadlines, and for all the fun we have had in the last four years.

Last but not the least, a special thanks to my family. Words cannot express how grateful I am to my mother, and father for all of the sacrifices that they've made on my behalf. Their prayers for me was what sustained me thus far.

The successful conduct of this research would not have been possible without the support of Universiti Sains Malaysia (USM). My appreciation to Institute of Postgraduate Studies (IPS), School of Pharmaceutical Sciences and the academic staff of Universiti Sains Malaysia for their support and assistance. I would also like to thank all of my friends who supported me in writing, and incented me to strive towards my goal in the following institution.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATION

A°	Angstrom
AIA	Acid insoluble ash content
$AlCl_3$	Aluminum chloride
ALP	Alkaline phosphatase
As	Arsenic
ALT	Aspartate transaminase
AST	Aspartate aminotransferase
BSA	Bovin serum albumin
BuOH	Butanol
C	Concentration
CCD-18Co	Human fibroblast cell line
Cd	Cadmium
CET	Cetrimide
CGT	C-glutamyl transpeptidase
CL	Clearance
C_{max}	Maximum concentration
CMC	Carboxymethyl cellulose
CN	Clinacanthus nutans
CO2	Carbon dioxide
CPK	Creatine phosphokinase enzyme
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOX	Doxorubicin hydrochloride
DPPH	2,2-diphenyl-1- picrylhydrazil
Ea	Activation energy

Et	Ethanol
EW	Ethanol water
F	Fraction
$FeCl_3$	Ferric chloride
FT-IR	Fourier transform infra-red
g	Gram
GA	Gallic acid
GSH	Glutathione enzyme
g/kg	Gram per kilogram
h	Hour
H_2O	Water
H_2O_2	Hydrogen peroxide
H3PO4	Orthosphoshoric acid
HA	Hippuric acid
Hb	Hemoglobin
HCl	Hydrochloric acid
HCT 116	Human colon cancer cell line
Hg	Mercury
HNO3	Nitric acid
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
HR	Heart rate
HSV	Herpes simplx virus
IC50	Half maximal inhibitory concentration
IV	Intravenouse
J	Joule
K562	Leukemia cell line

KBr	Potassium bromide
KCl	Potassium chloride
L	Litre
LDH	Lactate dehydrogenase enzyme
LOD	Limit of detection
log	Logarithm
LOQ	Limit of quantification
M	Molar
MCF7	Human breast adenocarcinoma cell line
mg	Milligram
Mg/g	Milligram per gram
mg/kg	Milligram per kilogram
min	Minutes
mL	Millilitre
MLT	Microbial limit test
mm	Millimeter
mM	Milimolar
MSA	Mannitol salt agar
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NaCl	Sodium chloride
ND_s	Naturopathic doctors
NP	Natural product
RVSEB	Rappaport vassiliadis salmonella enrichment broth
SCD	Soybean-Casein digest agar
SDA	Sabouraud dextrose agar
TA	Total ash content
TAMC	Total aerobic microbal count

TYMC	Total yeast and mold count
VZV	Varicella-zoster virus
WHO	World Health Organization
WSA	Water soluble ash
XLD	Xylose-lysine-deoxycholate

KESAN PERLINDUNGAN KARDIO EKSTRAK TERPIAWAI DAUN CLINACANTHUS NUTANS TERHADAP TOKSISITI-KARDIO TERARUH KEMOTERAPI

ABSTRAK

Tujuan utama tesis ini adalah untuk mengkaji kesan perlindungan-kardio ekstrak terpiawai daun *Clinacanthus nutans* (CN) terhadap toksisiti-kardio teraruh kemoterapi. Di Timur Jauh, CN telah digunakan secara tradisional untuk merawat pelbagai penyakit termasuk herpes, kanser, tulang retak, diabetes, diuretik, jaundis, gigitan ular dan kegagalan buah pinggang. Walaupun terdapat beberapa produk CN di pasaran, masih terdapat kekurangan maklumat saintifik dari segi pemiawaian. Kajian ini dijalankan untuk memberikan maklumat tersebut sebagai rujukan kepada penyelidik lain serta pengguna produk herba berasaskan potensi ekstrak daun CN. Kajian kualiti dan keselamatan bahan mentah tumbuhan telah diperiksa menggunakan analisis gravimetri dan ujian had mikrob (MLT). Pelbagai ekstrak tumbuhan (25% EW, 50% EW, 75% EW, 95% EW, W) telah dipiawaikan menggunakan kaedah spektroskopi (UV, IR) dan kromatografi kualitatif (HPTLC) serta kromatografi kuantitatif (HPLC). Metabolit primer dan sekunder ekstrak CN dianalisis bagi jumlah flavonoid, polifenol, tanin, protein, polisakarida dan glikosaponin. Komposisi fitokimia kesemua ekstrak ditentukan menggunakan pelbagai teknik kolorimetri dan gravimetri. Ekstrak terpiawai akueus CN (CN-W) menunjukkan kandungan glikosaponin dan polifenol tertinggi (masing-masing 45 ± 2.82 dan 4.08 ± 0.25 w/w%,). Vitexin, isovitexin, orientin, isoorientin, asid galik, asid kafeik dan asid klorogenik telah digunakan sebagai penanda kimia untuk kajian analitikal ekstrak CN. Hasil kajian menunjukkan bahawa asid galik merupakan komponen utama dalam

semua ekstrak CN, manakala asid kafeik merupakan komponen minor. Penentuan dan pengkuantitian 17 asid amino bebas dalam ekstrak CN telah dilakukan dengan kaedah HPLC. Hasil kajian menunjukkan bahawa L-alanine adalah asid amino bebas utama dalam CN-W ekstrak dengan 3.05 ± 0.09 nmol/mg manakala L-glutamik adalah asid amino bebas minor dengan 0.66 ± 0.04 nmol/mg. Di samping itu, stigmasterol telah dipencil dan dicirikan daripada ekstrak metanol daun CN. Pencirian struktur stigmasterol telah dijalankan menggunakan pelbagai teknik termasuk teknik kristalografi X-ray dan NMR. Kesan sitotoksik bagi ekstrak CN terhadap jalur sel kanser terpilih (MCF 7, HCT 116 dan K562) dan jalur sel normal (CCD-18Co) telah diuji menggunakan assai MTT dan keputusan menunjukkan bahawa semua ekstrak CN tidak mempamerkan kesan sitotoksik terhadap jalur sel normal dan kanser (nilai IC_{50} adalah lebih besar daripada 20 μg/mL). Assai radikal bebas difenilpikrihidrazil (DPPH) telah digunakan untuk menentukan kapasiti antioksidan daripada ekstrak tumbuhan. Hasil kajian menunjukkan bahawa, CN-W) mempamerkan perencatan tertinggi (dengan 94.33 ± 11.10% pada kepekatan akhir 62.5 μg/mL) dan nilai IC_{50} paling rendah (6.48 ± 1.18 μg/mL). Ekstrak CN-W dipilih untuk kajian perlindungan-kardio *in vivo* berdasarkan aktiviti antioksidan dan tahap kandungan polifenol yang tinggi. Kesan perlindungan-kardio bagi CN-W terhadap toksisiti teraruh kemoterapi telah dikaji menggunakan model toksisiti-kardio-teraruh-doksorubisin *in vivo* ke atas tikus Sprague Dawley (SD). Rawatan dos tunggal doksorubisin (25 mg/kg) ke atas tikus menyebabkan peningkatan aktiviti CPK dan LDH plasma (sebagai enzim jantung) ($P < 0.001$) berbanding kawalan manakala pra rawatan tikus yang dirawat doksorubisin dengan ekstrak CN-W (125, 250 dan 500 mg/kg) selama 28 hari, menunjukkan penurunan dalam aktiviti CPK dan LDH plasma ($P < 0.05$). Oleh itu, ekstrak CN-W pada semua dos mempunyai aktiviti perlindungan kardio yang tinggi

dalam model haiwan terhadap ketoksikan doksorubisin. Potensi (antagonis) ikatan penanda aktif CN (asid kaffeik, asid klorogenik, asid gallik, orientin, vitexin dan isovitexin) dan deksrazoksen (kawalan positif) dengan enzim CPK dan LDH telah dinilai dalam analisis dok untuk mengesahkan keupayaan mengikat sebatian penanda dengan enzim jantung secara teori. Hasil kajian menunjukkan bahawa, sebatian penanda daripada ekstrak CN mempunyai afiniti pengikatan yang lebih baik berbanding kawalan positif (deksrazoksen). Oleh itu, boleh dicadangkan bahawa sebatian penanda terpilih mempamerkan aktiviti perencatan terhadap CPK dan LDH dan boleh bertindak sebagai ejen perlindungan kardio. Untuk kajian kestabilan, CN-W telah didedahkan kepada empat keadaan penyimpanan bagi suhu dan kelembapan relatif yang berbeza 30°C/75% RH, 40°C/75% RH, 50°C/85% RH dan 60°C/85% RH untuk tempoh enam bulan. Hasil kajian menunjukkan bahawa semua sebatian penanda (asid kafeik, asid klorogenik, asid galik, orientin dan vitexin) adalah lebih stabil pada 30°C dan 40°C berbanding suhu 50°C dan 60°C.

CARDIO-PROTECTIVE EFFECT OF STANDARDIZED EXTRACT OF CLINACANTHUS NUTANS LINDAU AGAINTS DOXORUBICIN-INDUCED TOXICITY

ABSTRACT

The primary purpose of this thesis was to investigate cardio-protective effect of standardized leaf extract of *Clinacanthus nutans* (CN) against chemotherapy-induced cardio-toxicity. In the Far East, CN has been used traditionally to treat a wide range of diseases including herpes, cancer, fractures, diabetes, diuretic, jaundice, snake bites and kidney dysfunction. There are a number of CN products in market, however there is still lack of scientific evidence in terms of standardisation. Quality and safety of the plant raw material were examined using gravimetric analysis and microbial limit test (MLT). Various plant extracts (25% EW, 50% EW, 75% EW, 95% EW, W) were standardized using qualitative spectroscopic (UV, IR) and chromatographic (HPTLC) and quantitative chromatographic (HPLC) methods. Primary and secondary metabolites of CN extracts were analysed for their total flavonoids, polyphenols, tannins, proteins, polysaccharides and glycosaponins. Aqueous extract of CN (CN-W) showed the highest contents of glycosaponins and polyphenolics (45 ± 2.82 and 4.08 ± 0.25 w/w%, respectively). Vitexin, isovitexin, orientin, homoorientin, gallic acid, caffeic acid and chlorogenic acid were used as chemical markers for the analytical study of CN extracts. The results indicated that gallic acid is a major component in all CN extracts, while caffeic acid is the minor component. Determination and quantification of 17 free amino acids in CN extracts was done by HPLC method. The results showed that L-alanine with 3.05 ± 0.09 nmol/mg was the major free amino acid and L-glutamic with 0.66 ± 0.04 nmol/mg was the minor free amino acid in CN-W

extract. Stigmasterol was isolated and characterized from methanolic leaf extract of CN. The structural characterization of stigmasterol was carried out using X-ray crystallographic techniques and NMR. Cytotoxicity effect of CN extracts against selected cancer cell lines (MCF 7, HCT 116 and K562) and normal cell line (CCD-18Co) was tested by MTT assay and the results showed that all CN extracts did not exhibit any cytotoxicity against normal and cancer cell lines (IC_{50} values were greater than 20 µg/mL). Free radical diphenylpicryhydrazyl (DPPH) assay results showed that, CN-W exhibited highest inhibition (with 94.33 ± 11.10% at the final concentration of 62.5 µg/mL) and lowest IC_{50} value (4.74 ± 0.05 µg/mL). CN-W extract was selected for *in vivo* cardio-protective study due to its high antioxidant activity and high level of polyphenolics content. Cardio protective effect of CN-W against chemotherapy induced toxicity was studied in *in vivo* model in doxorubicin (DOX)-induced cardio-toxicity on Sprague Dawley (SD) rats. Treatment of rats with a single dose of DOX (25 mg/kg) resulted in increase plasma CPK and LDH (as cardiac enzymes) activities ($P < 0.001$) in compared to control while pretreatment of DOX-treated rats with CN-W extract (125, 250 and 500 mg/kg) for 28 days, resulted in a decreased in plasma CPK and LDH activities ($P < 0.05$). Therefore, CN-W extract at all doses had potent cardio-protective activity in animal models against DOX toxicity. Moreover, the pilot pharmacokinetics study was done on all marker compounds (vitexin, isovitexin, orientin, caffeic acid, chlorogenic acid and gallic acid) on Sprague Dawley (SD) rats. The results revealed that three glycoside flavonoids (vitexin, isovitexin, orientin) did not show any absorption in rat's plasma after oral administration of extract even at very high dose (5000 mg/kg). Therefore, three phenolic compounds (caffeic acid, chlorogenic acid and gallic acid) were used for further pharmacokinetics study and subsequently caffeic acid presented highest

absorption in rat's plasma after oral administration of CN-W extract. Binding (antagonistic) potential of CN active markers (caffeic acid, chlorogenic acid, gallic acid, orientin, vitexin and isovitexin) and dexrazoxane (positive control) with CPK and LDH enzymes were evaluated in the docking analysis to confirm the binding ability of marker compounds with cardiac enzymes theoretically. The results demonstrated that, all marker compounds from CN extracts had better binding affinity than the positive control (dexrazoxane). Therefore, it could be suggested that marker compounds presented in CN-W exhibited inhibitory activity against CPK and LDH and may act as cardioprotective agent. For stability studies, CN-W was exposed to four different storage conditions of temperatures and relative humidity 30°C/75% RH, 40°C/75% RH, 50°C/85% RH and 60°C/85% RH for six months' period. The results showed that marker compounds (caffeic acid, chlorogenic acid, gallic acid, orientin and vitexin) are more stable at 30°C and 40°C compared to 50°C and 60°C temperature.

CHAPTER 1
INTRODUCTION

1.1 Medicinal Plants

Medicinal plants have been probably used to heal or combat illness as old as humankind. For centuries Native peoples of various cultures have used plants as medicine for all sorts of healing. Plants were at the basis of Indian and Chinese medicine for millennia, and they still are to this day. It is from these roots that the Western pharmaceutical industry grew to be how and what it is today. Unfortunately, the modern view of plants is very different from what it was. We were once connected to nature, honored and respected nature, and tapped into its greatest potential, where plants were viewed and appreciated with utmost reverence. In modern times, we are greatly disconnected from nature, where we often either fear or disregard the presence and importance of plants. Most people cannot fathom using wild edibles today, whether for food or medicine. Likewise, most cannot be bothered to grow some of their own plants for culinary or medicinal purposes. What this all has also led to, is that we have decreased the quality of our environments and food supply and increased our rates of sickness and disease. This is why, today, a quiet, yet significant revolution is under way. More and more people are rekindling their connection to nature and combining the best of ancient wisdom and modern evolution by seeking the pure and unadulterated benefits that plants can offer us. During the past decades, public interest in natural therapies, namely herbal medicine, has increased dramatically not only in developing countries but mainly in industrialized countries. This has increased the international trade in herbal medicine enormously and has attracted most of the pharmaceutical companies, including the multinationals. Until a few years ago, only small

companies had interest in the marketing of herbal medicines. Currently, most large multinational companies are interested in commercializing herbal drugs. Phytotherapeutic agents or phytomedicines are standardized herbal preparations consisting of complex mixtures of one or more plants which are used in most countries for the management of various diseases. According to the World Health Organization (WHO1996a and b, 1992) definition, herbal drugs contain as active ingredients plant parts or plant materials in the crude or processed state plus certain excipients, i.e., solvents, diluents or preservatives. Usually, the active principles responsible for their pharmacological action are unknown. Whole herbs contain several hundred constituents of organic chemicals which may include fatty acids, sterols, alkaloids, glycosides, saponins, tannins and terpenes (Hill, 1952).

Quality control and standardisation of herbal medicines involve several steps. However, the source and quality of raw materials play a pivotal role in guaranteeing the quality and stability of herbal preparations. Other factors such as the use of fresh plants, temperature, light exposure, water availability, nutrients, period and time of collection, method of drying, packing, storage and transportation of raw material, age and part of the plant collected, etc., can greatly affect the quality and consequently the therapeutic value of herbal medicines (EMEA, 2005; WHO, 2002c, 1998c, 1996, 1991a, 1991b, 1990, 1988).

The general idea that herbal drugs are very safe and free from side effects is false. Plants have many constituents that some are very toxic such as the most cytotoxic anti-cancer plant-derived drugs, digitalis, the pyrrolizidine alkaloids, ephedrine, phorbol esters, etc. However, the adverse effects of most herbal drugs are relatively less frequent when the drugs are used properly compared with

synthetic drugs, but wellcontrolled clinical trials now confirm that they really exist (Bisset, 1994).

1.2 Standardisation and Quality Control of Herbal Crude Drugs

Generally, all medicines, whether they are synthetic or of plant origin, should fulfill the basic requirements of being safe and effective (EMEA, 2005; WHO, 2002c, 1998c, 1996, 1991a, 1991b, 1990, 1988). According to WHO (1996a and b, 1992), standardisation and quality control of herbals is the process involved in the physicochemical evaluation of crude drug covering aspects, such as selection and handling of crude material, safety, efficacy and stability assessment of finished product, documentation of safety and risk based on experience, provision of product information to consumer and product promotion. Attention is normally paid to such quality indices such as:

1) Macro and microscopic examination: For Identification of right variety and search of adulterants.

2) Foreign organic matter: This involves removal of matter other than source plant to get the drug in pure form.

3) Ash values: These are criteria to judge the identity and purity of crude drug such as: total ash, sulphated ash, water soluble ash and acid insoluble ash etc.

4) Moisture content: Checking moisture content helps reduce errors in the estimation of the actual weight of drug material. Low moisture suggests better stability against degradation of product.

5) Extractive values: These are indicative weights of the extractable chemical constituents of crude drug under different solvents environment.

6) Crude fibre: This helps to determine the woody material component, and it is a criterion for judging purity.

7) Qualitative chemical evaluation: This covers identification and characterization of crude drug with respect to phytochemical constituent. It employs different analytical technique to detect and isolate the active constituents. Phytochemical screening techniques involve botanical identification, extraction with suitable solvents, purification, and characterization of the active constituents of pharmaceutical importance.

8) Chromatographic examination: Include identification of crude drug based on the use of major chemical constituents as markers.

The processes mentioned above involves wide array of scientific investigations, which include physical, chemical and biological evaluation employing various analytical methods and tools. The specific aims of such investigation in assuring herbal quality are as varied as the processes employed. It is the process of developing and agreeing upon technical standards. Specific standards are worked out by experimentation and observations, which would lead to the process of prescribing a set of characteristics exhibited by the particular herbal medicine. Hence standardisation is a tool in the quality control process.

In this study, a local medicinal herb has been selected, namely *Clinacanthus nutans* (CN) which is locally known as ' Belalai gajah and Sabah snake grass' in

Malaysia, phaya yo or phaya plongtong in Thailand and Giro de flores, cocodrilo flor, e zui hua in Chinese language. *Clinacanthus nutans (Burm. f.) Lindau* is the accepted name of this species and *Clinacanthus nutans var. robinsonii Benoist, Clinacanthus burmanni Nees, Justicia nutans Burm. f.* are their synonyms. It is distributed in Thailand, Indonesia, Malaysia, Vietnam and China (Guangdong, Guangxi, Hainan, Yunnan). In Malaysia, the fresh leaves are boiled with water and consumed as herbal tea. It's also used for treating skin rashes and snake bites, lesions caused by herpes simplex virus, diabetic myelitis, fever and diuretics. In Thailand, an alcoholic extract of fresh leaves is used externally for treatment of skin rashes, snake and insect bite, herpes simplex virus (HSV), and varicella-zoster virus (VZV) lesions. The leaves can be consumed as raw material or mixed with other juices such as apple juice, sugar cane or green tea and served as fresh drink (Shahzad Aslam et al., 2015).

1.3 Justification of the Research

Clinacanthus nutans Lindau leaves (CN) have been used in traditional medicine for treating certain diseases such as skin rashes, scorpion and insect bites, diabetes mellitus, fever and diuretics but the therapeutic potential has not been explored for cancer prevention and treatment. Chemotherapy is the first line treatment of different types of cancer; however, occurrence of toxicities in patients with chemotherapy is a major concern. Therefore, the elimination of side effects and possible toxicities in cancer patients receiving chemotherapy has become a major task. Despite all the known biological activities from previous work, emerging lay testimonies and Malaysian newspaper reports suggested that *C. nutans* possesses anticancer effects. However, these testimonies were not supported by scientific evidence. Therefore, it can be hypothesized that *C. nutans* derivatives

could be a source of cytoprotective antioxidant based anticancer regimen. Hence, the main aim in this study was to examine the antioxidant and cytoprotective effects of *C. nutans* against chemotherapy-induced toxicity.

1.4 General Objectives

In general, this study seeks to standardize *C. nutans* leaves extracts by optimizing new analytical methods to measure the content of primary and secondary metabolites in *C. nutans* extracts. In addition, this work is designed to appraise the possible *in vitro* anti-cancer properties of the standardized *C. nutans* extracts against selected cancer cell lines and also evaluate their antioxidant properties to estimate the *in vivo* cardioprotective effect of the most active extract against induced DOX cardio-toxicity based on structure activity relationship of marker compounds and to perform stability studies of selected *C. nutans* extract.

1.5 Specific Objectives

1) To standardize *C. nutans* extracts using selected markers and to optimize analytical methods for standardisation of *C. nutans* extracts

2) To evaluate *in vitro* anti-cancer properties of the standardized *C. nutans* extracts versus selected cancer cell lines

3) To estimate antioxidant properties of the various standardized extracts of *C. nutans* for best extract selection to evaluate its *in vivo* cardioprotective potential against DOX-induced cardiotoxicity

4) To perform molecular docking study in order to evaluate cardioprotective property of *C. nutans* extract based on structure activity relationship of marker compounds

5) To perform stability of selected *C. nutans* extract

1.6 Hypotheses

C. nutans is a well-known medicinal plant in folklore medicine for the treatment of a variety of symptoms such as herpes infectious, inflammation, skin pruritis and insect bites. *C. nutans* contains high content of phenolics and flavonoids compounds and a positive correlation was observed between precense of high phenolic content and strong antioxidant activity (Manach et al., 2004). On the other hand, a natural antioxidant could be a potential therapeutic intervention (Rasmussen et al., 2005; Arts and Hollman, 2005; Hertog et al., 1994; Cole et al., 2005). Thus, it could be hypothesized that, pretreatment with standardised extract of *C. nutans* may have protective effect versus DOX-induced cardiac toxicity.

1.7 Significance of Study

The findings of present study provide knowledge on application of analytical methods for standardisation of plant materials and extracts to produce, safe and high quality herbal medicinal products for manufacturers and consumers in order to reduce cardiac toxicity in patients receiving anthracycline-based chemotherapy for cancer. Furthermore, this research fills the gaps between native herbal practices and contemporary medicinal sciences on protective effect of medicinal plant against cardiotoxicity.

CHAPTER 2
LITERATURE REVIEW

2.1 *Clinacanthus nutans*

2.1.1 Taxonomy

Family	Acanthaceae Juss
Order	Lamiales Bromhead
Genus	*Clinacanthus Nees*
Scientific name	*Clinacanthus nutans (Burm. f.) Lindau*

Clinacanthus nutans is a species of plant in *Acanthaceae* family. Acanthaceae, one of 24 families in the mint order (Lamiales) of flowering plants, containing approximately 220 genera and nearly 4,000 species distributed predominantly in tropical and subtropical regions of the world. The greater part of the *Acanthaceae* family are herbs or shrubs, but vines and trees occur as well. The range of habitats extends from marshes and estuaries to extremely dry situations, but most of these plants are found in damp tropical forests (Meyer et al., 2004).

A diverse family, *Acanthaceae* has few universal characteristics among its members. Most have simple leaves arranged in opposite pairs, with cystoliths (enlarged cells containing crystals of calcium carbonate) in streaks or protuberances in the vegetative parts. The bisexual flowers are frequently bilaterally symmetrical and are usually enclosed by leaflike bracts, often coloured and large. Sepals and petals number five or four each and are often fused into tubular structures. There are usually two or four stamens that extend beyond the mouth of the flower, often with one to three staminodes (sterile stamens).

The pistil is superior (i.e., positioned above the attachment point of the other flower parts) and generally consists of two fused carpels (ovule-bearing segments) enclosing two locules (chambers), each of which has two to many ovules in two rows along the central axis of the ovary. The fruits are often exploding capsules containing seeds borne on hooks on the placenta (Smith, 1991; Heywood, 1993; Mabberley, 1998; Bosser & Heine, 2000; Whistler, 2000).

Table 2.1: Common names of *Clinacanthus nutans* all around the world (Smitinand, 1980)

Common names	**Language**
Belalai gajah	Malay
Sabah snake grass	
Twist of flowers	Chinese
Alligator flower	
E zui hua	
Phaya yo	Thai
Phaya plongtong	

Table 2.2: Synonyms of *Clinacanthus nutans*

***Clinacanthus nutans* (Burm. f.) lindau**	**Accepted name**
Clinacanthus nutans var. robinsonii Benoist	Synonym
Clinacanthus burmanni Nees	Synonym
Justicia nutans Burm. f	Synonym

2.1.2 Ethnopharmacology

Clinacanthus nutans is popular due to its benefits in alternative medicine to cure various kinds of diseases. It has been used traditionally as antivenom, anti-inflammatory, analgesic, antidiabetic, antirheumatism, antiviral and antioxidant (Sakdarat et al., 2009; Sittiso et al. 2010; Wanikiat et al., 2008; Pannangpetch et al., 2007). In traditional medicine, fresh leaves of *C. nutans* has been suggested to be effective in treating poisonous snake and insect bites, burns, allergic reactions and

skin lesions caused by virus, diabetes mellitus, fever and diuretics, and Dengue disease (Lau et al., 2014; Kunsorn et al., 2013; Goonasakaran, 2013; Sakdarat et al., 2006; Sakdarat et al., 2009; Shim et al., 2013; Sujittapron et al., 2010; Tuntiwachwuttikul et al., 2004). It is believed that it has strong ability to neutralize the poison from the snake. The dried leaves are used to treat fever, diarrhea and dysuria (Uawonggul et al., 2011). Herpes genitalis is a sexually transmitted disease caused by the herpes simplex virus (HSV). This plant has long been traditionally used in herpes simplex virus (HSV) treatment in Thailand (Sangkitporn et al., 1993). *C. nutans* is commonly used in traditional Malaysian medicine for its nourishing and antioxidant properties. Recently, the leaf extracts have been used extensively as primary sources of complementary and alternative healthcare or as economical in-house regimens for cancer patients (Arullappan et al., 2014; Yong et al., 2013; Wang et al., 2013). Patients have claimed that they have recovered from cancer illness after consuming the leaves over a certain period of time. It is also commonly used for kidney cleanse or detoxifying purpose.

Figure 2.1: Pictures of *Clinacanthus nutans* Lindau leaves and flower

2.2 Review of Chemical Constituents of *Clinacanthus nutans*

Clinacanthus nutans contains lupeol (1), β-sitosterol (2) (Dampawan et al., 1977), stigmasterol (3) (Dampawan 1976) Botulin (Lin et al., 1983) and myricyl alcohol (Boongerd 1967; Dampawan et al., 1996). It also contain six known C-glycosyl flavones including vitexin (4), isovitexin, shaftoside (5), isomollupentin

7-O-b –glucopyranoside (6), orientin (7) and homoorientin isolated from the n-BuOH and water soluble portion of the methanolic extract of the stems and leaves of *C. nutans* collected in Thailand (Teshima et al., 1997). Five sulfur-containing glucosides were isolated from the n-BuOH soluble portion of a methanolic extract of the stems and leaves of plant material (Teshima et al., 1998). A mixture of cerebrosides (8) and a monoacyl monogalactosyl glycerol [(2S)-1-O-linolenoyl- 3-O-b-Dgalactopyranosylglycerol] (9) were isolated from the EtOAc-soluble fraction of the ethanolic extract of the fresh leaves of *C. nutans* (Tuntiwachwuttikul et al., 2004). 13-hydroxy- (13-S)-phaeophytin b, Pupurin-18-phytyl ester and Phaeophorbide were isolated from leaves of hexane and chloroform extract of *C. nutans* (Ayudhya et al., 2001). Trigalactosyl and digalactosyl diglycerides (10) were isolated form the leave extract and possess anti-herpes simplex virus effect (Janwitayanuchit et al., 2003). Hexane and chloroform leave extract of *C. nutans* contain, 132-hydroxy- (132-S) -chlorophyll-b, 132-hydroxy- (132-R)-chlorophyll-b,132- hydroxy-(132-S)- phaeophytin-b,132-hydroxy-(132-R)- phaeophytin-b, 132-hydroxy-(132-S)-phaeophytin-a,132-hydroxy-(132-R)- phaeophytin-a, purpurin-18- phytyl ester and phaeophorbide-a (Sakdarat et al., 2006). Three chlorophyll derivatives (phaeophytins) (11-13) were isolated from the chloroform extract of *Clinacanthus nutans* Lindau leaves. Three of these were known compounds with structures related to chlorophyll a and chlorophyll b namely 132-hydroxy- (132-R)- phaeophytin b, 132-hydroxy- (132-S)- phaeophytin-a and 132-hydroxy-(132-R) -phaeophytin (Sakdarat et al., 2009).

Table 2.3: Chemical structures of *Clinacanthus nutans*

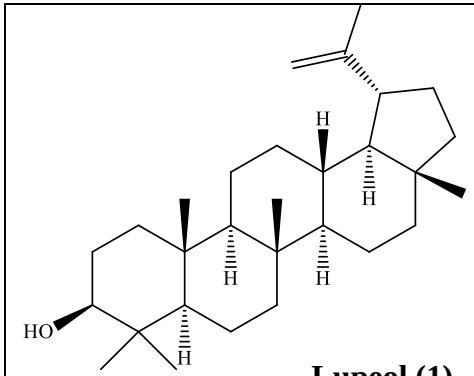
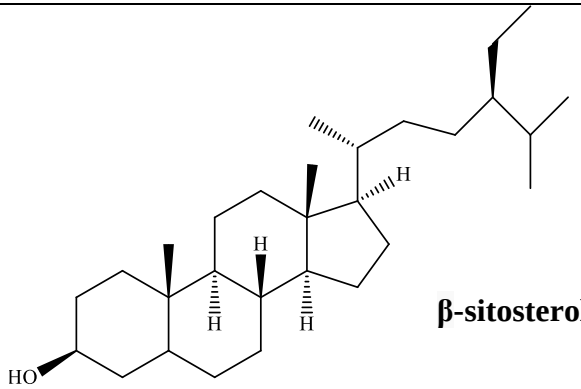
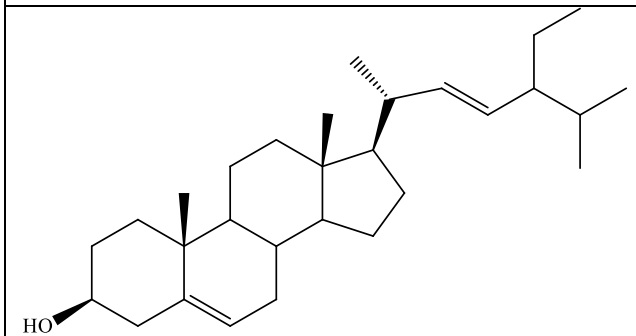
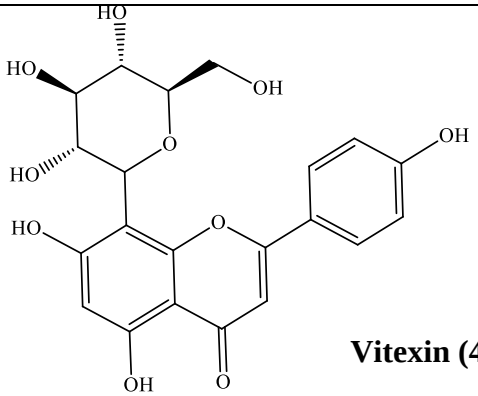
 **Lupeol (1)**	 **β-sitosterol (2)**
 **Stigmasterol (3)**	 **Vitexin (4)**

Table 2.3: Continued

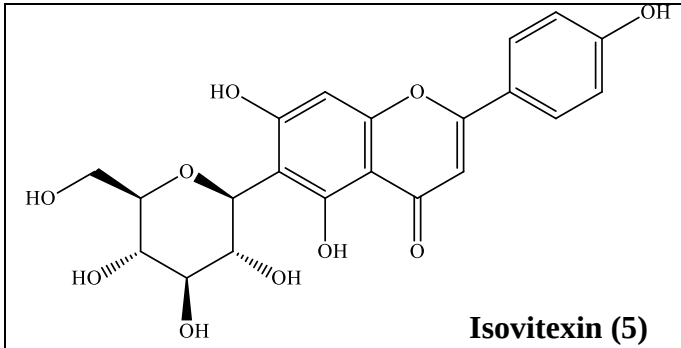
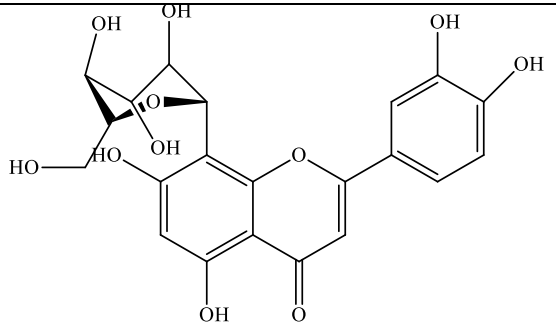
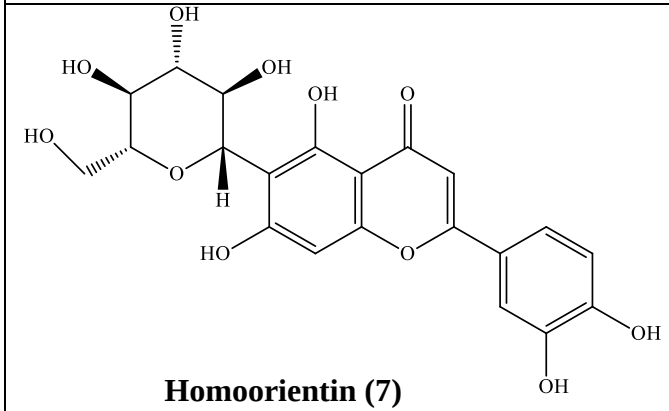
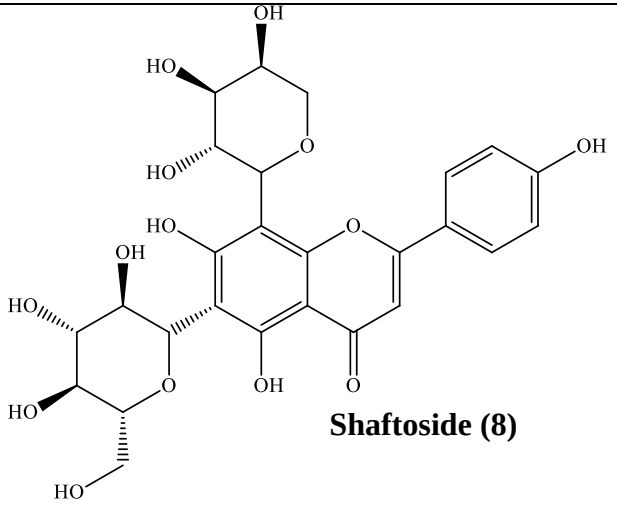
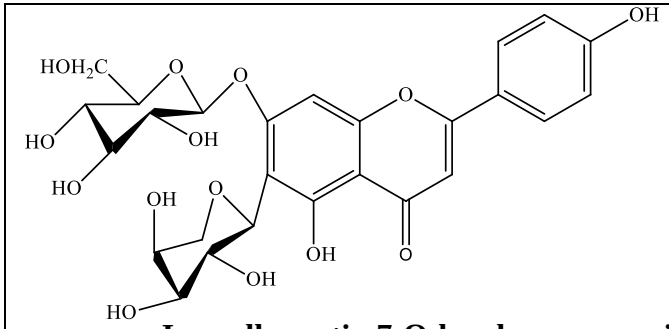
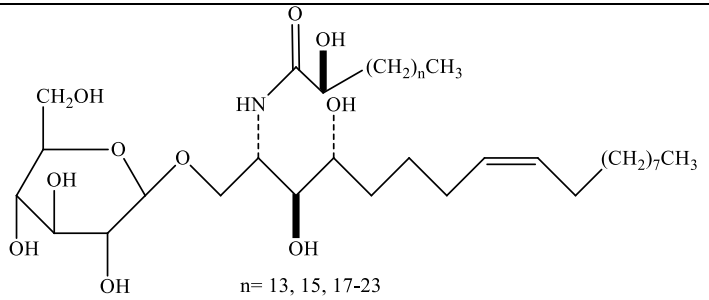
 **Isovitexin (5)**	 **Orientin (6)**
 **Homoorientin (7)**	 **Shaftoside (8)**

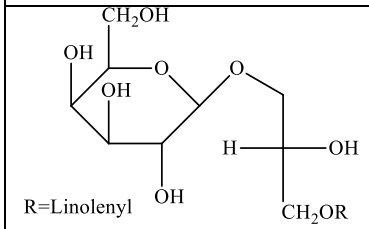
Table 2.3: Continued



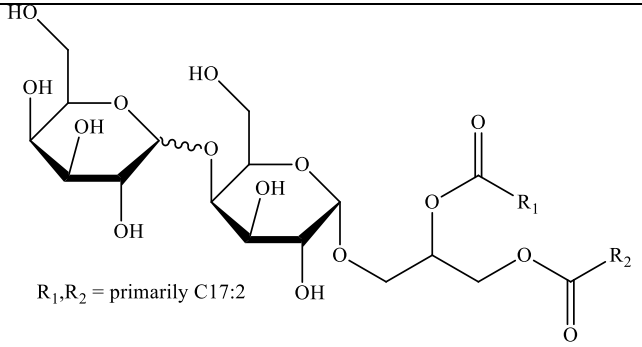
Isomollupentin 7-O-b –glucopyranoside (9)



Cerebrosides (10)

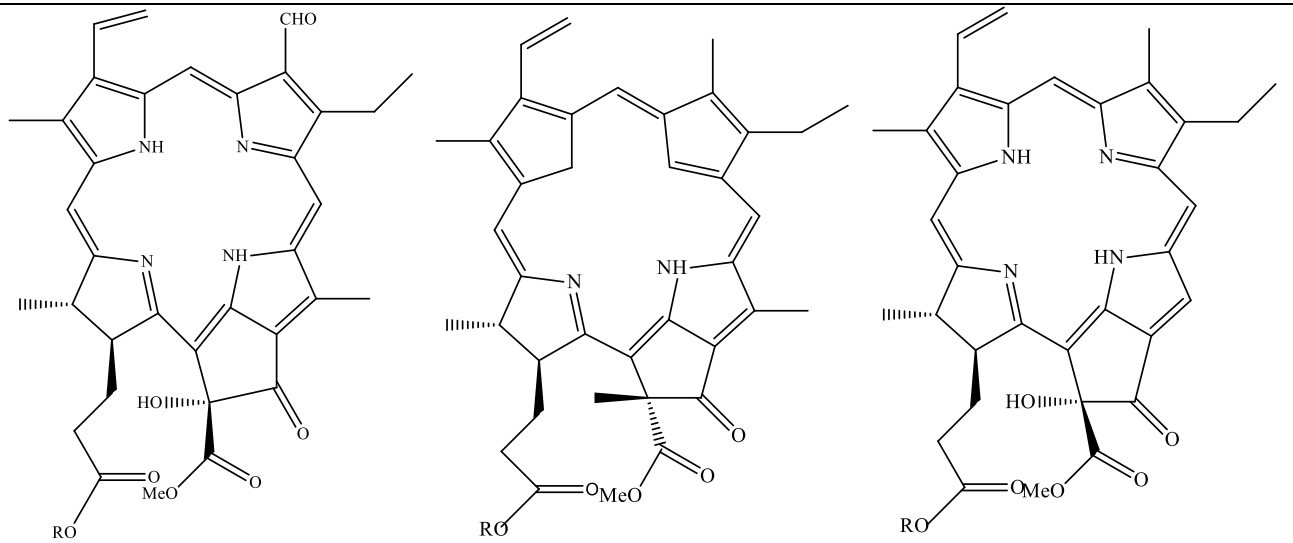


Monoacyl monogalactosyl glycerol[(2S)-1-O-linolenoyl- 3-O-b-Dgalactopyranosylglycerol] (11)



Trigalactosyl and digalactosyl diglycerides (12)

Table 2.3: Continued



Three chlorophyll derivatives (phaeophytins) (13-15)

2.3 Review of Biological and Pharmacological Activities of *Clinacanthus nutans*

Clinacanthus nutans is one of the medicinal plants easily grown in Southeast Asia such as Malaysia, Thailand and China. This plant has attracted public interest due to its high medicinal values in treating skin rashes, insect and snake bits, skin lesions caused by virus, diabetes mellitus, fever and diuretics, and dengue disease (Lau et al., 2014; Kunsorn et al., 2013; Goonasakaran, 2013; Sakdarat et al., 2006; Sakdarat et al., 2009; Shim et al., 2013; Sujittapron et al., 2010; Tuntiwachwuttikul et al., 2004). The possibility of employing the *C. nutans* extract acted as an antioxidant substance to ameliorate the oxidative damage (Pannangpetch et al., 2007). Eight compounds namely 132-hydroxy-(132-S)- chlorophyll B, 132-hydroxy-(132-R)-chlorophyll B, 132-hydroxy-(132-S)-phaeophytin B, 132-hydroxy- (132-R)-phaeophytin B, 132-hydroxy-(132-S)- phaeophytin A, 132-hydroxy-(132-R)-phaeophytin A, purpurin 18 phytyl ester and phaeophorbide A have been discovered (Sakdarat et al., 2006). Recent study found that, *C. nutans* extracts are antioxidant with antiproliferative effect on cultured human cancer cell lines (Arullappan et al., 2014; Yong et al., 2013). The anti-cancer role of *C. nutans* extracts was discovered in China (Wang et al., 2013). The leaves of *C. nutans* were found to be rich in amino acids, trace elements and bioactive chemical constituents in China suggesting a high nutritional value of *C. nutans* (Yi et al., 2012). In China, the rare *C. nutans* is usually propagated by cutting propagation, which is low proliferation freqency. In Malaysia, Ying (2013) and Gunasekaran (2014) succeeded in callus induction but no result in plantlet regeneration.

2.3.1 Antioxidant Effects

The ethanol extract of the dried leaves was tested for antioxidant activity through DPPH, FRAP and the intracellular antioxidant activity assays. Moreover, the protective effect of the extract on rat RBC against 2,2″-azobis(2- amidinopropane) hydrochloride (AAPH) was studied. The results clearly demonstrated significant antioxidant activity of the extract. Furthermore, the extract protected RBCs against AAPH-induced hemolysis with an IC50 of 359.38±14.02 mg/ml, suggesting that *C. nutans* may protect oxidative damage to cell components in diseased conditions (Direkbusarakom et al., 1998). In another experiment, the antioxidant activity of different extracts of *C. nutans* was also reported by Pannangpetch et al. (2007). Yuan et al. (2012) studied the ethanol extract of the leaves for antioxidant activity and its protective effects on the integrity of plasmid DNA in Escherichia coli. The results revealed better retention of the integrity of super-coiled plasmid DNA under riboflavin photochemical treatment when compared with the extracts of green tea.

2.3.2 Antiviral Activity

The effect of ethyl acetate extract of the leaves was tested on HSV type 1 strain F (HSV-1F) using plaque reduction assay. The extract completely inhibited the HSV-1F plaque formation with IC50 value of 7.6 µg/ml. The study further suggested that the antiviral mode of action of the crude extract may be due to the virucidal activity through inhibition of the viral attachment or the penetration (Thongchai et al. 2008). In another study by Direkbusarakom et al. (1998) the antiviral activity of ethanolic extract of *C. nutans* on yellow-head rhabdovirus (YRV) in black tiger shrimp (Penaeus monodon) was investigated. The results indicated that the extract inhibited YRV *in vitro* with a minimum concentration of 1µg/ml suggesting that *C. nutans* extract (1g/kg) mixed with pallet feed could be ideal to control YRV infection in shrimps.

Kunsorn et al. (2013) studied the difference between *C. nutans* and *C. siamensis* by assessing characteristics, molecular aspect through genomic DNA extraction and evaluation of their anti-herpes simplex virus (HSV) type 1 and type 2 activities. The antiviral activity of n-hexane, dichloromethane and methanol extracts was performed using plaque reduction assay and their cytotoxicity was studied on Vero cells through MTT assay. The results revealed that the combination of macroscopic, microscopic and biomolecular methods will be helpful for their identification and both the plants were found to possess anti HSV activity against type 1 and type 2 virus.

2.3.3 Cytotoxicity Activity

The aqueous extract of the *C. nutans* leaves is reported to possess cytotoxicity effect on vero cell cultures with a CD50 of 2828 mcg/ml (Yong et al. 2013). The *in vitro* cytotoxic, antioxidant and antimicrobial activities of the petroleum ether, ethyl acetate and methanol extracts and semi-fractions of *C. nutans* leaves were studied by several researchers (Arullappan et al. 2014) against HeLa and K-562 cell lines by MTT assay and antioxidant activity by DPPH assay. The sub fractions collected from ethyl acetate extract were tested against *Bacillus cereus, Escherichia coli, Salmonella enterica Typhimurium* and *Candida albicans*. The results revealed strongest cytotoxic activity of the petroleum ether extract against HeLa and K-562 cells with IC50 of 18.0 and 20.0 μg/mL, respectively and highest radical scavenging activity among other extracts. In MIC assay, all the extracts and fractions showed inhibition against all tested microorganisms.

2.3.4 Anti-inflammatory and Analgesic Effects

The possible anti-inflammatory activities of methanolic extracts of *Barleria lupulina* and *C. nutans* was studied by Wanikiat et al. (2008) through two neutrophil-

dependent acute inflammatory models (carrageenan-induced paw oedema and ethyl phenylpropiolate-induced ear oedema) in rats. The study was further extended to investigate the effects of the tested extracts on human neutrophil responsiveness, in order to elucidate underlying cellular mechanisms. The results revealed significant inhibitory dose-dependent effects in both the models with a significant inhibition of myeloperoxidase (MPO) activity in the inflamed tissues. The anti-inflammatory activity of the extracts is believed to be associated with reduced neutrophil migration. In a separate study performed by Satayavivad et al. (1998) the significant anti-inflammatory and analgesic activities of the butanol extracts of *C. nutans* leaves is also reported.

2.3.5 Immunomodulatory Effects

The immunomodulatory capabilities of the ethanolic extract of the leaves of *C. nutans* were investigated on cell-mediated immune response (CMIR) through its effects on lymphocyte proliferation, natural killer (NK) cell activity and cytokine production of human peripheral blood mononuclear cells. The findings of the study indicated that the lymphocyte proliferation was significantly increased with decrease in activity of NK cells and increase in the level of IL-4. Further, the findings suggested that the effect of the extract on human CMIR may be partially because of the release of IL-4 from peripheral blood mononuclear cells (Sriwanthana et al. 1996). Another study performed by Thamaree et al. (2001) reported the interleukin-1-beta (IL-1β) release inhibition of the ethanol extract of the leaves when tested on human blood.

2.3.6 Antidiabetic Effects

Wong et al. (2014) were investigated the antioxidant and antiglucosidase activities of six tropical medicinal plants including *C. nutans*. Results of the study exerted that *C. nutans* possesses antioxidant and α-glucosidase inhibitory activity.

2.3.7 Wound Healing Effects

Roeslan et al. (2012) performed *in vitro* wound healing assay through migration rate of human gingival fibroblast (HGF) supplemented with hexane and chloroform extracts of *C. nutans.* The results indicated that the extracts did not give any effect on HGF proliferation, but enhanced the migration rate, suggesting the potential therapeutic effect on periodontal disease.

2.3.8 Anticancer Activity

The antiproliferative effects of chloroform, methanol, and aqueous extracts of the *C. nutans* leaves were performed by Yong et al. (2013) on HepG2, IMR32, NCL-H23, SNU-1, Hela, LS-174T, K562, Raji, and IMR32 cancer cells using MTT assay. The chloroform extract exhibited the highest antiproliferative effect on all cell lines in a concentration dependent manner except on IMR-32 cells. In another study done by Na-Bangchang et al. (2012) the anticancer and immunostimulating activities of one of the most popular Thai folkloric remedies were investigated for cancer treatment. The remedy included of a mixture of parts from five different plants (namely whole part of *Polygala chinensis, Ammania baccifera,* stems and leaves of *C. nutans,* rhizomes of *Canna indica* and *Smilax corbularia*), and five animals (namely scales of Manis javanica, spines of Hystrix brachyuran, Damonia subtrijuga and sternums of Trionyx cartilajineus) respectively. In this study decoction of the remedy was examined for the activities. The cytotoxic activity was investigated *in vitro* in KB cells and *in vivo* in

mammary cancer-bearing rat (induced by 7, 12 DMBA at a single oral dose of 150 mg/kg body weight) and in cervical cancer xenograft nude mouse models. Acute and subacute toxicity tests were performed following intraperitoneal and/or oral dose administration. Therefore, it was concluded that the remedy was well tolerated in both acute and subacute toxicity tests and possess significant anticancer activity and activated NK cell activity. Furthermore, in the study conducted earlier by Thongrakard and Tencomnao (2010) the modulatory effects of ethanolic extract of *C. nutans* on IFN-γ/TNF-α caused HaCaT apoptosis and correlate with the natural phenolic contents. The results demonstrated that the *C. nutans* extract (1 and 100 μg/ml), significantly inhibited the IFN-γ/TNF-α induced HaCaT apoptosis but these findings might not be directly implicated to its natural phenolic content.

2.4 Definition of Cardiac Toxicity

Cardiac toxicity is damage to the heart by harmful chemicals. Cardiotoxicity is one of the most important adverse reactions of chemotherapy which is used for cancer treatment, leading to an important increase of morbidity and mortality (Cardinale et al., 2010; Steinherz et al., 2007). Cardiotoxicity includes a wide range of cardiac effects from small changes in blood pressure and arrhythmias to cardiomyopathy. In the literature different mechanisms of chemotherapy induced cardiotoxicity are postulated including cellular damage due to the formation of free oxygen radicals and the induction of immunogenic reactions with the presence of antigen presenting cells in the heart. Moreover, the influence of the cytotoxic agent on certain phospholipids, especially cardiolipin, may also explain the development of cardiotoxicity (Hatch and McLarty, 2006).

Myocardial damage is commonly known as cardiotoxicity, cardiomyopathy or cardiotoxicity, a medical emergency that occurs when blood supply to the part of heart is interrupted. The resulting ischemia causes damage and death of heart tissue. It is commonly seen with chemotherapy and medications taken to control existing diseases. Myocardial toxicity may cause arrhythmias or it can develop into heart failure, means the heart muscle cannot pump with enough force to supply the body with blood containing essential oxygen and nutrients. In more serious cases it results in congestive heart failure, heart attack, or death (Marieb and Nicpon, 2003).

Data on the mechanism of the appearance of cardiac dysfunction during chemotherapy and the susceptibility of patients to develop cardiotoxicity are scarce (Brana and Tabernero, 2010; Khakoo et al., 2010). Some studies suggest that patients without known cardiovascular history may develop symptomatic heart failure in direct connection to the cumulative dose received, affirmation which has led to the use of reduced doses of chemotherapy and, therefore, to a reduction in their efficiency (Jensen, 2006). But also under these circumstances, there is a risk of cardiotoxicity induced by chemotherapy, risk which cannot be foreseen by the cumulative dose. Moreover, the cardiac alteration is very frequently subclinical and it can appear early (during therapy), late (during the first year after therapy) or very late (more than one year after finishing therapy) (Jiji et al., 2012). Consequently, better understanding of pathophysiology and early diagnosis of subclinical cardiac dysfunction in patients under chemotherapy, as well as the close cardiac monitoring during antineoplastic treatment is essential in order to reduce cardiotoxicity. Since, cardiotoxicity resulting from direct myocyte damage and has been a known complication of cancer treatment. Therefore, better understanding of cancer definition and its treatment might be useful way for studying the acute and long-term cardiotoxicity in cancer treatment modalities.

2.5 Definition of Cancer

Cancer is the name given to a collection of related diseases. In all types of cancer, some of the body's cells begin to divide without stopping and spread into surrounding tissues. Cancer can start almost anywhere in the human body, which is made up of trillions of cells. Normally, human cells grow and divide to form new cells as the body needs them. When cells grow old or become damaged, they die, and new cells take their place. When cancer develops, however, this orderly process breaks down. As cells become more and more abnormal, old or damaged cells survive when they should die, and new cells form when they are not needed. These extra cells can divide without stopping and may form growths called tumors (Cella and Tulsky, 2009). Many cancers form solid tumors, which are masses of tissue. Cancers of the blood, such as leukemias, generally do not form solid tumors. Cancerous tumors are malignant, which means they can spread into, or invade, nearby tissues (Fearon and Vogelstein, 1990). Moreover, as these tumors grow, some cancer cells can break off and travel to distant places in the body through the blood or the lymph system and form new tumors far from the original tumor. Unlike malignant tumors, benign tumors do not spread into, or invade, nearby tissues (Gallup and Talledo, 1987). Benign tumors can sometimes be quite large. However, when they are removed, they usually don't grow back, whereas malignant tumors sometimes do (Brown et al., 1993). Unlike most benign tumors elsewhere in the body, benign brain tumors can be life threatening (Samii, 1992; DeMonte et al., 1994; Coulwell et al., 1996, Samii et al., 1996, 1997; George, 1997; Natarajan et al., 2007). Cancer cells differ from normal cells in many ways that allow them to grow out of control and become invasive. One important difference is that cancer cells are less specialized than normal cells. That is, whereas normal cells mature into very distinct cell types with specific functions, cancer cells do not. This is