TRANSCRIPTOME ANALYSIS OF CERVICAL CANCER CELLS (HeLa) TREATED WITH *Clinacanthus nutans* EXTRACT

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TRANSCRIPTOME ANALYSIS OF CERVICAL CANCER CELLS (HeLa) TREATED WITH *Clinacanthus nutans* EXTRACT

by

NOR HASYIMAH BINTI HARON

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ABTS	2,2'-azinobis (3-ethylbenzthiazoline-6-sulphonic acid)
AlCl3	Aluminium chloride
AMDI	Advanced Medical & Dental Institute
ANOVA	Analysis of variance
BAD	Bcl2-associated agonist of cell death
BAM	Binary Alignment Map
BAX	BCL2-associated X protein
Bcl2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
BGI	Beijing Genome Institute
bp	Base pair
BP	Biological process
CC	Cellular component
cDNA	Complementary deoxyribonucleic acid
ChIP	Chromatin immunoprecipitation
CLiP	Cross-linking and immunoprecipitation
CN	*Clinacanthus nutans*
CN30	30% ethanol extract of CN
CO_2	Carbon dioxide
CVD	Cardiovascular disease
DCM	Dichloromethane
DDB2	DNA damage-binding protein 2
DEG	Differential expression genes
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
dT	Deoxythymine
DV2	Serotype 2 of dengue virus
EB	Elution buffer
ELISA	Enzyme-linked immunosorbent assay

EPP	Ethylphenylpropiolate
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FC	Fold change
FITC	Fluorescein isothiocyanate
FOXP3	Forkhead box P3
FPKM	Fragments Per Kilobase of transcript per Million mapped reads
FRAP	Ferric reducing antioxidant power
GA-1000	Gentamicin-amphotericin-B
GAE	Gallic acid equivalent
GC-MS	Gas Chromatography Mass Spectrometry
GFP	Green fluorescent protein
GO	Gene ontology
GRCh37	Genome Reference Consortium Human Build 37
GTF	Gene transfer format
GUI	graphical user interface
H&E	Hematoxylin and eosin
HeLa	Human cervical cancer cell line
HMDB	Human metabolome database
HPLC	High pressure liquid chromatography
HPLC-DAD	High-performance liquid chromatography with diode-array detection
HPV	Human papilloma virus
HSV	Herpes simplex virus
HSV-2	Herpes simplex virus type 2
IC_{50}	The half maximal inhibitory concentration
ICR	Institute of Cancer Research
IFN-γ	Interferon gamma
IL-10	Interleukin 10
IL-17A	Interleukin 17A
IL-2	Interleukin 2
IL-4	Interleukin 4
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC	Liquid chromatography
LCTOF-MS	Liquid Chromatography-Time-of-Flight Mass Spectrometry

LLE	Liquid-liquid extraction
lncRNAs	Long non-coding RNAs
LSGS	Low serum growth supplement
MAE	Microwave assisted extraction
MEBM	Mammary epithelial cell growth medium
MF	Molecular function
MOHE	Ministry of Higher Education
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2–5-diphenyltetrazolium bromide
N	Unknown base
NA	Not available
NCI	National Cancer Institute of the United States
NIST	National Institute of Standards and Technology
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NTC	No template control
p-AKT	Protein kinase B
PBS	Phosphate buffered saline
PCA	P-coumaric acid
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PI	Propidium iodide
PI3K-Akt	Phosphoinositide-3-kinase–protein kinase B
PLS	Partial Least Squares
PPI	Protein–protein interactions
QC	Quality control
QE	Quercetin equivalent
RT-qPCR	Quantitative Real-Time-PCR
RNA	Ribonucleic acid
RNA-seq	RNA-sequencing
ROS	Reactive oxygen species
RPKM	Reads Per Kilobase of transcript per Million mapped reads
RPM	Revolutions per minute
SAGE	Serial analysis of gene expression

SAM	Sequence Alignment Map
SD	Standard deviation
SFE	Supercritical fluid extraction
SI	Selective index
SRB	Sulforhodamine B
T1	Treated sample biological replicate 1
T2	Treated sample biological replicate 2
TAE	Tris-acetate-EDTA
TCA	Trichloroacetic acid
TE	Trolox equivalent
TFC	Total Flavonoid Content
TLC	Thin-layer chromatography
TNF-α	Tumor necrosis factor alpha
TPC	Total Phenolic Content
TPM	Transcripts per million
UHPLC	Ultra-high-performance liquid chromatography
UPLC	Ultra performance liquid chromatography
UPLC-MS/MS	Ultra performance liquid chromatography - tandem mass spectrometer
UT1	Untreated sample biological replicate 1
UT2	Untreated sample biological replicate 2
UV	Ultraviolet
VZV	Varicella zoster virus
WHO	World Health Organization
YBV	Yellow head baculovirus

LIST OF APPENDICES

ANALISIS TRANSKRIPTOM SEL KANSER SERVIKAL (HeLa) YANG DIRAWAT DENGAN EKSTRAK *Clinacanthus nutans*

ABSTRAK

Clinacanthus nutans Lindau (CN) adalah spesies herba dalam keluarga Acanthaceae. Tumbuhan ini telah digunakan oleh pengamal perubatan alternatif di beberapa kawasan di Asia kerana kepelbagaian potensi perubatannya termasuklah rawatan kanser. Ekstrak CN telah menunjukkan kesan antiproliferatif terhadap pelbagai jenis titisan sel kanser. Bagi menentukan mekanisme molekul yang mendasari proses kematian sel-sel kanser dirawat dengan CN, kajian ini menggunakan sel-sel kanser serviks manusia (HeLa) sebagai model berdasarkan kajian terdahulu yang menunjukkan CN berkesan dalam merawat penyakit jangkitan virus herpes simpleks (HSV) dan virus varisela-zoster (VZV) melalui aplikasi topikal. Kanser serviks kebanyakannya disebabkan oleh virus papilloma manusia (HPV), justeru satu kajian susulan perlu dibuat untuk mengaitkan keberkesanan CN dalam merawat penyakit ini. Dalam kajian ini, sel-sel kanser serviks manusia (HeLa) dirawat dengan ekstrak CN pada kepekatan yang berbeza antara 15.63 hingga 250 μg/ml (untuk fraksi heksana dan diklorometana) dan 250 hingga 4000 μg/ml (untuk ekstrak air dan 80% metanol; dan fraksi air) bagi tempoh setiap 24, 48 dan 72 jam, untuk menyaring ekstrak terbaik dengan bacaan kepekatan perencatan median (IC_{50}) terendah. Fraksi diklorometana (DCM) CN telah menunjukkan bacaan IC_{50} terendah dalam tempoh masa 48 jam rawatan terhadap sel-sel HeLa. Keadaan ini telah digunakan untuk menentukan mekanisme yang mendasari proses apoptosis bagi sel-sel kanser dirawat dengan CN melalui analisis transkriptomik. Dalam analisis ini, sel-sel HeLa yang tidak dirawat turut disediakan sebagai kawalan. Dengan

menggunakan penjujukan RNA, perbandingan transkrip telah mengenalpasti 421 gen yang diekspresi secara berbeza (*DEGs*) keseluruhannya ($p < 0.05$) dalam sel HeLa dirawat dengan CN berbanding HeLa yang tidak dirawat. Analisis laluan KEGG menunjukkan ekspresi gen pembezaan (*DEGs*) bagi kawal atur menaik terlibat dalam laluan pelbagai jenis tumor di mana mekanisme kematian sel berlaku dalam laluan tumor itu sendiri. Ini disokong oleh analisis ontologi gen (*GO*) untuk proses biologi yang kebanyakannya melibatkan mekanisme apoptosis (10 daripada 147 gen yang terlibat dalam kawal atur menaik, 6.80%). Ini termasuk pengaktifan aktiviti endopeptidase jenis sisteina dalam proses apoptosis, kawal-atur positif potensi membran luar mitokondria dalam laluan isyarat apoptosis dan tindak balas protein terungkap yang mungkin mencetuskan tekanan retikulum endoplasma bagi mendorong laluan isyarat apoptosis jenis intrinsik. Selain itu, laluan isyarat apoptosis jenis ekstrinsik juga didorong melalui reseptor domain kematian. Kawal atur menaik gen-gen MYC, DDB2 dan BAD sebagai gen yang kerap terlibat dalam laluan-laluan ini adalah selari dengan keputusan analisis RT-qPCR. Sebagai kesimpulan, ekstrak CN boleh menunjukkan potensi kesan antiproliferatif terhadap percambahan sel-sel HeLa melalui mekanisme yang tertentu.

TRANSCRIPTOME ANALYSIS OF CERVICAL CANCER CELLS (HeLa) TREATED WITH *Clinacanthus nutans* EXTRACT

ABSTRACT

Clinacanthus nutans Lindau (CN) is a herb species belongs to the Acanthaceae family. It has been used among the folklore healers in some regions in Asia for its diverse medicinal potential including cancer treatments. CN extracts have been demonstrated to exhibit potential antiproliferative efficacy against various cancer cell lines. To determine the underlying molecular mechanisms involved in CN anticancer activity, this study used human cervical cancer cell line (HeLa) as a model based on previous findings that CN was effective in treating viral infections diseases such herpes simplex virus (HSV) and varicella-zoster virus (VZV) lesions through topical application. Cervical cancer is mostly caused by human papilloma virus (HPV), so a further study is needed to link the effectiveness of CN in treating the disease. In this study, human cervical cancer cell line (HeLa) was treated with CN at different concentration ranging from 15.63 to 250 µg/ml (for hexane and dichloromethane fractions) and 250 to 4000 µg/ml (for water and 80% methanol crude extracts; and aqueous fraction) for 24, 48 and 72 hours, respectively to screen the best representative extract with the least half maximal inhibitory concentration (IC_{50}) reading. The dichloromethane (DCM) fraction of CN showed the least IC_{50} reading (70 µg/ml) within 48 hours of treatment against HeLa cells. This condition has been used to determine the mechanisms underlying the cell death process of CN-treated HeLa cells through transcriptome analysis. In this analysis, untreated HeLa cells were also used as a control. Using RNA-sequencing, transcriptome comparisons were performed, which identified 421 differentially expressed genes overall ($p <$

0.05) in HeLa treated with CN extract versus untreated HeLa. KEGG pathway analysis demonstrated that upregulated DEGs involved in various types of tumours where the cell death mechanism occured within the tumour pathways itself. This was supported by GO analysis of biological process which mostly involved apoptosis mechanism (10 out of 147 genes from upregulated DEGs, 6.80%). These included activation of cysteine-type endopeptidase activity in apoptotic process, positive regulation of mitochondrial outer membrane potential in apoptotic signalling pathway and unfolded protein response which might trigger endoplasmic reticulum stress to induce intrinsic apoptotic signalling pathway. Besides, extrinsic apoptotic signalling pathway was also induced via death domain receptors. Upregulation of MYC, DDB2 and BAD genes as common genes involved in the pathways were parallel with the RT-qPCR analysis. As a conclusion, CN extract may exhibit a potential antiproliferative effect on HeLa cells proliferation.

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Clinacanthus nutans (CN) is a popular medicinal plant native to Southeast Asia which has been used traditionally as natural medicine to treat fever, dysentery, skin rashes, scorpion bites, snake venom, diabetes mellitus and diuretics in Malaysia, Indonesia and Thailand (Daduang *et al.*, 2005; Sakdarat *et al.*, 2006; Tuntiwachwuttikul *et al.*, 2003; Uawonggul *et al.*, 2006). A report by Siew *et al.* (2014) showed that CN had the highest number of users compared to other fresh medicinal plants in Singapore. It was mostly taken in juice and decoction for treating diverse medicinal and general health purposes. However, few of these were supported by clinical and scientific data as shown by the use of CN to treat Herpes virus infections (Kongkaew & Chaiyakunapruk, 2011; Kunsorn et al., 2013; Lipipun et al., 2011; Yoosook et al., 1999). This condition draws the need of further research to evaluate its potential health benefits and risks.

In Malaysia and Singapore, CN is gaining popularity as claims of its anticancer properties. Thus, a lot of commercial products in a form of teas, drinks and powders are available in the market to suit the need of general health purposes including treating or preventing cancer. So far, there was scientific evidence to support its efficacy as an anticancer herb. The previous study has demonstrated that CN could be effective against certain, but not all cancer cell lines. A study has demonstrated that the chloroform extract of CN showed the highest antiproliferative effect against human erythroleukemia K-562 cell line (IC_{50} value: 47.70 μg/ml) and human Burkitt's lymphoma Raji cell line (IC_{50}: 47.31 μg/ml), but not on human neuroblastoma IMR-32 cell line (Yong *et al.*, 2013). Other study by Arullappan *et al.*

(2014) have shown that petroleum ether extracts of CN demonstrated the strongest cytotoxic activity against HeLa and K-562 cells with IC_{50} of 18.0 and 20.0 μg/ml, respectively. Che Sulaiman *et al.* (2015) found that ethyl acetate and ethanol extracts of CN were cytotoxic against estrogen-dependent breast cancer cells (MCF-7) with IC_{50} 24.04 ± 1.7 and 28.90 ± 2.1 μg/ml respectively, while Khiru Nasir and Mohd Bohari (2015) found that methanol leaves extract was cytotoxic against estrogen.

The plant showed its anticancer effect via the active compounds present which were influenced by the types of extractions, solvents used, plant age as well as plant part that were used. For example, the presence of terpenoids and sulfur-containing glucosides in the dried leaf extracts of CN were the main components responsible for the antioxidant and α-glucosidase inhibitory via nuclear magnetic resonance (NMR) metabolomics analysis and the Partial Least Squares (PLS) biplot mode (Khoo *et al.*, 2015). Besides, six known C-glycosyl flavones (vitexin, isovitexin, shaftoside, isomollupentin 7-O-ß-glucopyranoside, orientin and iso-orientin) were identified from the butanol and water-soluble portion from the methanol extract of CN (Teshima *et al.*, 1997). Based on the reports of these compounds, this plant possessed important biological activities such as antimicrobial, hepatoprotective, antioxidant, anti-inflammatory and anti-neoplastic.

1.2 Problem Statements

The molecular mechanisms and cellular actions underlying CN anti-cancer property remained elusive. Thus, a lot of work should be carried out to find more therapeutic values of the plant especially the mechanism in cell death. A study by Huang *et al.* (2015) provided a clear example of the anticancer mechanism of CN 30% ethanol extract on mice hepatoma *in vivo*. The finding of size and weight

reduction from the treatment showed condensation of cytoplasm and pyknosis of nuclei by H & E staining. This supported the reduction of tumour volume and growth rate prior the treatment. Besides, decreased expression of proliferating markers such proliferating cell nuclear antigen (PCNA) and phosphorylated Akt (*p*-AKT) showed that the process of malignant transformation of cancerous cells was affected upon treatment. Other than that, decreased expression of Bcl2 and increased expression of both BAX and cleaved-caspase-3 showed that apoptosis was induced in hepatoma cells. The significant increase of IL-2 and IFN-γ as well as decreased of IL-4 serum level in CN-treated tumour showed that the antitumour activity was enhanced by promoting the immune response.

Ng et al. (2017) on the other hand, found that CN hexane extract induced apoptosis at the sub-G1 population at A549, CNE1 and HepG2 cells in a dose dependent manner while the G0/G1 and G2/M population showed a corresponding decrease. The extract induced apoptosis activity by upregulating caspase 3/7, 8, and 9 across all three cell lines with the most prominent in HepG2 followed by A549 and CNE cells. Another study by Ng *et al.* (2018) suggested that CN water extract has a potential as anti-angiogenic effects by suppressing human umbilical veins endothelial cell (HUVEC) proliferation and migration in both absence and presence of vascular endothelial growth factor (VEGF), a factor that promotes endothelial cell proliferation, invasion, migration and capillary tube formation within tumour microenvironment. However, inability of the extract to suppress VEGF production from human oral squamous cell carcinoma (HSC-4) raised further investigation as to confirm whether CN water extract is acting through the VEGF receptor.

A study by Lu *et al.* (2018) showed that CN acetone extract induced apoptosis in human lymphoma cell line (SUP-T1) by caspase-3, -7 and -8 activation,

mitochondria-dependent pathway through decreased mitochondrial membrane potential, endoplasmic reticulum (ER) stress through overexpression of IRE-1α and CHOP proteins and glycolysis inhibition by decreased Hexokinase II expression. Besides, a recent study by Nik Zainuddin *et al.* (2020) found that CN standardized fraction arrested human cervical cancer cell line (SiHa) at G1/S cell cycle phase and induced apoptosis via upregulation of p53.

The anti-cancer potential of CN was also related to the moderate antioxidant activity (Kamarudin et al., 2017) as determined by various assays including 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azinobis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), nitric oxide (NO) and ferric reducing antioxidant power (FRAP) radical scavenging. Thus, antioxidants ability of CN might suppress intracellularly reactive oxygen species (ROS) and NO which then further suppresses the progression of tumorigenesis. However, this information was inadequate to illustrate cancer progression which is a complex process. Therefore, a comprehensive approach to discover the therapeutic values of CN for human cancers and other malignancies are needed.

Here, I further investigate transcriptome changes induced by CN extracts in order to determine the major cause of cell death in HeLa cervical cancer cells. Transcriptome analysis using high-throughput next-generation sequencing technologies has been widely used in cancer biology for the identification of novel biomarkers, mutations and even novel transcripts in different cancer types such as lncRNAs,. In this study, transcriptome changes induced by CN extracts to determine the major cause of cell death in HeLa cervical cancer cells has been investigated. The transcriptome analysis was used to identify potential molecular mechanisms of CN *in vitro*. The potential gene targets of CN were then identified based on differential

gene expression as well as the potential biological pathways targeted by CN. These results will be able to provide an important starting point for subsequent experimental functional validation.

1.3 Limitation of Study

The annotated genes from differential expression gene (DEG) list that involved in enriched biological process as well as KEGG pathways were too little. This situation made the information of definite pathways that involved were a little bit harder. For example, a gene which annotated in a cell death pathway does not have relation with downstream genes as obtained in DEGs.

1.4 Research Hypotheses

1.4.1 Null Hypotheses

Transcriptomic analysis of HeLa cells showing the mechanism of cell death was induced by CN extracts.

1.4.2 Alternative Hypotheses

Transcriptomic analysis of HeLa cells showing the mechanism of cell death was not induce by CN extracts.

1.5 Objective of the Study

To determine antiproliferation mechanism of CN extracts in HeLa cells using transcriptomic approach.

1.5.1 Specific Objectives

1.5.1(a) To extract CN leaves using water, 80% methanol and liquid–liquid extraction (LLE) from 80% methanol using hexane and dichloromethane (DCM).

1.5.1(b) To determine phytochemical profile of selected CN extract by using LC-TOF/MS and GC-MS.

1.5.1(c) To determine the antiproliferative activity of HeLa cells treated with CN extracts.

1.5.1(d) To determine effect of HeLa cells treated with CN extracts using flow cytometry.

1.5.1(e) To suggest antiproliferation mechanism of CN extracts in HeLa cells using transcriptomic approach.

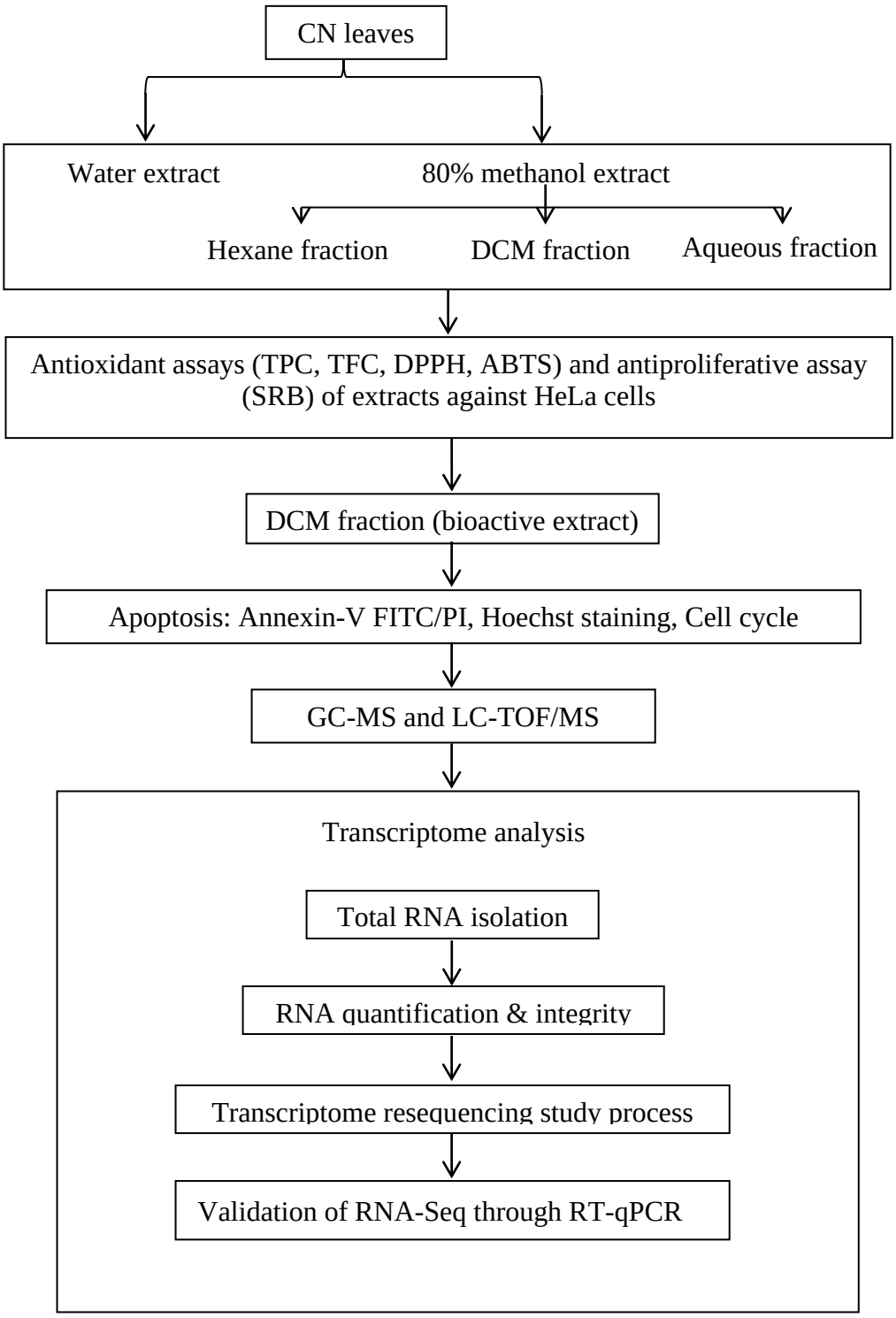

Figure 1.1 Flow chart of study

CHAPTER 2 LITERATURE REVIEW

2.1 Cancer Overview

Cancer is a group of diseases that involve abnormal growth of cells due to multiple changes in gene expression which lead to a dysregulated balance of cell proliferation and cell death. This imbalanced cell population has the potential to invade tissues or spread to other parts of the body to cause morbidity and mortality to the host if untreated (Ruddon, 2007). Cancer is the leading cause of death in the world and has become the second-highest ranking of death which has contributed 9.6 million deaths in 2018. The highest cancer cases among men are lung, prostate, colorectal, stomach and liver cancer while women often suffer from breast cancer, colorectal, lungs, cervix and thyroid (International Agency for Research on Cancer, 2018). However, survival rates are improving for most types of cancers due to the improvements in cancer screening and treatment.

Some of the common factors that are responsible for cancer development, includes personal habits and lifestyles, genetics, health and environment. Common personal habits and lifestyle factors are tobacco consumption or smoking, obesity, alcohol consumption, diet and stress. Cigarette smoking causes heart, respiratory and chronic diseases, stroke, cirrhosis and cancer of the liver (Boyle, 1997). Lack of physical activity, obesity and diet are related to 30-35% of cancer deaths where overnutrition in the diet contributes can form cancer. Excessive intake of alcohol contributed to the head and neck (Suzuki *et al.*, 2008), esophageal (Yang *et al.*, 2005), liver, breast (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2010) and colorectal cancer (Seitz *et al.*, 2006) while frequent use of pickled vegetables has significantly increased the risk of gastric cancer (Strumylaite *et al.*, 2006). Environmental factors such as exposure to the ionizing and non-

ionizing ultraviolet radiation, when combines with the tobacco smoke increased the risk of cancer (Little, 2000).

Surgery and radiotherapy are the successful treatment of cancers in many cases while chemotherapy is moderately efficacious for some advanced cancers. A better knowledge of the molecular and cellular basis will eventually open the door to successful treatment of the primary carcinomas, as will the development of new drugs and new therapies base on the results of molecular biological cancer research.

2.2 Cervical Cancer

Cervical cancer is the fourth most common cancer among women globally where about 570,000 new cases were reported in 2018 representing 6.6% of all female cancers (International Agency for Research on Cancer, 2018). In Malaysia, cervical cancer is the third most common malignancy in women which consists of 12% of total female cancers after breast and colon (Feizal & Abdul Hakim, 2018). It is a type of cancer that develops in the tissues of the cervix and usually has no symptoms at the beginning due to its slow-growing characteristic. About 99% of cervical cancer was detected with human papillomavirus (HPV). The main cause of cervical cancer is often transmitted during vaginal or anal intercourse or even the touch to the genitals (Marth *et al.*, 2017). The current recommended treatment for this cancer involves surgery, chemotherapy or radiation (International Agency for Research on Cancer, 2018).

2.3 Cervical Cancer Cell Line (HeLa)

HeLa cells are a line of human cells derived from cervical cancer. The first cultured cancer cell was taken from Henrietta Lacks in 1951 (Scherer *et al.*, 1953) which then led to the establishment and propagation of hundreds of cancer cell lines

either *in vitro* as monolayer cultures or *in vivo* as xenografts in mice (Mattern *et al.*, 1988). HeLa is one of the fundamental models which has been used in laboratories to study the biology of cancer and to test the therapeutic efficacy of anticancer agents (Sharma *et al.*, 2010).

2.4 Plant Sources as Anticancer Agents

The increase in the global mortality rate due to cancer has led to efforts to curb it using chemotherapy methods, namely the use of drugs to destroy cancer cells from growing, dividing, and making more cells. Although clinical research has produced many new chemotherapy agents, the problem of toxicity effects still exists. For example, 5-fluorouracil, a common chemotherapeutic agent for solid carcinomas, is known to cause myelotoxicity (Garg *et al.*, 2012), cardiotoxicity (Rexroth & Scotland, 1994) and has even been shown to act as a vasospastic agent in rare but documented cases (Rastogi *et al.*, 1993),

Another widely used chemodrug, doxorubicin causes cardiac toxicity (Kilickap *et al.*, 2005), renal toxicity (Manil *et al.*, 1995) and myelotoxicity (Gibaud *et al.*, 1994). Similarly, bleomycin a well-known chemotherapeutic agent, is known for its pulmonary (Karam *et al.*, 1995) and cutaneous toxicity to cause human pulmonary fibrosis and scleroderma respectively due to the deficiency of bleomycin hydrolase enzyme (Yamamoto, 2006). Cyclophosphamide, a nitrogenous mustard associated with cytotoxic or cytostatic drugs integrates a subgroup of alkylating agents, which effective against slow-growing tumours that damage cells at any phase of cellular growth (Kanno *et al.*, 2009). It possesses a wide spectrum of toxicities such damaging the bladder (haemorrhagic cystitis), immunosuppression and alopecia

as the most significant as well as cardiotoxicity a possible toxicity at a very high dose (Fraiser *et al.*, 1991).

The toxicity of chemotherapeutic drugs sometimes creates a significant problem in the treatment of cancer using allopathy or established medicine. Various therapies have been propounded for the treatment of cancer, many of which use plant-derived products. The endless diversity of the plant kingdom makes plant derived products commonly used for clinical trials development of new drugs. Besides, they give chemoprotective potential with non-toxic effects on normal cells and cytotoxic effects on cancer cells. This condition put them in high demand in certain continents of the world such Africa and Asia where herbal therapies are practiced and medicinal plants are relied upon for primary treatment (Ochwang'i *et al.*, 2014).

Plant derived products also become an alternative to limit the emergence and spread of cancer (Saini *et al.*, 2012). They increase the efficacy of conventional chemotherapeutic drugs as well as interfere with the specific stage of carcinogenesis (Wang *et al.*, 2012). On top of that, isolated compounds from plants are natural and have an advantage over synthetic chemical compounds as they are readily available in nature. Thus, the problem of acquiring resistance against these natural compounds is minimized to a very great extent (Arpita & Bharadvaja, 2017).

The use of plants in cancer treatment has started a long time ago where more than 3000 plant species have been reported in cancer treatment. However, based on the poorly defined cancer by folklore and traditional medicine, only visible conditions were observed and treated such as abscesses, calluses, corns, hard swellings, polyps, tumours or warts (Forero & Hartwell, 1989). Nevertheless, plants have played an important role as an effective source of anticancer agents in which

more than 60% of anticancer agents used today come from natural resources including plants, marine organisms and micro-organisms (Cragg & Newman, 2005).

The intensive search for anticancer agents from plant sources began in the 1950s with the discovery and development of vinca alkaloids, vinblastine and vincristine, as well as isolation of cytotoxic podophyllotoxins. As a result, the National Cancer Institute of the United States (NCI), began a large-scale of plant-gathering programme in 1960 to discover possible novel chemotypes with various cytotoxic activity (Cragg & Newman, 2005). In 30 years (1960-1990), taxanes and camptothecins have been developed into an active clinical. However, this plant collection programme was terminated in 1982 but the development of new screening technologies led to the revival of collections of plants and other organisms in 1986, with a focus on the tropical and sub-tropical regions of the world.

2.5 The Plant: *Clinacanthus nutans* (CN)

Clinacanthus nutans (Burm.f.) Lindau shares the same genus with *Clinacanthus siamensis* Bremek. The genus Clinacanthus belongs to the family Acanthaceae with 250 genera and 2500 species belongs to a taxon of dicotyledonous flowering plants and are mostly tropical herbs, shrubs while some of them are epiphytes. The plant is commonly known as Sabah snake grass in Malaysia and other vernacular names; belalai gajah (Malay), dandang gendis (Javanese), phayo or phyaya plongtong (Thai) and zui hua or you dun cao (Chinese). CN is distributed in Yunnan, Guangxi, Guangdong, Hainan provinces of China, Thailand, Vietnam, Malaysia, Indonesia and other parts of southern Asia (Yahaya *et al.*, 2015).

2.5.1 Taxonomy of CN

Kingdom : Plantae

Phylum : Tracheophyta

Class : Magnoliopsida

Order : Lamiales

Family : Acanthaceae

Genus : Clinacanthus

Species : nutans

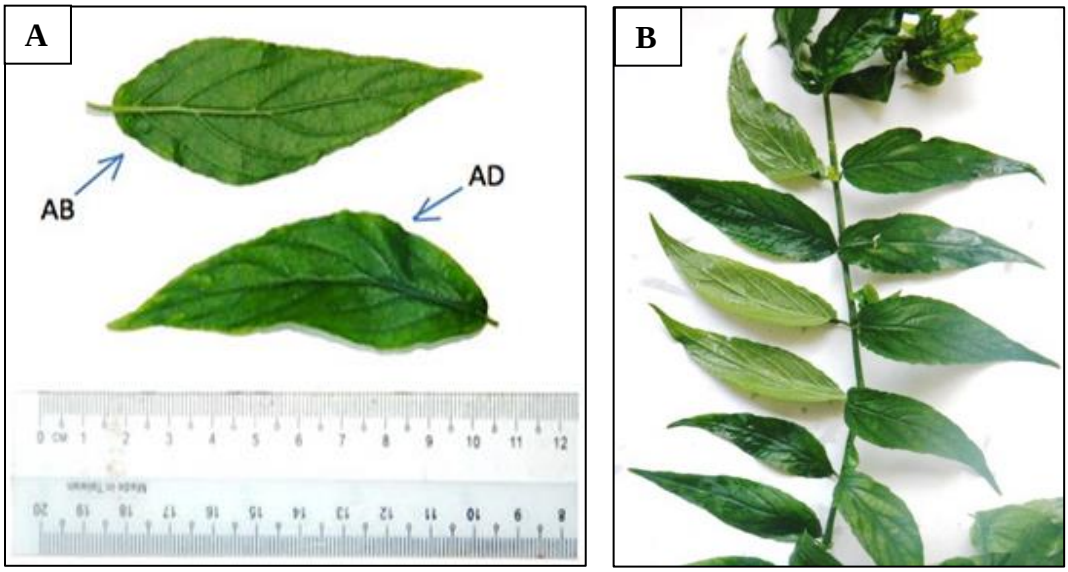

Figure 2.1 *Clinacanthus nutans*, A: Abaxial (AB) and adaxial (AD) leaves, B: leaves showing oppositely arrangement
Source: World Wide Web Globinmed, 2016

2.5.2 Plant Morphology

CN can grow to one meter tall high with pubescent branches (Available from World Wide Web Globinmed, 2016). The stems were striate, glabrescent and slightly curved which resembles the curve of an elephant's trunk. Therefore, it is also known as Belalai Gajah (elephant's trunk) in Malay language. The leaves were papery with

lanceolate or ovate-lanceolate in shape and pale green in colour. They are in pairs opposite arrangement with 2.5 to 13 cm long and 0.5 to 4 cm wide. The flowers are dull red with a green base in colour and arranged in dense cymes (five to eight flowered) at the top of the branches.

2.5.3 Plant Growth Habit or Cultivation

CN cultivation is not influenced by the soil or edaphic requirement. Loam (ordinary garden soil) or sandy loam is enough to support the growth. Normally, CN can grow in soil mixture which contains garden soil, animal manure or compost and river sand at a defined portion. This mixture is filled in a polyethylene bag before CN's aerial is planted. The plant is cultivated under the half-shaded area to obtain quality leaves (Available from World Wide Web The Miracle of Sabah Snake Grass: *Clinacanthus Nutans*, 2012).

2.5.4 Ethnomedicinal use of CN and its supported bioactivity

CN has been used traditionally among the folklore healers in some regions in Asia for certain medicinal purposes. It has been used as a remedy for poisonous snake and insect bites, burns and allergic reaction (Sriwanthana *et al.*, 1996). Clinical study has supported that anti-snake venom activity of CN was originated from anti-cell lysis activity (Uawonggul *et al.*, 2006) and not from anti-neuromuscular blockage (Cherdchu *et al.*, 1977) or administered the extract directly by intraperitoneal injection in the tested mice (Levey, 1969) as reported before.

CN has also been used as a topical treatment of *Herpes zoster* in Thailand (Charuwichitratana *et al.*, 1996). *In vitro* study has supported that CN was virucidal

against herpes simplex virus (HSV) (Jayavasu, Balachandra, *et al.*, 1992; Jayavasu, Dechatiwongse, *et al.*, 1992) and varicella-zoster virus (VZV) (Thawaranantha *et al.*, 1992). In a clinical study, CN extract was effective in treating genital herpes (Jayavasu, Dechatiwongse, *et al.*, 1992). Besides, CN extract showed good efficacy in shortening the duration of HSV-2 infection and reduction in its severity (Jayavasu, Dechatiwongse, *et al.*, 1992). This antiviral activity was prominent as CN ethanol extract has been reported to inhibit the growth of yellow head baculovirus (YBV) in black tiger shrimp (Direkbusarakom *et al.*, 1998) while a compound from the CN fraction was able to inhibit the production of viral RNA as well as viral protein when A549 cells which have been infected with a serotype 2 of dengue virus (DV2) were cultured in it (Sittiso *et al.*, 2010). Another study by (Sookmai *et al.*, 2011) demonstrated that anti-HPV16 PsV infection (ability to inhibit infection of a synthetic HPV particle which contains L1 and L2 proteins as virulence factor of HPV16, a HPV strain which responsible for cervical cancer) of CN compounds (136C and 136D) that inhibit the early step of infection by direct binding between HPV particles and host cell receptor and also prevent HPV16 PsVs internalization. These suggested the potential role of the CN compounds on prevention of HPV infection.

In Indonesia, Malaysia, and Thailand, CN is also used to treat diabetes (Roosita *et al.*, 2008). Previously, a research conducted by Sugiri in 1980 showed that the water extract of CN leaves was able to lower the blood glucose level of the tested animal by glucose tolerance method with an active value of 64.77% compared to tolbutamid, a first-generation drug to treat type 2 diabetes (Nurulita *et al.*, 2008). Later, Nurulita *et al.* (2008) found that ethanol fraction of CN leaves was able to significantly decreased blood glucose serum level of mice after glucose

administration by the same method. On the other hand, the *in vitro* method reported by Alam *et al.* (2017a) revealed the highest potential α-glucosidase inhibitory activity. It was found that the butanol and ethyl acetate fractions showed the inhibition of 72.16 ± 1.0041% and 70.76 ± 0.4974%, respectively, at 200 µg/ml.

CN has been reported to treat various infectious or non-infectious diseases by ameliorating its anti-inflammatory an antioxidant activity. These include anaemia, diuretics, dysentery, dysuria, eye diseases, fever, hepatoma, jaundice, rashes or allergic responses, rheumatism and wounds from falls, fractures and contusions (Sakdarat *et al.*, 2006; Shim *et al.*, 2013; Tuntiwachwuttikul *et al.*, 2003; Uawonggul *et al.*, 2011). Results from *in vitro* and *in vivo* pharmacological investigation supported the claim of the notable dose-dependent activity of the CN in suppressing the inflammation inducer, ethylphenylpropiolate (EPP) and carrageenan (Wanikiat *et al.*, 2008). It was also reported that the effect of CN to neutrophil function and migration was due to the reduction of myeloperoxidase, an enzyme released in the body to aid in microbial killing in the EPP-induced rat ear edema model. Apart from that, CN has also been used traditionally, in Asia, to treat oxidative stress-related diseases such as cardiovascular disease (CVD), diabetes, and various kinds of cancers (Sarega *et al.*, 2016).

The leaves of CN were traditionally consumed as herbal tea or as a mixture in sugarcane juice, apple juice, and green tea (Alam *et al.*, 2016; Shim *et al.*, 2013). A study by Poonthananiwatkul *et al.* (2015) reported that CN has been used as part of a famous Thai traditional anticancer remedy known as “Ya-tan-mareng” in the province of Singburi. It was able to improve the blood profile, appetite, and sleeping pattern of patients. As for the high-value medicinal use, CN was reported as an endemic medicinal plant to Asia, which has prompted the National Drug Committee

of Thailand to recognize it as one of Thailand's National List of Essential Medicines (Saokaew *et al.*, 2015).

2.5.5 CN phytochemistry

Before explained in depth of CN phytochemistry, it is good to understand the main factors that determined the class of compound or phytochemicals from the plant prior extraction. The extraction yield and biological activity of the resulting extract is affected by the extraction method and the solvents used for extraction (Ajanal *et al.*, 2012). Many solvents, including methanol, ethanol, acetone, and water, have been used for extracting bioactive compounds from the plant material. Solvents used for the extraction of biomolecules from plants are chosen based on the polarity of the compound of interest. The presence of various compounds with different polarities and chemical characteristics may or may not be soluble in a particular solvent (Turkmen *et al.*, 2006). A solvent of similar polarity to the compound will properly dissolve the compound. Multiple solvents can be used sequentially to limit the number of analogous compounds in the desired yield. The polarity, from least polar to most polar, of a few common solvents is as follows: Hexane < Chloroform < Ethylacetate < Acetone < Methanol < Water (Altemimi *et al.*, 2017).

Polar solvents are frequently used for recovering polyphenols from plant matrices. The most suitable solvents are aqueous mixtures containing ethanol, methanol, acetone and ethyl acetate. Ethanol has been known as a good solvent for polyphenol extraction and is safe for human consumption. Methanol has been generally found to be more efficient in extracting lower molecular weight polyphenols, whereas aqueous acetone is good for extracting higher molecular weight flavanols (Dai & Mumper, 2010).

Non-polar solvent such hexane is suitable for extracting non-polar compounds like aliphatic hydrocarbons while ether and ethyl acetate are suitable for relatively polar compounds containing oxygen. Dichloromethane has high extraction efficiency for a wide range of non-polar to polar compounds. Dichloromethane is suitable for simultaneous analysis because it has low boiling point and easy to reconcentrate after extraction, non-flammable and easy to separate from water because of its higher specific gravity (Available from World Wide Web Standard Guidelines for the Environmental Monitoring of Chemicals, no date). Table 2.1 shows a summary of various extraction methods for natural products as explained by (Zhang *et al.*, 2018).

CN contains a wide range of bioactive compounds based on previous phytochemical investigation of this plant. Early phytochemical studies of CN by Boongerd, 1967 revealed the presence of myricyl alcohol (Kamarudin *et al.*, 2017). This was followed by finding of stigmasterol by Dampawan in 1976, lupeol, β-sitosterol (Dampawan *et al.*, 1977) and belutin (Kamarudin *et al.*, 2017). Six compounds of known C-glycosyl flavones involving isoorientin (homoorientin/luteolin-6-C-beta-D-glucopyranoside), isomollupentin 7-*O*-b-glucopyranoside, isovitexin (saponaretin/6-beta-D-glucopyranosyl-4',5,7-trihydroxyflavone), orientin (lutexin/luteolin), schaftoside and vitexin (apigenin/8-beta-D-glucopyranosyl-5,7-dihydroxy-2-(4hydroxyphenyl)-4*H*-1-benzopyran-4one) have been isolated from CN leaves and stem extractions using n-butanol and water-soluble portions of methanol (Sakdarat *et al.*, 2006; Teshima *et al.*, 1997).

In continuation of the finding, five sulfur-containing glucosides, namely clinacoside A, clinacoside B, clinacoside C, cycloclinacoside A1, and cycloclinacoside A2 were identified from the leaves and stems of CN

Table 2.1 A summary of various extraction methods for natural products

Method	**Solvent**	**Temperature**	**Pressure**	**Time**	**Volume of organic solvent consumed**	**Polarity of natural products extracted**
Maceration	Water, aqueous and non-aqueous solvents	Room temperature	Atmospheric	Long	Large	Dependent on extracting solvent
Percolation	Water, aqueous and non-aqueous solvents	Room temperature, occasionally under heat	Atmospheric	Long	Large	Dependent on extracting solvent
Decoction	Water	Under heat	Atmospheric	Moderate	None	Polar compounds
Reflux extraction	Aqueous and non-aqueous solvents	Under heat	Atmospheric	Moderate	Moderate	Dependent on extracting solvent
Soxhlet extraction	Organic solvents	Under heat	Atmospheric	Long	Moderate	Dependent on extracting solvent
Pressurized liquid extraction	Water, aqueous and non-aqueous solvents	Under heat	High	Short	Small	Dependent on extracting solvent
Supercritical fluid extraction	Supercritical fluid (usually S-CO2), sometimes with modifier	Near room temperature	High	Short	None or small	Nonpolar to moderate polar compounds
Ultrasound assisted extraction	Water, aqueous and non-aqueous solvents	Room temperature, or under heat	Atmospheric	Short	Moderate	Dependent on extracting solvent
Microwave assisted extraction	Water, aqueous and non-aqueous solvents	Room temperature	Atmospheric	Short	None or moderate	Dependent on extracting solvent
Pulsed electric field extraction	Water, aqueous and non-aqueous solvents	Room temperature, or under heat	Atmospheric	Short	Moderate	Dependent on extracting solvent
Enzyme assisted extraction	Water, aqueous and non-aqueous solvents	Room temperature, or heated after enzyme treatment	Atmospheric	Moderate	Moderate	Dependent on extracting solvent
Hydro distillation and steam distillation	Water	Under heat	Atmospheric	Long	None	Essential oil (usually non-polar)

Source: Zhang *et al.*, 2018

Teshima *et al.* (1997). The following finding reported two glycoglycerolipids (Satakhun *et al.*, 2001), β-galactosyl diglycerides (Janwitayanuchit *et al.*, 2003), a mixture of nine cerebroside and a monoacyl monogalatosylglycerol [(2S)-1-*O*-linolenoyl-3-*O*-b-D-galactopyranosyl glycerol] were isolated from the ethyl acetate-soluble fraction of the ethanol extract of CN fresh leaves (Tuntiwachwuttikul *et al.*, 2004). This was followed by the finding of chlorophyll derivatives, phaeophytins (Panyakom, 2006; Sakdarat *et al.*, 2009; Sittiso *et al.*, 2010).

Four new sulfur-containing compounds (clinamides A, clinamides B, clinamides C, and 2-cis-entadamide A) were isolated and identified together with three known sulfur-containing compounds (entadamide A, entadamide C and *trans*-3-methylsulfinyl-2-propenol) from the aerial parts of CN of the ethanol extract (Tu *et al.*, 2014). This was followed by rapid identification and quantification of the flavones C-glycosides through a two-step method using thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC) to identify shaftoside, isoorientin, orientin, isovitexin and vitexin of CN (Chelyn *et al.*, 2014). Later, seven compounds from a 30% ethanol extract were identified involving 6,8-apigenin-C-α-L-pyranarabinoside, apigenin 6,8-di-C-α-L-arabinopyranoside, isoorientin, isovitexin, orientin, shaftoside and vitexin (Huang *et al.*, 2015). Recently, two new sulfur-containing compounds, clinamide D and E were isolated from methanolic extracts of CN (Hamid *et al.*, 2016).

Through phytochemistry screening, CN has been reported to have various bioactive compounds. Alam *et al.*, (2017b) found major phytochemicals in the methanol extract of CN and its fractions (except for residual aqueous) such as α-hydroxylacids, dicarboxylic acids, terpenes, phenolics, inositols, fatty acids, coumarins, glycosides, phytosterols, polysiloxanes and polyols. Abdul Rahim *et*

al. (2016) reported that the UHPLC phytochemical screening analysis of CN methanol showed the presence of flavonoids of which the flavone C-glycoside was the major component, alongside with saponins, steroids and triterpenes.

Meanwhile, Othman *et al.* (2016) discovers that the ethanol extract of CN contained lupeol, lup-20(29)-en-3-one, β-amyrin, linoleic acid ethyl ester and squalene by GC-MS analysis. Khoo *et al.* (2015) found several newly reported compounds, identified using tandem mass spectrometry, included gendarucin A, gendarucin A isomer, 3,3-di-*O*-methylellagic acid, ascorbic acid and two isomeric oxoprolinates by UPLC-MS/MS analysis. In addition, Mustapa *et al.* (2015) carried out GC-MS analysis against the microwave assisted extraction (MAE), supercritical fluid extraction (SFE) and conventional Soxhlet extraction of the CN ethanolic extracts. Other than that, UPLC analysis was performed against MAE and pressurised-MAE of CN at different concentrations of ethanol (50 and 86% v/v). GC-MS analysis discovered the presence of diterpene, triterpene, fatty acids and other bioactive compounds where phytol was found as the major compound in MAE and Soxhlet while palmitic acid was the major component in the SFE extract. UPLC analysis detected high phenolic compounds, terpene glycosides and oxygenated fatty acids where identified phenolics belong to several classes of flavonoids, mainly phenolic acids, flavones, flavonol, flavanone, isoflavones, flavan- 3-ol, C-glycosylated flavone and anthocynidin. Both extracts of 50 and 86% v/v contained compounds of shaftoside, vitexin, isoorientin, with exception of 50% v/v which only present with catechin and gallic acid while 86% v/v present with kaempferol-7-neohesperidoside only.

Yong *et al.* (2013) performed GC-MS on chloroform extract of CN and reported the major constituent of 2-benzenedicarboxylic acid, mono-(2-ethylhexyl)

ester. Later, Ng *et al.* (2017) reported that (*Z,Z*)-9,12-octadecadienoic acid was the major constituent from the hexane extract of CN. HPLC-IT/MS analysis of the methanol extract of CN by Quah *et al.* (2017) identified the presence of schaftoside, vitexin and orientin. HPLC-DAD detected protocatechuic acid, cinnamic acid, gallic acid, caffeic acid, ferulic acid and *p*-coumaric acid in the hot water fraction. The cinnamic acid, gallic acid and *p*-coumaric and chlorogenic acid were not found in its crude. The 80% methanol fractions also showed the presence of vanillic acid, gallic acid and *p*-coumaric acid, which were not found in its crude extract. Both the crude extracts of water and 80% methanol showed high amounts of *p*-coumaric acid with their ethyl acetate fractions showing the *p*-coumaric acid concentrations around 6- and 3.6-fold higher, respectively (Sarega *et al.*, 2016). Teoh *et al.* (2017) found that ethyl acetate root extract of CN consisted of more compounds compared to the methanol root extract by GC-MS analysis with lupeol as the most abundant compound in both root extracts.

2.5.6 Cytotoxic and anticancer properties of CN

Some reported studies have demonstrated that CN might be effective against certain cancer cell lines. The chloroform extract of CN has been reported to show the highest antiproliferative effect against human erythroleukemia K-562 cell line (IC_{50} of 47.70 μg/ml) and human Burkitt's lymphoma Raji cell line (IC_{50} of 47.31 μg/ml) but not on the human neuroblastoma IMR-32 cell line (Yong *et al.*, 2013). Another study reported that the methanol extract of CN showed cytotoxic activity against Hep G2 and MDA-MB-231 cells with IC_{50} of 13.3 and 18.7 μg/ml, respectively, but not with A549, HT-29, MCF-7 and CRL 1739 (Quah *et al.*, 2017). Nasir and Mohd Bohari (2015) found that methanol extract of CN inhibited proliferation of MDA-MB

123 (IC_{50}: 170 µg/ml 170) at moderate rate as compared to normal cells, CHO (IC_{50}: 240 µg/ml 170). Wang *et al.* (2019) found that ethyl acetate fraction of CN gave the least IC_{50} values among other fractions when tested against various cancer cell lines with the most potent activity against HCT116 cell line.

Other than that, different plant parts involving leaf, bark, stem and roots may differ in their efficacy against cancer cells. A study reported by Arullappan *et al.* (2014) showed that petroleum ether extract from the leaves of CN was cytotoxic against HeLa (IC_{50}: 18 µg/mL) but not from the stem (IC_{50}: >100 µg/ml). However, ethyl acetate extract from the stem was cytotoxic (IC_{50}: 20 µg/ml) compared to the leaf (IC_{50}: 92 µg/ml) against the same cells. Another study by Teoh *et al.* (2017) found that ethyl acetate and methanol extract from the CN root were effective against MCF-7 cells with IC_{50} at 30 and 35 µg/mL each compared to HeLa that slightly showed growth inhibition at about 20 to 40% for concentrations of 40 to 50 µg/ml each which did not reach 50% at the tested concentrations.

Besides that, different solvent extracts contribute to different cytotoxic activity. Kunsorn *et al.* (2013) found that DCM extract of CN leaf gave the least IC_{50} result (869 µg/ml) compared to n-hexane and methanol extracts (>1600 µg/ml each). Hamid and Yahaya (2016) on the other side used hexane, ethyl acetate, chloroform and methanol extracts of CN leaf against HepG2 cells to obtain IC_{50} values of 68.38, 62.07, 55.61 and 43.93 µg/ml each. Haron *et al.* (2019) tested hexane, DCM, aqueous, 80% methanol and water extracts of CN against HeLa cells at three time points (24, 48 and 72 hours each) to obtain IC_{50} values of 200, 185 and 170; >4000, 1700 and 700; 1300, 650 and 650; and 4000, 1800 and 1500 µg/ml each. Ismail *et al.* (2020) tested hexane, DCM, chloroform, n-butanol, aqueous and 80% methanol

extracts of CN against MCF7 cells at 72 hours to obtain IC_{50} values of 50.34±0.11, 65.95±0.14, 67.52±0.17, 111.50±0.20, 496.50±0.45 and 398.00±0.24 µg/ml each.

Besides these, certain purified compounds from CN crude extracts has been used to test against certain cell lines. For example, Janwitayanuchit *et al.* (2003) tested synthesized compound from monoglycosyl diglycerides of CN against Vero cells to obtain IC_{50} of >100 µg/ml. Besides, Pongmuangmul *et al.* (2016) isolated monogalactosyl diglyceride (MGDG) and digalactosyl diglyceride (DGDG) from chloroform crude extract of CN to obtain IC_{50} values of 955.00 ± 7.00 and 922.00 ± 4.00 ug/ml each including the crude (523.00 ± 4.00). Huang *et al.* (2015) showed that polysaccharide fraction from CN (CNP-1-2) showed 37.88 ± 1.28% to 92.34 ± 0.94% inhibitory percentage as applied at the range of 50 to 200 µg/ml against SGC-7901 cells.

Other than that, other studies tested specific cancer cell line with specified CN extracts for obtaining IC_{50} value, such as Liew *et al.* (2012) (Saos-2; Methanol leaf extract; IC_{50}: not toxic – tested until 2000 µg/ml), Fong *et al.* (2019) (D24; Water leaf extract; IC_{50}: 950 and 770 µg/ml at 24 and 72 hours each), Fazil *et al.* (2016) (A549; Water leaf extract; IC_{50}: 138.82 ± 0.60 µg/ml), Abdul Ghani *et al.* (2015) (A549; Acetonitrile leaf extract; IC_{50}: 20 mg/ml), Yoosook *et al.* (1999) (Vero; Whole plant methanol extract; IC_{50}: 6670 µg/ml) and Na-Bangchang *et al.* (2012) (KB; Water extract from remedies containing CN; IC_{50}: >100 µg/ml).

Other than these, the age of CN plant is another factor to be considered as study by Ghasemzadeh *et al.* (2014) has reported that younger plant age of CN exhibited the most cytotoxic activity against HeLa whenever the IC_{50} value for 6-month and 1-year-old buds were of 56.8 and 110.4 µg/ml, respectively. Besides this, more recent studies of CN cytotoxicity were focused on combining CN extract with