Faculty of Medicine and Health Sciences

In-vitro Study of Cytotoxicity and Apoptosis Effects of *Clinacanthus nutans* (Burm.f.) Lindau Extracts on Colorectal Cancer Cell Lines, HT-29 and HCT-116

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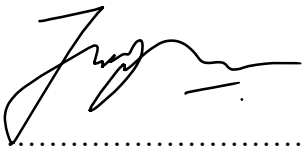
DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.



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ABSTRACT

Cancer is one of the leading causes of mortality in Malaysia, with colorectal cancer as the most common cancer in males and the second most common cancer in females. Colorectal cancer causes high mortality as it is hardly detected during the early stage yet has a 15-year window of intervention. Hence, the discovery of the chemoprevention method is important to delay carcinogenesis and prevent the recurrence of cancer. *Clinacanthus nutans* (Burm.f.) Lindau (*C. nutans*), from *Acanthaceae* family, is known as Sabah Snake Grass or '*Belalai Gajah*' in Malaysia. It is popular in Southeast Asia for its medicinal properties such as anticancer, anti-inflammation and antiviral properties. Therefore, this study aimed to determine the total phenolic content (TPC), total flavonoid content (TFC), antioxidant activity, and anticancer properties of *C. nutans* leaves extracts on human colorectal cancer cell lines, HCT-116 and HT-29 in dose- and time-dependent manners. The ground *C. nutans* leaves were extracted with methanol, chloroform, and acetone for 30 minutes and 24 hours, respectively. The TPC and TFC were determined spectrophotometrically using the Folin-Ciocalteu and aluminium chloride colourimetric methods. 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay was used to determine the antioxidant activity. The antiproliferation and apoptotic capabilities were studied using MTT and apoptosis (Annexin V-FITC and PI staining method) assays, respectively. *C. nutans* methanol extracts and 24 hours of extraction time resulted in higher extraction yield (7.65g ± 0.13) than its counterparts. Acetone extracted *C. nutans* at 30 minutes and 24 hours resulted in the highest TPC values of 3.06 mg GAE/g and 3.18 mg GAE/g, respectively. On the other hand, methanol extracted *C. nutans* at 30 minutes and 24 hours exhibited the highest TFC (0.49 mg QE/g and 0.50 mg QE/g, respectively) and strongest antioxidant activity (IC_{50} values at 24.25 μg/mL and 19.67 μg/mL; AAEAC at 22.14% and 27.31%, respectively) than

chloroform and acetone extracts. The methanol extracts were selected for *in vitro* study. HCT-116 cells revealed higher antiproliferative effects than HT-29 cells using methanol extracted *C. nutans* at 30 minutes when treated for 24 hours and 72 hours. On the contrary, HCT-116 cells exhibited lower antiproliferative effects when treated for 48 hours. Methanol extracted *C. nutans* at 24 hours exhibited stronger antiproliferation activity when treated on HT-29 cells with less cytotoxicity to normal cells for 24, 48, and 72 hours. In addition, a low dose of 250 μg/mL methanol extracted *C. nutans* with 24 hours extraction time induced early and late apoptosis in HCT-116 and HT-29 cells after treatment for 72 hours. The methanol extract increased the late apoptosis of HCT-116 cells from 18.88% to 35.66% when treatment from 24 hours to 72 hours, respectively. The late apoptotic rate of HT-29 cells was increased from 14.28% to 20.67% when treatment was prolonged from 24 hours to 72 hours, respectively. In conclusion, methanol extracted *C. nutans* that underwent 24 hours extraction contained high TFC, exhibited high antioxidant activity, and able to inhibit the early and late stages of colorectal cancer cell growth by triggering apoptosis in dose- and time-dependent manners. The findings suggested that *C. nutans* possessed anticancer properties against colorectal cancer cell lines, HCT-116 and HT-29 through the apoptosis pathway. The findings provided new perspective for the future study to prove *C. nutans* as an alternative treatment against early and late stages colorectal cancer.

Keywords: *Clinacanthus nutans*, colorectal cancer, antioxidant, anticancer, apoptosis

Kajian In-vitro tentang Kesan Sitotosisiti dan Apoptosis Clinacanthus nutans (Burm.f.) Lindau pada Sel Kanser Kolorektal, HT-29 dan HCT-116

ABSTRAK

Kanser adalah salah satu punca utama kematian di Malaysia dengan kanser kolorektal sebagai kanser paling biasa di kalangan lelaki dan kanser kedua paling biasa di kalangan wanita. Kanser kolorektal menyebabkan kematian yang tinggi kerana ia jarang dikesan pada peringkat awal tetapi mempunyai tempoh intervensi selama 15 tahun. Oleh itu, penemuan kaedah kemopencegahan adalah penting untuk melambatkan karsinogenesis dan mencegah berulangnya kanser. Clinacanthus nutans (Burm.f.) Lindau (C. nutans), daripada keluarga Acanthaceae, dikenali sebagai 'Sabah Snake Grass' atau Belalai Gajah di Malaysia. Ia popular di Asia Tenggara dengan khasiat perubatannya seperti antikanser, anti-radang dan antivirus. Oleh itu, kajian ini bertujuan untuk menentukan jumlah kandungan fenolik (TPC), jumlah kandungan flavonoid (TFC), aktiviti antioksidan, dan sifat antikanser ekstrak daun C. nutans pada sel kanser kolorektal manusia, HCT-116 dan HT-29 dengan cara yang bergantung kepada dos dan masa. Daun C. nutans yang telah dikisar, diekstrak dengan metanol, kloroform, dan aseton selama 30 minit dan 24 jam. TPC dan TFC ditentukan secara spektrofotometri dengan menggunakan kaedah Folin-Ciocalteu dan kolorimetrik aluminium klorida. Ujian penghapusan radikal 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) digunakan untuk menentukan aktiviti antioksidan. Kemampuan antiproliferasi dan apoptosis telah dikaji melalui ujian MTT dan apoptosis (kaedah pewarnaan Annexin V-FITC dan PI). Ekstrak metanol C. nutans dan 24 jam masa pengekstrakan menghasilkan hasil pengekstrakan yang lebih tinggi (7.65g ± 0.13). Ekstrak aseton C. nutans pada 30 minit dan 24 jam menghasilkan nilai TPC tertinggi iaitu 3.06 mg GAE/g dan 3.18 mg GAE/g, masing-masing. Sebaliknya, ekstrak metanol C. nutans pada 30 minit dan 24 jam

menunjukkan TFC tertinggi (0.49 mg QE/g dan 0.50 mg QE/g) dan aktiviti antioksidan tertinggi (IC_{50} pada 24.25 μg/mL dan 19.67 μg/mL; AAEAC pada 22.14% dan 27.31%, masing-masing) daripada ekstrak kloroform dan aseton. Ekstrak metanol kemudiannya dipilih untuk meneruskan kajian in vitro. HCT-116 mendedahkan kesan antiproliferasi yang lebih tinggi berbanding sel HT-29 menggunakan ekstrak metanol C. nutans dalam 30 minit apabila dirawat selama 24 jam dan 72 jam. Sebaliknya, sel HCT-116 mempamerkan kesan antiproliferasi yang lebih rendah apabila dirawat pada 48 jam. Selain itu, ekstrak metanol C. nutans dalam 24 jam menunjukkan aktiviti antiproliferasi yang lebih kuat apabila dirawat pada sel HT-29 dengan kurang toksik kepada sel normal selama 24, 48, dan 72 jam. Di samping itu, dos rendah 250 μg/mL ekstrak metanol C. nutans dalam 24 jam menyebabkan apoptosis awal dan lewat dalam sel HCT-116 dan HT-29 selepas rawatan selama 72 jam. Ekstrak metanol meningkatkan apoptosis lewat sel HCT-116 daripada 18.88% kepada 35.66% apabila rawatan daripada 24 jam kepada 72 jam, masing-masing. Selain itu, apoptosis lewat sel HT-29 meningkat daripada 14.28% kepada 20.67% apabila rawatan daripada 24 jam kepada 72 jam, masing-masing. Kesimpulannya, ekstrak metanol C. nutans pada 24 jam mengandungi TFC yang tinggi dan mempamerkan aktiviti antioksidan yang tinggi di samping dapat menghalang peringkat awal dan akhir pertumbuhan sel kanser kolorektal dengan mencetuskan apoptosis dalam cara yang bergantung kepada dos dan masa. Penemuan mencadangkan bahawa C. nutans mempunyai sifat antikanser terhadap garisan sel kanser kolorektal, HCT-116 dan HT-29 melalui laluan apoptosis. Penemuan ini memberikan perspektif baru untuk kajian masa depan untuk membuktikan C. nutans sebagai rawatan alternatif terhadap kanser kolorektal peringkat awal dan akhir.

Kata kunci: *Clinacanthus nutans, kanser kolorektal, antioksidan, antikanser, apoptosis*

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LIST OF ABBREVIATIONS

ABTS	2, 2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
C. nutans	*Clinacanthus nutans*
CRC	Colorectal cancer
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
FRAP	Fluorescence recovery after photobleaching
IFN-γ	Interferon gamma
IL	Interleukin
HCT-116	Human epithelial colorectal carcinoma cell line
HT-29	Human epithelial colorectal adenocarcinoma cell line
LPS	Lipopolysaccharide
MDA	Malondialdehyde
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NO	Nitrite oxide
PBS	Phosphate-buffered saline
PI	Propidium iodide
PS	Phospholipid phosphatidylserine
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SRB	Sulforhodamine B

TFC	Total flavonoid content
TLR-4	Toll-like receptor 4
TNF-α	Tumour necrosis factor alpha
TPC	Total phenolic content

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Cancer is one of the leading causes of non-communicable disease mortality with approximately 10 million mortalities worldwide in 2020 (WHO, 2022). World Health Organisation (WHO) reported that colorectal cancer was the third most common cancer and second most common cause of global mortality in 2020 (WHO, 2022). In Malaysia, colorectal cancer was the most common cancer in males and the second most common cancer in females, as stated in the Malaysia National Cancer Registry Report 2012-2016 (Azizah et al., 2019). Due to the hidden nature of its site, it is often left undetected until it is too far along with its progression and formation of hyperplastic and gradually dysplastic cells. These cells eventually become aberrant crypt foci (ACF) and polyps. Undetected polyps lead to a later stage of malignant carcinomas, also known as colorectal cancer (Pandurangan et al., 2018). At present, the routine treatment and management regime include surgical removal of tumour, chemotherapy, radiotherapy, or a combination of these three procedures. These procedures come with massive burdens in terms of human capital, cost of treatment and hospitalisation, and oftentimes, lives (Veettil et al., 2017; Costea et al., 2018). Therefore, it is pertinent to searching for alternative, less invasive treatments with low or no side effect intervention as a chemoprevention method against colorectal cancer.

1.2 Plants as Chemopreventive Agents

Chemoprevention is known to slow down and inhibit carcinogenesis or prevent the reappearance of cancer by using natural products or synthetic molecules (Siddiqui et al., 2015; Melo et al., 2018). Chemopreventive agents inhibit carcinogens from affecting DNA, aid in repairing DNA, delay the cell cycle process, and inhibit metastasis by affecting the related events (Melo et al., 2018).

There are three types of chemopreventive agents such as cancer formation inhibitors, suppressing agents, and blocking agents (**Figure 1.1**). Chemopreventive agents that act as inhibitors prevent the formation of cancer while suppressing agents exhibit antiproliferation effects by triggering apoptosis, necrosis, and autophagy to suppress the cancer progression. Blocking agents interfere with the carcinogenesis pathways and capture certain reactive oxygen species (ROS) by inducing the detoxification process (George et al., 2021). Chemopreventive compounds scavenge free radicals or activate enzymes that require scavenge free radicals to inhibit DNA alteration by a carcinogen (Melo et al., 2018). ROS and reactive nitrogen species (RNS) are intracellular free radicals usually produced in cell metabolic activities as by-products (Yong et al., 2013). Oxidative stress is occurred when ROS production is increased due to environmental factors and intracellular ROS (Aggarwal et al., 2019). Oxidative stress tends to cause cancer, diabetes mellitus, chronic inflammation, and atherosclerosis (Valko et al., 2007).

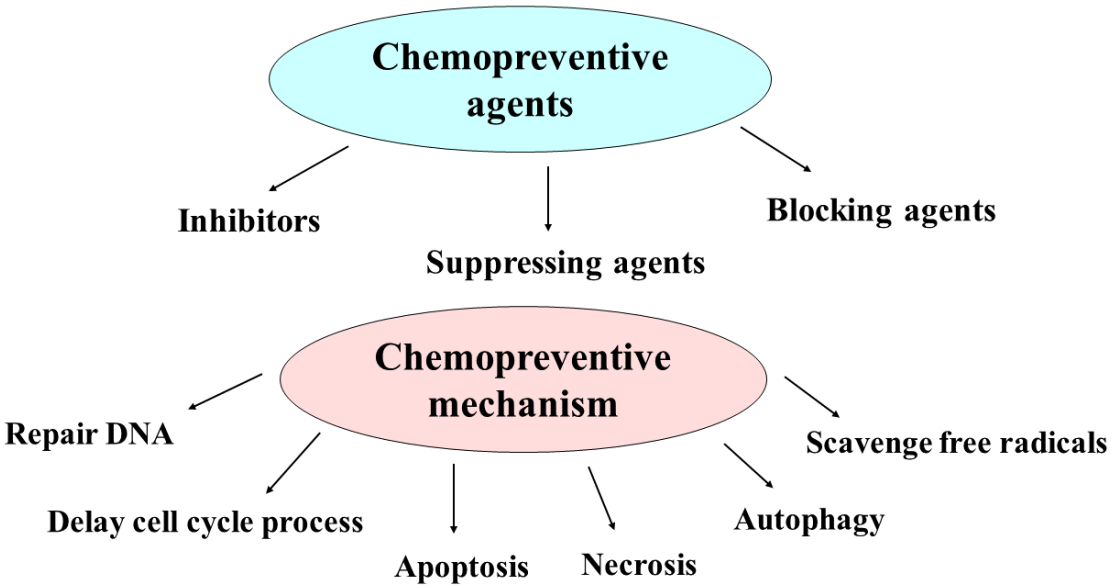


Figure 1.1: Types of chemopreventive agents and examples of chemopreventive mechanism.

Medicinal plants have been reported to elicit numerous efficacious benefits to our health (Zulkipli et al., 2017). When these are consumed or applied topically, a plethora of phytochemicals or naturally produced secondary metabolites exhibit medicinal properties, including anticancer, anti-inflammatory, and antimicrobial activities (Zulkipli et al., 2017). Some phytochemicals are natural antioxidants that work as free radical acceptors and hydrogen donors to suppress the oxidation reaction (Shahidi & Zhong, 2015). The antioxidant activity of phytochemicals is beneficial to our health as it can eliminate the excess ROS or RNS and stabilise the oxidant level in our body. Furthermore, bioactive compounds in plants have also been proven to trigger apoptosis, suppress cancer growth, and arrest the cell cycle (Zlotogorski et al., 2013; Schaffer et al., 2015; Sheikh et al., 2017a; Sheikh et al., 2017b; Tungmunnithum et al., 2018). Paclitaxel, also known as Taxol, is a common chemo drug that is derived from the bark of *Taxus brevifolia* Nutt. (Ashraf, 2020). Another example of anticancer compound that is derived from medicinal plant is vinblastine

from *Catharanthus roseus* G. Don. (Ashraf, 2020). Vinblastine in combination with vincristine and other chemo drugs is commonly used in a wide range of cancers especially lung cancer, breast cancer, and leukaemia (Ashraf, 2020). Therefore, the search for natural promising chemopreventive agents against cancer formation and metastasis provides an alternative to these expansive colorectal cancer treatments is strongly justified.

Phenolic compounds play important roles in inhibiting carcinogenesis stages; they are popular in chemoprevention (George et al., 2021). Phenolic compounds are common secondary metabolites in plants such as vegetables, fruits, and tea. The structure of phenolic compounds contains non-polar and polar components. The non-polar component is an aromatic ring with an attachment of polar part, which is single or multiple hydroxyl groups (Queimada et al., 2009). Phenolic compounds have strong antioxidant properties, and they are divided into flavonoid, and non-flavonoid based on the chemical structure (Costea et al., 2018). Examples of flavonoids are flavanones, isoflavonoids, flavonols, flavones, anthocyanidins, and flavonols (Manach & Donovan, 2004; Durazzo et al., 2019; Mark et al., 2019), whereas non-flavonoids are lignans, tannins, stilbenes, and phenol carboxylic acids (Caleja et al., 2017; Domínguez-Avila et al., 2017; Costea et al., 2018). Quercetin, a type of flavonol contained in apples and onion, has been reported to possess anticancer effects against colorectal cancer by downregulating altered genes and the synergistic manner in combination with chemo drugs (Costea et al., 2018).

1.2.1 Clinacanthus nutans

Clinacanthus nutans (*C. nutans*), from the Acanthaceae family, is also known as Sabah Snake Grass in English and '*Belalai Gajah*' in Bahasa Malaysia. *C. nutans* is a perennial herb that can grow between 1 to 3 m tall with pubescent branches and smooth,

striate, and cylindrical stems. It can be widely found in Southeast Asia and China (Yahaya et al., 2015; Alam et al., 2016; Zulkipli et al., 2017). The leaves are long and narrowly V-shaped with alternate opposite leave arrangements (Alam et al., 2016; Zulkipli et al., 2017). It is a reputable medicinal plant in Southeast Asia and is widely used in Thailand and Malaysia in treating inflammation, some cancers, and *Herpes simplex* virus infections (Zulkipli et al., 2017). It is commonly brewed and consumed as herbal tea in Malaysia, particularly treat diabetes (Alam et al., 2016). In addition, fresh leaves are also blended with apple, green tea, or sugarcane prior to consumption (Yahaya et al., 2015). It is also used topically as anti-snake venom in Thailand as it has anti-cell lysis properties (Alam et al., 2016). The ethnobotany uses of *C. nutans* from different geographical regions were summarised in **Table 1.1**.

Figure 1.2: *Clinacanthus nutans*

Table 1.1: Ethnobotany uses of *C. nutans* from different geographical regions

Preparation method	Effect	Regions	Reference
Boiled fresh leaves with water.	Treatment for diabetes	Malaysia	Alam et al., 2016 P'ng et al., 2013 Yahaya et al., 2015
Served as juice by blending the leaves.	Anti-cancer		
Alcohol extraction of leaves are applied on skin.	Treatment for varicella-zoster virus (VZV), herpes simplex virus (HSV) lesions, skin rashes, and insect and snake bite.	Thailand	Sookmai et al., 2011 Alam et al., 2016
Consumed as raw or blended with sugarcane, juice, or green tea	Anti-snake venom		
	Treatment for scorpion bites and nettle rash		
A handful of leaves is cooked in five glasses of water.	Treatment for dysentery, diabetes, dysuria, and fever	Indonesia	Alam et al., 2016
About 7 to 21 leaves is boiled in two glasses of water.			
Decoction of leaves.			

1.3 Problem Statement

Colorectal cancer is one of the leading causes of death in worldwide. The development of polyp to malignant colorectal cancer takes 10 to 15 years (Hossain et al., 2022). From this long-term malignancy development, it is believed that there is an opportunity for immediate treatment before progressing into late stage of cancer. Previous expansive studies on *C. nutans* revealed its pharmacological activities, such as antioxidant, antiviral, anti-inflammation and anticancer (Zulkipli et al., 2017). For example, previous studies showed that *C. nutans* extract inhibited the cell growth of colorectal cancer cell line, HCT-116 and induced apoptosis in cervical cancer cell, HeLa (Esmailli et al., 2016; Haron et al., 2019). Therefore, it is important to study the purported medicinal potential of *C. nutans* in treating colorectal cancer by determining the anticancer effects on early and late stage of colorectal cancer cells.

1.4 Objectives of Study

This study has achieved the following specific objectives:

i. To determine the total phenolic content (TPC), total flavonoid content (TFC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of different *C. nutans* solvent extracts.

ii. To assess the antiproliferation activity of *C. nutans* extracts against HCT-116 and HT-29 cell lines using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in time- and dose-dependent manners.

iii. To investigate the cytotoxicity effects of *C. nutans* extracts on the early and late apoptotic pathways in HCT-116 and HT-29 cells using Annexin V-FITC and PI staining method in dose- and time-dependent manners.

1.5 Chapter Summary

Colorectal cancer (CRC) is one of the leading causes of cancer mortality in Malaysia. *Clinacanthus nutans* (*C. nutans*) is one of many herbal plants which its natural products have been reported to demonstrate antioxidant, antiviral, anti-inflammation, and anticancer activities. Hence, it is important to study the effects of *C. nutans* on two human colorectal cancer cell lines of different progression stages in dose- and time-dependent manners. Therefore, this study aimed to determine the antioxidant properties and anticancer activities against HCT-116, and HT-29 cell lines, which may indicate *C. nutans* as promising alternative chemotherapy agents for CRC treatment.

CHAPTER 2

LITERATURE REVIEW

2.1 Carcinogenesis

Many factors induce cancer formation. The initiation of carcinomas or tumours is due to DNA alteration. It is difficult to predict the exact cause of DNA or gene alteration. However, some conditions such as underlying cancer history of the family, unhealthy lifestyle or environmental exposure, and virus infections are the common causes of cancer formation (Melo et al., 2018). The DNA alteration of cells inactivates tumour suppressor gene, activates the oncogene, and inhibits the DNA repair mechanism, such as apoptosis mechanisms (Hanahan & Weinberg, 2000; Hanahan & Weinberg, 2011; Fouad & Aanei, 2017). This causes the continuous proliferation of cells and forms tumour, which may become metastatic and spread to other parts of the body through blood or lymph.

Cancer formation, carcinogenesis, is divided into four stages: initiation, promotion, progression, and metastasis. DNA alteration occurs in the initiation stage of carcinogenesis either triggered spontaneously or by carcinogenic agents. After altering gene, for instance, suppressor gene, the cells are actively proliferating. These mutated cells are called preneoplastic cells. In the promotion stage, the accumulation of mutations in preneoplastic cells occurs. Subsequently, in the progression stage, these preneoplastic cells become neoplastic cells that multiply rapidly. These become either benign or malignant. In some severe advanced cases, the metastasis stage occurrs. In this stage, the cancer cells break off and spread to the other part of the body from the original place through the blood or lymph system (Siddiqui et al., 2015).

Different types of cancer are formed by different types of cells, such as squamous cells or epithelial cells. The most common cancer type is carcinoma, which originated from epithelial cells. There are a few types of carcinomas; adenocarcinoma, squamous cell carcinoma, basal cell carcinoma, and transitional cell carcinoma. Adenocarcinoma is formed in glandular tissues, which consist of epithelial cells that secrete mucus and fluid. Examples of adenocarcinomas are colorectal, breast, and pancreatic cancers. Squamous cell carcinoma or epidermoid carcinoma originated from squamous cells. Squamous cells are epithelial cells that grow on the outermost layer of the skin (National Cancer Institute, 2021).

On the other hand, basal cell carcinomas are formed in the bottom layer of the epidermis. Transitional cell carcinoma is developed in the transitional urothelium or epithelium tissue. For example, transitional cell carcinomas are kidney and bladder cancers (National Cancer Institute, 2021).

2.2 Colorectal Cancer

Colorectal cancer (CRC) is a type of cancer that forms in the colon or rectum. CRC is classified into five stages depending on the severity. Stage 0 involves the cancerous tumour that grows in the inner lining of the mucosa. Stage I denotes when the cancer cells grow through the mucosa into the submucosa in the muscular region of the colon. Stage II can be divided into three types depending on the sites; the colon lining, muscular lining of the colon, and neighbouring tissues. In Stage III, the cancerous cells start to spread into lymph nodes through the muscular layer of the colon. Lymph nodules are formed and categorised into three types: IIIA, IIIB, and IIIC based on the number of nodules. Stage IV

is the severe stage when the cancerous cells become metastatic and spread to the other part of the body (Pandurangan et al., 2018).

Furthermore, CRC stages are also classified by using Dukes' staging system, such as Dukes' A, B, C, and D. Dukes' A is when the tumour is located in the inner layer of the bowel. Dukes' B is when the tumour grows bigger and crosses the muscle layer. Dukes' C is when the tumour has started to proliferate to the lymph node near the bowel. Dukes' D is when the tumour becomes metastatic and is similar to stage IV cancer (Cancer Research UK, 2018).

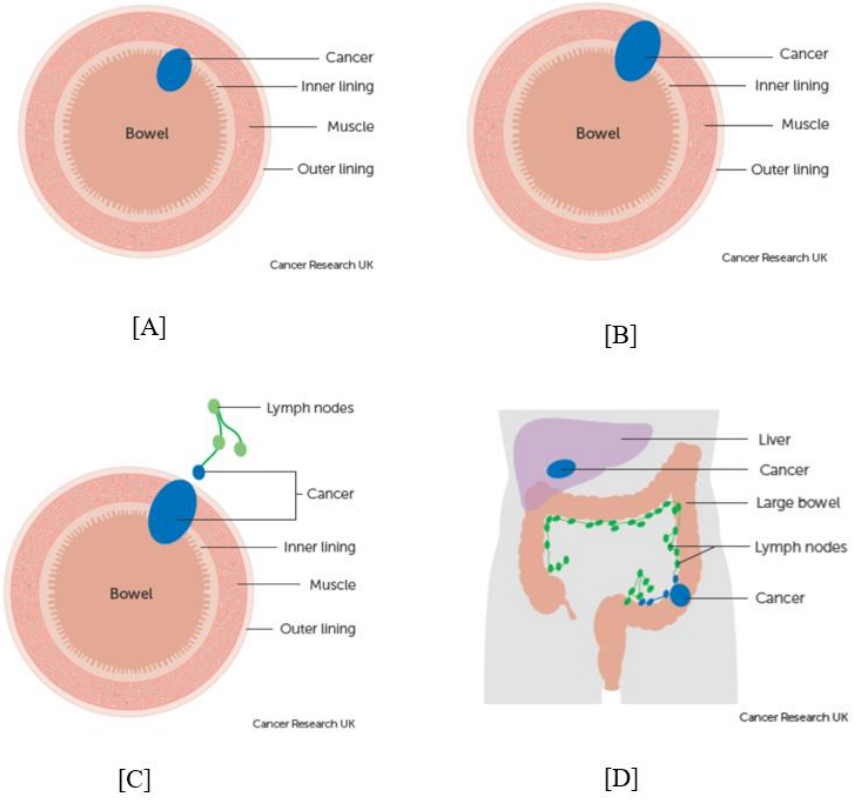


Figure 2.1: Dukes' Staging System that classified the stages of colorectal cancer.

[A] represents Dukes' A; [B] represents Dukes' B; [C] represents Dukes' C; [D] represents Dukes' D. Pictures are adapted from Cancer Research UK (2018).

2.2.1 Colorectal Cancer HCT-116 Cell Line

HCT-116 is a human colorectal carcinoma cell line with adherent culture properties and epithelial morphology (ATCC, 2021). HCT-116 is originated from the large intestine tissue of an adult male with colorectal cancer (ATCC, 2021). HCT-116 is commonly used for *in-vitro* studies on colorectal cancer. A study by Tragulpakseerojn et al. on chemoprevention of *Moringa oleifera Lam* leaf extract on HCT-116 showed that *M. oleifera* leaf extract inhibited phosphorylation of ERK1/2, reduced AKT expression, and caused antiproliferation of HCT-116 (Tragulpakseerojn et al., 2017). Nobiletin, a polymethoxyflavone is one of the flavonoids exclusively extracted from citrus peel. Nobiletin and its derivatives significantly inhibited HCT-116 cells growth by inducing cell cycle arrest and apoptosis (Goh et al., 2019). Moreover, luteolin, a type of flavonoid commonly found in vegetables and fruits, effectively inhibited HCT-116 cells in a dose-dependent manner (Zuo et al., 2018). Additionally, luteolin enhanced the inhibition of HCT-116 cells growth in combination with chemotherapy drug, oxaliplatin (Jang et al., 2019). In short, HCT-116 cell line is widely used in studying anticancer properties of natural products and phytochemicals on colorectal cancer.

2.2.2 Colorectal Cancer HT-29 Cell Line

HT-29 is a colorectal adenocarcinoma cell line that originated from the primary tumour of a 44-year-old Caucasian female in 1964 (Fogh & Trempe, 1975). HT-29 is a Dukes' C stage of adenocarcinoma with T2-3 N1 M0 grade where T denotes size and extension of the tumour, N denotes the degree of node spread, and M denotes the presence of metastasis (Techawathanawanna et al., 2012; Armistead et al., 2020). HT-29 is also

commonly used to test the anticancer effects of functional foods and phytochemicals. Metformin, a biguanide derivative, inhibited HT-29 cells by apoptosis and autophagy in time- and dose-dependent manners (Sena et al., 2018). Studies also had been conducted on HT-29 using silymarin and capsaicin by Ginghină and the team. The study reported a decrease in the cell viability of HT-29, changes in morphology, and apoptosis was triggered by treating these cells with silymarin and capsaicin separately (Ginghină et al., 2017). Furthermore, HT-29 cell proliferation has been reported to be suppressed by luteolin in a dose-dependent manner (Zuo et al., 2018).

2.3 Acanthaceae Family with Anticancer Effects

The Acanthaceae family is from the Lamiales order. It consists of 220 genera and 4000 species (Manjula & Sankar, 2021). Most of the plants in the family possess therapeutic functions including anti-diabetic, wound healing, antibacterial, anticancer, and anti-inflammation (Manjula & Sankar, 2021). Besides *C. nutans* , *Acanthaceae* family consists of various species that are commonly used in traditional home remedy such as *Graptophyllum pictum*, *Justicia gendarussa*, *Strobilanthes crispus*, and *Andrographis paniculata*.

Graptophyllum pictum (L.) Griff is a native plant from Papua New Guinea. It is often used in Southeast Asia, particularly in Thailand and Indonesia. In Indonesia, it is known as '*daun ungu*' and '*Bai Ngeoun*' in Thailand (Jiangseubchatveera et al., 2017; Amin et al., 2020). *G. pictum* is used as a part of traditional remedies to treat bowel problems such as haemorrhoids, constipation, and gall stone. The leaves are also made into a paste to aid wound healing, cuts, and ulcers (Jiangseubchatveera et al., 2017). The leaves' hexane, ethyl

acetate, and aqueous fractions exhibited cytotoxic effects on the human breast adenocarcinoma cell line MCF-7 without being harmful to normal cells, Vero cells (Jiangseubchatveera et al., 2017). Additionally, the hexane fraction of *G. pictum* leaves extract showed the most cytotoxic among the other solvent extracts on human colon cancer cell line, WiDr, using MTT assay (Amin et al., 2020).

Justicia gendarussa Burm F. is a medicinal plant from the Acanthaceae family that is widely found in Asia, namely Malaysia, Indonesia, Thailand, and China (Putri et al., 2020). *J. gendarussa* was reported to be able to treat cancer, fever, jaundice, and respiratory problems (Subramanian et al., 2012). Few studies have proved the anticancer properties of *J. gendarussa*. The ethanol extract of *J. gendarussa* leaves was found to be cytotoxic on human cervical cancer cell line HeLa and liver carcinoma cell line HepG2 (Mangai, 2017). Moreover, methanol extracted *J. gendarussa* leaves showed antiproliferative activity on breast cancer cell lines, MDA-MB-468 and MDA-MB-231 (Ayob et al., 2014).

Strobilantus crispus, one of the plants from the Acanthaceae family, is commonly used as traditional medicine in Malaysia and Indonesia (Ng et al., 2021). *S. crispus* is usually consumed as a tea or eaten fresh (Ng et al., 2021). *S. crispus* is purported to provide several advantages to health such as treatment for constipation, diabetes, and prevention of cancer by boosting immunity (Ng et al., 2021). *S. crispus* was broadly studied on its *in vitro* and *in vivo* anticancer effects against various types of cancers including cancer of the breast, liver, colon, prostate, cervical, nasopharyngeal, and lung (Ng et al., 2021). Ethanol extract of *S. crispus* leaves possessed anticancer effect on breast cancer cell line, MCF-7 through apoptosis by upregulating the level of p53, caspase 3/7 and cdk2 protein (Chong et al., 2012). Hexane extract of *S. crispus* stems exhibited cytotoxic on liver cancer cell line, HepG-2 and

proved to induce cell cycle arrest at G0/G1 phase and caspase 8-mediated apoptosis (Koh et al., 2017). *S. crispus* isolated γ-sitosterol exhibited IC_{50} value of 8.3 μg/mL and induced apoptosis by inhibiting c-myc gene expression on human colorectal adenocarcinoma cell line, Caco-2 (Endrini et al., 2014).

On the other hand, another plant with anticancer properties from the *Acanthaceae* family is called *Andrographis paniculata* (Burm.f.) Nees. The taste of *A. paniculata* is exceptionally bitter, therefore, it is also known as 'king of bitters' (Thakur et al., 2015). *A. paniculata* is broadly used in Asia countries, particularly China and India, due to its great medicinal properties (Thakur et al., 2015). Many studies reported its medicinal properties, such as their anti-inflammatory, anticancer, and antiviral properties (Calabrese et al., 2000; Shen et al., 2006; Churiyah et al., 2015; Khan et al., 2018).

Andrographolide is a diterpenoid lactone and the main phytochemical in *A. paniculata* (Khan et al., 2018). When they are used with chemotherapeutic agents, they decrease their side effects and enhance efficacy (Yuan et al., 2016). Additionally, andrographolide was a potential chemopreventive agent, as it exhibits anticancer ability (Varma et al., 2011). For instance, andrographolide exerted growth inhibition potential on nasopharyngeal carcinoma through cell cycle arrest, apoptosis, and down-regulation of NF-kβ target genes (Peng et al., 2015). In addition, andrographolide isolated from *A. paniculata* demonstrated antiproliferative effects on human breast cancer cell lines with the lowest IC_{50} value at 20.3 ± 2.35 µM on MDA-MB-231 cells. Apoptosis assay was also conducted on MDA-MB-231 cells and showed that andrographolide induced apoptosis through dose- and time-dependent manners (Peng et al., 2018). Similarly, the growth of HT-29 cells was inhibited by andrographolide in a dose- and time-dependent manners (Khan et al., 2018).

The IC_{50} values of andrographolide were 59.8 μM and 33.63 μM at 24 and 48 hours of treatment, respectively. In addition, apoptosis was induced by andrographolide, and intracellular ROS level was increased with the interference of the mitochondrial membrane (Khan et al., 2018). The species from the *Acanthaceae* family that possessed anticancer properties are summarized in **Table 2.1**.

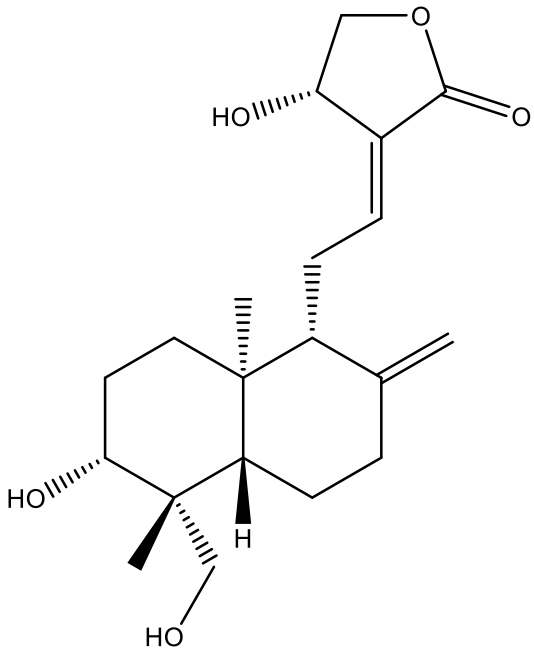


Figure 2.2: Chemical structure of andrographolide.

Table 2.1: Plants from the Acanthaceae family that possessed anticancer effects

Species	Extract / Phytochemical Compound	Anticancer effects	Reference
Graptophyllum pictum	Hexane, ethyl acetate, and aqueous fractions of leaves extract	IC_{50} on MCF-7 cells = 38.66, 26.01 and 20.41 µg/mL.	Jiangseubchatveera et al., 2017
	Hexane fraction of leaves extract	The lowest IC_{50} value on WiDr cells = 195.61 µg/mL	Amin et al., 2020
Justicia gendarussa	Leaves ethanol extract	IC_{50} on HeLa cells = 43.8 µg/mL IC_{50} on HepG2 cells = 19.8 µg/mL	Mangai, 2017
	Leaves methanol extract	IC_{50} on MDA-MB-468 cells = 23 µg/mL IC_{50} on MDA-MB-231 cells = 40 µg/mL	Ayob et al., 2014
Strobilanthes crispus	Leaves ethanol extract	IC_{50} on MCF-7 cells = 30 µg/mL Induced apoptosis by increasing the level of p53, caspase 3/7 and cdk2 protein.	Chong et al., 2012

Table 2.1 continued

	Stems hexane extract	IC_{50} on HepG-2 cells = 38.80 $\pm$ 8.50 µg/mL Induced cell cycle arrest at G0/G1 phase and caspase 8-mediated apoptosis.	Koh et al., 2017
Strobilanthes crispus	γ-sitosterol isolated from leaves extract	IC_{50} on Caco-2 cells = 8.3 µg/mL Activated apoptosis through inhibition of c-myc gene expression.	Endrini et al., 2014
Andrographis paniculata	Andrographolide	Inhibition of nasopharyngeal carcinoma through cell cycle arrest, apoptosis, and down-regulation of NF-kβ target genes.	Peng et al., 2015
		IC_{50} on MDA-MB-231 cells = 20.3 $\pm$ 2.35 µM. Apoptosis was resulted 4.9 – 15.1% at 24 hours and 5.4 – 31.95% at 48 hours.	Peng et al., 2018
		IC_{50} on HT-29 cells = 59.8 µM and 33.63 µM at 24 and 48 hours. Induced apoptosis by increasing intracellular ROS level.	Khan et al., 2018

2.4 Previous Studies on *Clinacanthus* genus

Clinacanthus genus, belongs to the *Acanthaceae* family, and consists of four species, namely *Clinacanthus nutans* (Burm.f.) Lindau, *Clinacanthus siamensis* Bremek., *Clinacanthus robinsoni* (Benoist) Bongch. & I. Darbysh., and *Clinacanthus spirei* Benoist (POWO, 2021). Studies have been done on *Clinacanthus nutans* and *Clinacanthus siamensis* except for *Clinacanthus robinsoni* and *Clinacanthus spirei*.

2.4.1 Previous Studies on *Clinacanthus siamensis* Bremek.

In Thailand, *C. siamensis* is commonly found and named as '*Lin nguu hao*'. It is used as folk remedies to relieve skin rashes, swelling, and treat insect bites (Wirotesangthong et al., 2009; Kunsorn et al., 2013). The *n*-butanol-soluble fraction from leaves of *C. siamensis* had isolated two new sulfur-containing compounds which are *trans*-3-methylsulfinyl-2-propenol **(1)** and *trans*-3-methylsulfonyl-2-propenol **(2)**, as well as entadamide A **(3)**, entadamide C **(4)**, and *trans*-3-methylthioacrylamide **(5)** (Tuntiwachwuttikul et al., 2003). *C. siamensis* was found to have anti-inflammatory and antimycobacterial activity. The entadamide A, isolated from *C. siamensis* leaves extract, exhibited anti-inflammatory activity as it could suppress the 5-lipoxygenase activity when treated on RBL-1 lymphoblast cancer cells. The isolated *trans*-3-methylthioacrylamide from *C. siamensis* leaves extract exhibited antimycobacterial activity against *Mycobacterium tuberculosis* H37Ra with a minimum inhibitory concentration of 200 µg/mL (Tuntiwachwuttikul et al., 2003). *In vivo* study of anti-influenza virus activity of *C. siamensis* was carried out: The results showed that *C. siamensis* leaves extract induced IgG_1 and IgA antibodies production in broncho-alveolar lavage of female BALB/c mice (Wirotesangthong et al., 2009). Moreover, *C.*

siamensis possessed anti-herpes simplex virus (HSV) activity as the methanol extract exhibited IC_{50} value of 37.39 ± 5.85 µg/mL against HSV-1, and *n*-hexane extract resulted in an IC_{50} value of 46.52 ± 4.08 µg/mL against HSV-2 (Kunsorn et al., 2013).

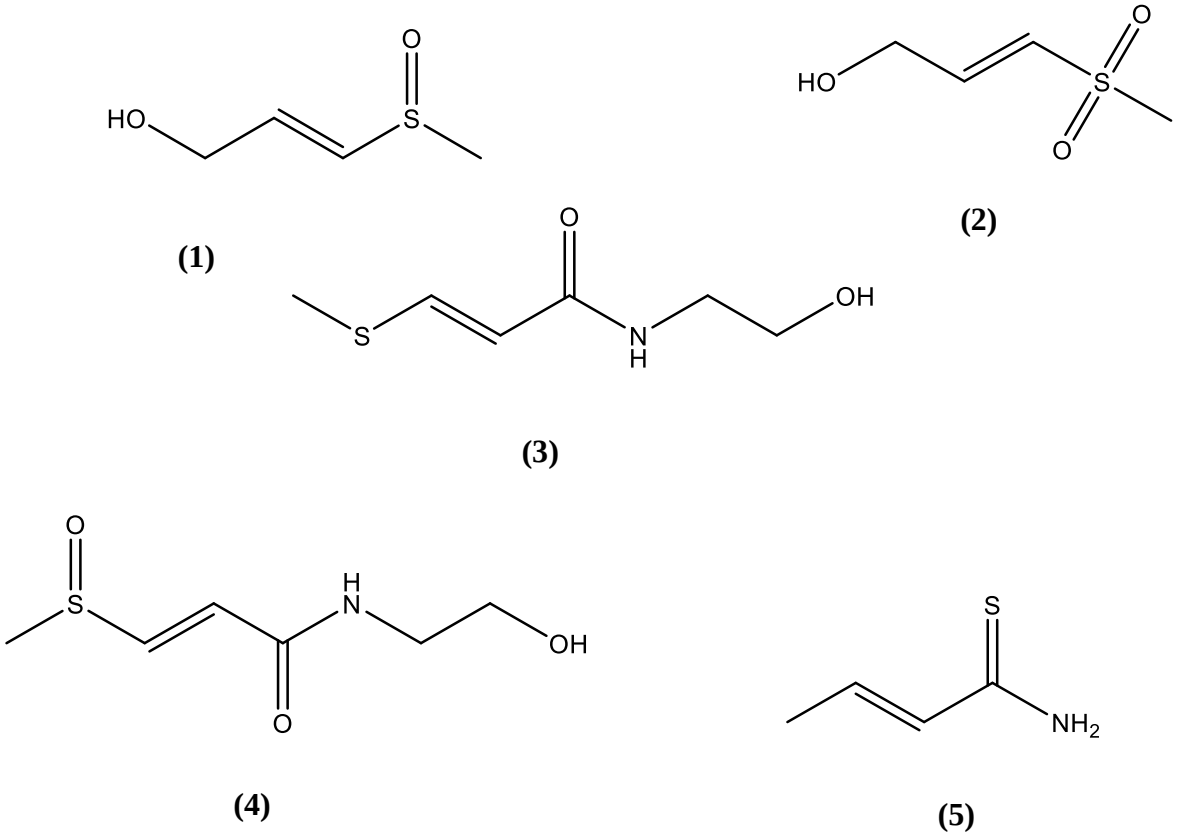


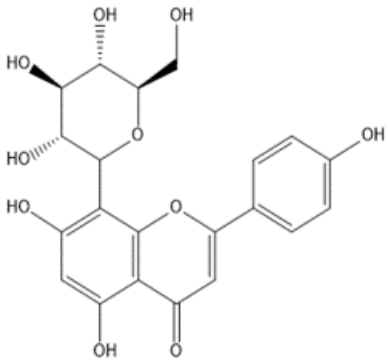
Figure 2.3: Chemical structure of *trans*-3-methylsulfinyl-2-propenol **(1)**, *trans*-3-methylsulfonyl-2-propenol **(2)**, entadamide A **(3)**, entadamide C **(4)**, and *trans*-3-methylthioacrylamide **(5)**.

2.5 Previous Studies on *Clinacanthus nutans* (Burm.f.) Lindau

2.5.1 Chemical Profiles

Since *C. nutans* (*C. nutans*) is popular in its medicinal uses, the phytochemical compounds of *C. nutans* are widely studied and identified. Different parts of *C. nutans* were reported to contain different bioactive compounds. The aerial parts of *C. nutans*

consisted of flavone-C-glycosides such as vitexin (**1**), orientin (**2**), isovitexin (**3**), shaftoside (**4**), isoorientin (**5**), and apigenin 6,8-C-a-L-pyranarabinoside (**6**) (Huang et al., 2015; Teshima et al., 1998). Moreover, the aerial parts of *C. nutans* also contained clinamides A (**7**), B (**8**), and C (**9**), 2-cis-entadamide A (**10**), trans-3-methylsulfinyl-2-propenol, entadamide A and entadamide C (Tu et al., 2014; Tuntiwachwuttikul et al., 2003; Ikegami et al., 1985; Ikegami et al., 1989). Besides that, cerebrosides (**11**) and monoacylmonogalactosylglycerol were isolated from *C. nutans* leaves extract (Tuntiwachwuttikul et al., 2004). *C. nutans* leaves extract was also found to contain chlorophyll A (**12**) and chlorophyll B (**13**) (Sakdarat et al., 2006; Sakdarat et al., 2009). Chemical structure of bioactive compounds isolated from *C. nutans* are shown in **Figure 2.2**.



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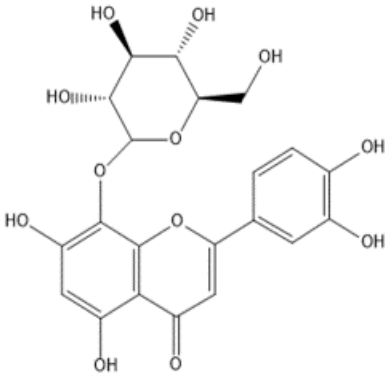

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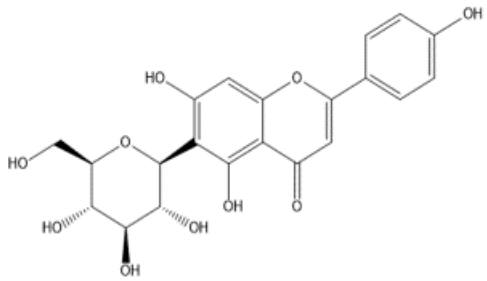

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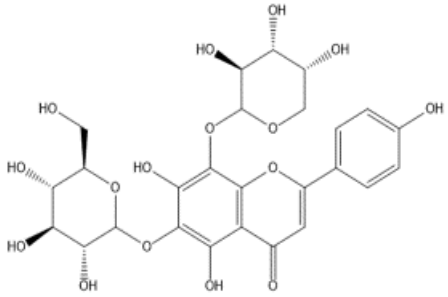

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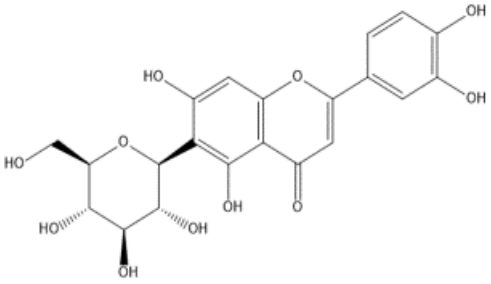

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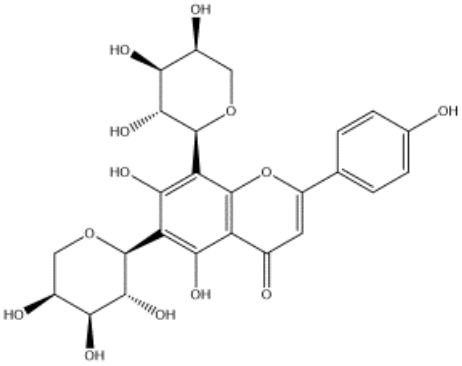

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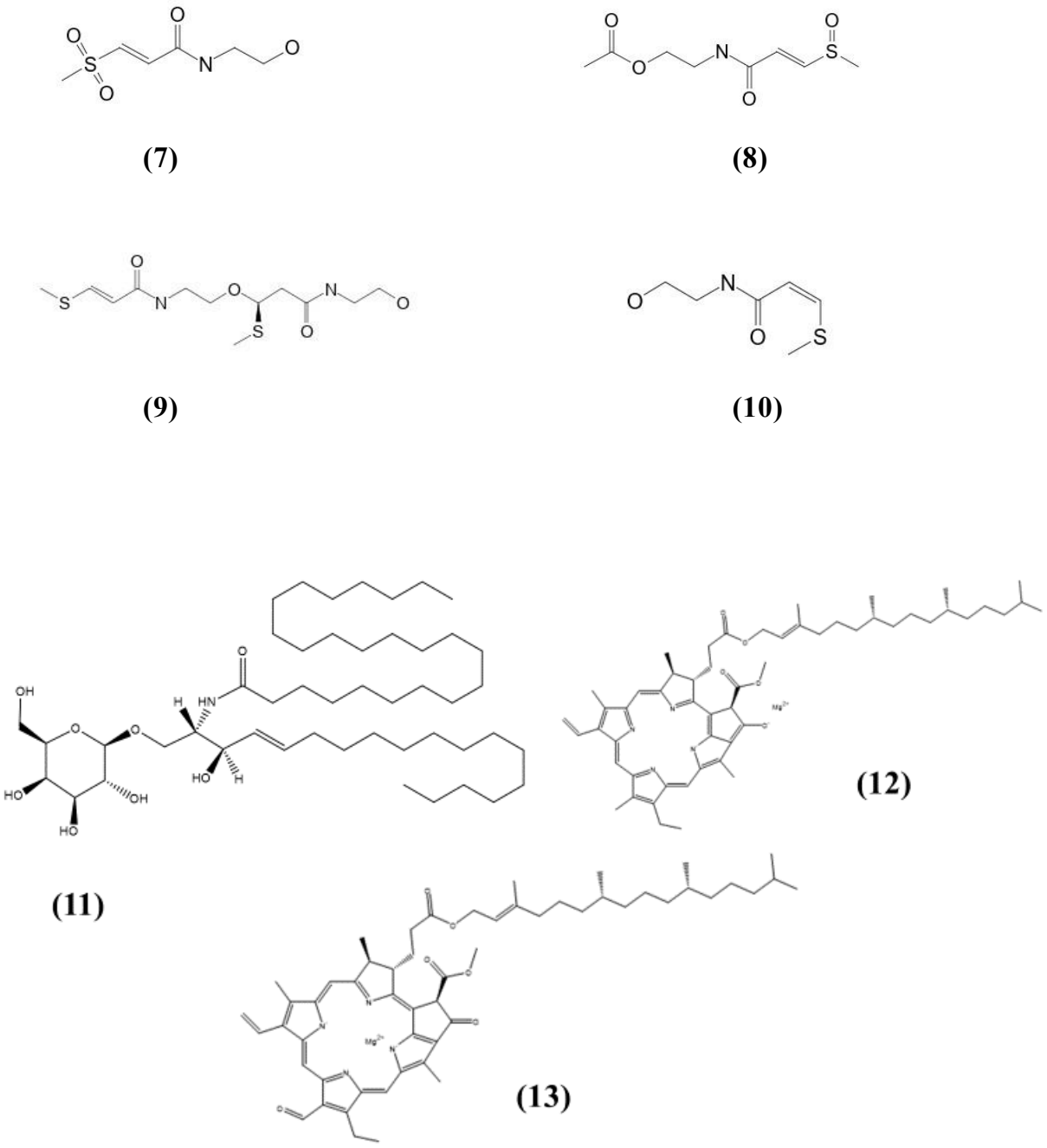


Figure 2.4: Chemical structure of bioactive compounds isolated from *C. nutans*. Vitexin (**1**), orientin (**2**), isovitexin (**3**), shaftoside (**4**), isoorientin (**5**), and apigenin 6,8-C-a-L-pyranarabinoside (**6**), clinamides A (**7**), B (**8**), and C (**9**), 2-cis-entadamide A (**10**), cerebrosides (**11**), chlorophyll A (**12**), and chlorophyll B (**13**).

2.5.2 Phytochemical Contents and Antioxidant Activity of *Clinacanthus nutans*

C. nutans leaves are rich in phytochemical contents that contribute to the antioxidant activity. Polar solvent, ethanol, extracted *C. nutans* leaves contained the highest total phenolic, tannin, and flavonoid contents and exhibited antioxidant activity when determined through the oxygen radical absorbance capacity (ORAC) assay. In addition, the ethyl acetate leaves extract of *C. nutans* showed the highest antioxidant activity through the DPPH assay (Sulaiman et al., 2015). On the other hand, Jusoh and his research team studied the different forms of insoluble bound, soluble bound and free phenolic from the *C. nutans* leaves. The insoluble bound phenolic *C. nutans* leaves extract had the highest radical scavenging activity ($73.3 \pm 0.82\%$) and IC_{50} value (0.60 ± 0.006 mg/mL) than free phenolic and soluble bound phenolic extracts, which indicated that insoluble bound phenolic of *C. nutans* contributed to the highest antioxidant activity (Jusoh et al., 2019). The *in vivo* study of antioxidant properties of methanol extracted *C. nutans* fresh leaves in breast cancer cell line found that *C. nutans* treated groups with both low-dose and high-dose were able to decrease nitrite oxide (NO) and malondialdehyde (MDA) in the blood. The NO level in the high-dose treated group was 0.044 ± 0.010 μM/mg, and in the low-dose treated group was 0.054 ± 0.013 μM/mg while 0.080 ± 0.025 μM/mg in the untreated group. The level of MDA was decreased from 0.017 ± 0.001 nM/mg in the untreated group to 0.013 ± 0.001 nM/mg in low-dose of treatment and 0.0083 ± 0.001 nM/mg in high-dose treatment groups (Nik Abd Rahman et al., 2019). A comparison of the antioxidant activity of ethanol extract of *C. nutans* leaves was studied between different extraction methods, such as maceration, magnetic stirring, and soxhlet extraction methods. This study showed that the phenolic from the maceration extraction method accounted for higher antioxidant activity than the others. In comparison, flavonoids from the magnetic stirring and soxhlet extraction methods accounted

for higher antioxidant activity than the other methods (Chuah et al., 2020). Another study comparing the antioxidant activities between tissue-cultured and conventionally propagated *C. nutans* leaves with different temperatures of aqueous extraction was reported by Haida et al. (2020). The results showed that the aqueously extracted tissue-cultured *C. nutans* leaves at 100^{o}C exhibited the strongest antioxidant activity based on 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, fluorescence recovery after photobleaching (FRAP), decolourisation 2, 2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) assay, and superoxide anion radical scavenging assay. In contrast, the tissue-cultured *C. nutans* leaves at 25^{o}C resulted in the highest iron (II) chelating activity (Haida et al., 2020).

2.5.3 Anticancer Activities of *Clinacanthus nutans*

The *in vivo* and *in vitro* anticancer activities of *C. nutans* were studied to determine the cytotoxic, apoptosis, and antitumor properties. Several methods are used to determine the anticancer activities of *C. nutans* extracts, including MTT assay, sulforhodamine B (SRB) assay, and Annexin V-FITC and PI staining assay. MTT assay is based on the reduction of MTT to purple insoluble formazan crystals formed by the enzyme activity of mitochondria in the living cells (Esmailli et al., 2019). Hence, the MTT assay is a standard method to analyse the cytotoxicity of natural products or drugs as it measures the cell proliferation rate affected by the cytotoxic agents (Esmailli et al., 2019). During apoptosis, the membrane phospholipid phosphatidylserine (PS) of the cell is exposed to the outer layer from the inner layer of the plasma membrane. Annexin V has a high affinity to PS and can easily bind to PS to indicate the cells undergoing apoptosis. Annexin V is linked with fluorochromes, fluorescein isothiocyanate (FITC), a sensitive probe to analyse apoptosis

cells using flow cytometry. Propidium iodide (PI) is usually applied together with Annexin V-FITC to differentiate early and late apoptosis. PI is only permeable to damaged and dead cell membranes. Therefore, viable cells would not be stained with Annexin V-FITC and PI. Cells undergoing early apoptosis would be stained with Annexin V-FITC, but cells undergoing late apoptosis or necrosis would be stained with both Annexin V-FITC and PI (Hingorani et al., 2011).

2.5.3.1 Antiproliferation Activity

The anticancer effects of *C. nutans* leaves collected from different locations were studied on the D24 melanoma cells by Fong et al. (2016). The methanol extracted *C. nutans* leaves, collected from a place with higher elevation and cooler climates, exhibited a time-dependent antiproliferative effect on D24 melanoma cells. The cytotoxic effect was significantly different when 2 mg/mL of the extract was treated on D24 melanoma cells for 24 and 72 hours (Fong et al., 2016). In addition, *C. nutans* leaves inhibited the cell proliferation of cervical cancer (HeLa), when extracted with water and dichloromethane (Zakaria et al., 2017; Haron et al., 2019). Another study by Esmailli et al. (2019) stated that methanol extracted *C. nutans* leaves possessed the lowest IC_{50} value at 52.90 ± 1.79 μg/mL on the colorectal cancer cell line, HCT-116 without cytotoxic to normal colon fibroblast, CCD-18Co. One of the fractions (F14) of methanol extract accounted for the most cytotoxicity with IC_{50} of 10.78 ± 1.46 μg/mL (Esmailli et al., 2019). Moreover, the ethyl acetate fraction of *C. nutans* leaves extracts showed the lowest IC_{50} value at 48.81 ± 1.44 μg/mL on HCT-116 cells with less cytotoxicity to normal colon cell line, CCD-18Co (Wang et al., 2019). On the other hand, the dichloromethane fraction of *C. nutans* leaves extract was less toxic to normal cells as it exhibited the highest selective index of 1.48, as the IC_{50} on

breast cancer cell line, MCF7 was 65.95 ± 0.14 µg/mL and IC_{50} on the normal breast cell line, MCF 10A was 100.20 ± 2.88 µg/mL (Ismail et al., 2020). The ethanol extracted *C. nutans* leaves demonstrated the most cytotoxic to nasopharyngeal carcinoma cell, HONE-1 as it decreased 89 ± 4.85% of cell viability, which was comparable with the chemotherapy drug, doxorubicin, which inhibited 91.8 ± 2.68% of cells (Roeslan & Fatima, 2020). Similarly, the leaves and stems of *C. nutans* were found to inhibit the cell viability of lung and liver cancers with the hexane and chloroform extracts with the lowest IC_{50} on the non-small cell lung cancer cell line, A549 (74 µg/mL) and liver cancer cell line, HepG2 (25 µg/mL), respectively (Ng et al., 2017).

2.5.3.2 Induction of Apoptosis, Autophagy, and Cell Cycle Arrest

Apoptosis is a natural programmed process of cell death to maintain tissue homeostasis and commonly happens in embryonic development (Hingorani et al., 2011). Recently, most cancer research has focused on apoptosis, especially in the chemoprevention study of natural products. Apoptosis is an effective method to inhibit continuous cancer growth without affecting other cells. *C. nutans* methanol leaves extract was found to cause apoptosis in D24 melanoma cells in the dose- and time-dependent manners as it showed early apoptotic cells at a low dose (1 mg/mL) and late apoptotic cells at high dose (2 mg/mL) at 72 hours (Fong et al., 2016). The water extract of *C. nutans* leaves induced apoptosis in HeLa cells with condensation of chromatin, apoptotic bodies, and fragmentation of nuclear (Zakaria et al., 2017).

Furthermore, *C. nutans* dichloromethane fraction induced apoptosis in HeLa cells in a dose-dependent manner as most cells underwent early apoptosis in the lowest dose (62.5

μg/mL) and late apoptosis in the highest dose (250 μg/mL) at 48 hours (Haron et al., 2019). The study also analysed the cell cycle arrest of dichloromethane fraction, the S and G2/M phase was arrested for 24 hours, and the S-phase arrest after treatment for 48 hours (Haron et al., 2019). The apoptosis of HCT-116 cells was induced by ethyl acetate fraction through the intrinsic mitochondrial pathway. It increased the pro-apoptotic proteins like Bax and Bak whereas decreased the anti-apoptotic proteins such as Bcl-xL and Bcl-2 in a dose-dependent manner. Both intrinsic and extrinsic pathways were induced in HCT-116 cells by ethyl acetate fraction as the caspase-3, -8, -9, and -10 proteins were decreased, and cleaved caspase-9 and death receptor 5 were increased at high dose treatment (200 μg/mL). Besides that, the level of LC3-II protein was increased, and p62 protein was decreased at high dose treatment (200 μg/mL) which indicated the evidence of autophagy. The accumulation of ROS induced both apoptosis and autophagy as the ROS inhibitor, N-acetyl cysteine, was used and it repealed the activation of caspase-3 and -9 by ethyl acetate fraction (Wang et al., 2019). In another study, apoptosis was initiated by treatment of hexane *C. nutans* extract as the lung, nasopharyngeal, and liver cancer cells (A549, CNE1, and HepG2, respectively) increased at sub-G1. They decreased at G0/G1 and G2/M in a dose-dependent manner. The apoptotic effect of hexane extract might be due to the intracellular oxidative stress as the reactive oxygen species (ROS) levels were increased by 2.7-fold in A549 and 1.78-fold in HepG2 compared with untreated cells. The concentration of hexane extract of more than 100 μg/mL also increased caspase 3/7, 8, and 9 in A549 cells and indicated the ability to induce intrinsic and extrinsic caspase pathways (Ng et al., 2017).

2.5.3.3 Antitumour Effect

An *in vivo* study of *C. nutans* on antitumour effect of HepA tumour-bearing mouse models revealed that a 30% ethanol fraction of *C. nutans* aerial part extract (CN30) reduced the tumour size and weight in a dose-dependent manner. At 3 mg/kg doses of CN30, the inhibition ratio was 8.2%, while 10 mg/kg doses accounted for an inhibition ratio of 58.6% (Huang et al., 2015). In addition, 10 mg/kg doses of CN30 reduced tumour size and weight by causing tumour cell damage indicated by cytoplasm condensation. The tumour cell apoptosis was performed and found that the apoptosis pathway was induced by cleavage of caspase-3, increased pro-apoptotic protein BAX, and decreased level of anti-apoptotic protein Bcl2 (Huang et al., 2015).

2.5.3.4 Synergistic Effects of Chemotherapy Drugs

There was a synergistic effect when n-hexane fraction was combined with doxorubicin. Exposure to ethanol, ethyl acetate, and n-hexane fractions of *C. nutans* leaves extracts respectively resulted in low cytotoxicity on breast cancer cell lines, MCF7 and T47D. Still, they showed potent cytotoxicity in these cells when n-hexane fraction was combined with 0.1 µg/mL of chemotherapy drug, doxorubicin. For instance, the cell viability of T47D was at 22.87% when the n-hexane fraction was combined with doxorubicin (Widjaja & Rusdiana, 2021). In addition, studies have been conducted on the synergistic effect of *C. nutans* stem with chemotherapy drugs against cancer cells. The non-polar stem extracts of *C. nutans* reduced dosage 2.38-5.28-fold of a chemotherapy drug, namely gemcitabine, without affecting the drug effects on human pancreatic ductal adenocarcinoma, PDAC (Hii et al., 2019).

2.5.4 Anti-inflammatory Activity of *Clinacanthus nutans*

The anti-inflammatory assay showed that the 80% ethanol extract of *C. nutans* aerial part possessed the highest inhibitory effect of elastase release at 68.33% with a concentration of 10 μg/mL (Tu et al., 2014). Thus, non-polar leaves extracts, polar leaves extracts, non-polar stem extracts, and polar stem extracts demonstrated anti-inflammatory activities and were found to inhibit the production of lipopolysaccharide (LPS)-induced nitric oxide in a dose-dependent manner. Polar leaves extract resulted in the lowest IC_{50} value at 18.9 ± 3.6 μg/mL. For the inhibition of TLR-4 activation induced by LPS, all extracts exhibited inhibition ability in a dose-dependent manner, while polar leaves extract showed the lowest IC_{50} value at 21.3 ± 5.0 μg/mL. The polar and non-polar leaves extracts resulted in lower levels of LPS-induced IFN-γ, IL-6, IL-17, IL-1β, IL12p40, and TNF-α, which indicated the inhibition ability on the production of LPS-induced cytokines. The anti-inflammatory activity of polar leaves extracts on inhibition of LPS-induced TLR-4 activities was further proven by showing that 20 μg/mL of polar leaves extracts reduced the phosphorylation of JNK1/2, ERK1/2, IRF3, p38, and p65 induced by LPS (Mai et al., 2016). In addition, the molecular docking of phytochemical components of *C. nutans* were analysed. These phytochemicals included cycloclinacoside, clinacoside A, B, and C, orientin, isoorientin, vitexin, isovitexin, shaftoside, β-sitosterol, and lupeol inhibited enzymes are involved in inflammation. Isoorientin and isovitexin were found to bind all the enzymes tested, including human neutrophil elastase, nitric oxide synthase, squalene synthase, xanthine oxidase, matrix metalloproteinase 2, and matrix metalloproteinase 9 (Narayanaswamy et al., 2016). This indicated that phytochemicals of *C. nutans* were able to inhibit the pro-inflammatory enzymes and enhance anti-inflammation ability.

2.5.5 Modulation of Immune Response

Several studies assessed the immune response modulation potential of *C. nutans*. The results showed that a low concentration of 80% ethanol extract (0.1 μg/mL) increased the level of IFN-γ whereas at a high concentration of 100 μg/mL, it decreased the IFN-γ level (Tu et al., 2014). On the other hand, treatment with a 30% ethanol fraction of *C. nutans* aerial part enhanced the activity of $CD8^+$T cells as it boosted the penetration of $CD8^+$T cells into HepA tumour cells compared to the untreated sample. The 30% ethanol fraction of *C. nutans* extract also increased the level of IFN- γ and IL-2 in HepA tumour-bearing mice. The intracellular staining showed that the 30% ethanol fraction increased the proportion of IFN-γ^+ $CD4^+$T cells and decreased the ratio of IL-4^+ $CD4^+$T cells in the spleen of HepA tumour-bearing mice. *C. nutans* extract was proven to increase immune cytokines in the serum, modulated immune response in tumour-bearing mice, and enhance the antitumour ability (Huang et al., 2015). The polysaccharide fraction of *C. nutans* leaves possessed a significant ability to stimulate macrophage activity. It increased the nitric oxide production in mouse macrophages, RAW264.7 cells in a dose-dependent manner after 48 hours of treatment (Huang et al., 2016). In order to inhibit the metastasis of triple-negative breast cancer, the immunomodulatory potential of *C. nutans* by targeting the tumour microenvironment and the immune system has been studied. Ethanol extracted *C. nutans* leaves inhibited the production of IL-1β and IL-6 at concentrations 25 and 100 μg/mL, respectively, in the co-culture of triple-negative breast cancer cell line, MDA-MB-231, with human macrophage-like cells, THP-1. Both ethanol and water extracted *C. nutans* leaves inhibited the expression of TNF-α cytokine. The ability of *C. nutans* leaves extracts in suppression of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) indicated that *C. nutans* could modulate

the cytokines in the tumour microenvironment and promote antitumour activity (Nordin et al., 2021).

2.6 Chapter Summary

In short, there are five stages of CRC progression. It is classified using Dukes' staging system such as Dukes' A, B, C, and D. The primary colorectal cancer cell line, HCT-116, and Duke's C stage colon adenocarcinoma cell line, HT-29, were commonly used in cancer studies. Plants from the Acanthaceae family have attracted the interests of researchers due to their purported and proven pharmacological efficacies in *in vitro* assays and *in vivo* models. Previous studies have revealed several plants, such as *G. pictum*, *J. gendarussa*, *S. crispus*, and *A. paniculata* exhibited significant anticancer activities against several human cancer cell lines: breast (MCF-7), colon (HL-60), nasopharyngeal (HONE-1), and cervical (HeLa).

C. nutans and *C. siamensis* were the two common species from *Clinacanthus* genus that have been reviewed for their pharmacological activities. *C. nutans* was found to contain numerous bioactive compounds that exhibited antioxidant, anticancer, anti-inflammatory, and immune response modulation and yielded the promising pharmacological advantages. Besides, *C. nutans* has also been revealed to show anticancer activities through cell cycle arrest as well as extrinsic and intrinsic apoptosis pathways. It worked well with chemotherapy drugs, such as gemcitabine and doxorubicin, to form synergistic effects against pancreatic ductal adenocarcinoma and breast cancer cell lines. In addition, *C. nutans* extracts affected the immune system and enzyme production causing anti-inflammatory activities, whereas modulated immune response could aid in preventing tumorigenesis.

CHAPTER 3

MATERIALS AND METHODS

3.1 General Experimental Procedures

This study used *Clinacanthus nutans* (*C. nutans*) dried powdered leaves with different polarity solvents and different extraction times to assess the phytochemical contents, antioxidant activity, and anticancer activities on two human colorectal cancer cell lines, HCT116 and HT-29. The phytochemical contents, namely the total phenolic (TPC) and flavonoid contents (TFC) were determined spectrophotometrically using the Folin-Ciocalteu and aluminium chloride colorimetric method, respectively. The antioxidant activity was assessed by 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay. In addition, the antiproliferation activity was evaluated *in vitro* via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. Besides, its ability to induce early and late apoptosis was also assessed in dose- and time-dependent manners based on apoptosis assay by Annexin V-FITC and PI staining. The experimental procedures are summarised in **Figure 3.1**.

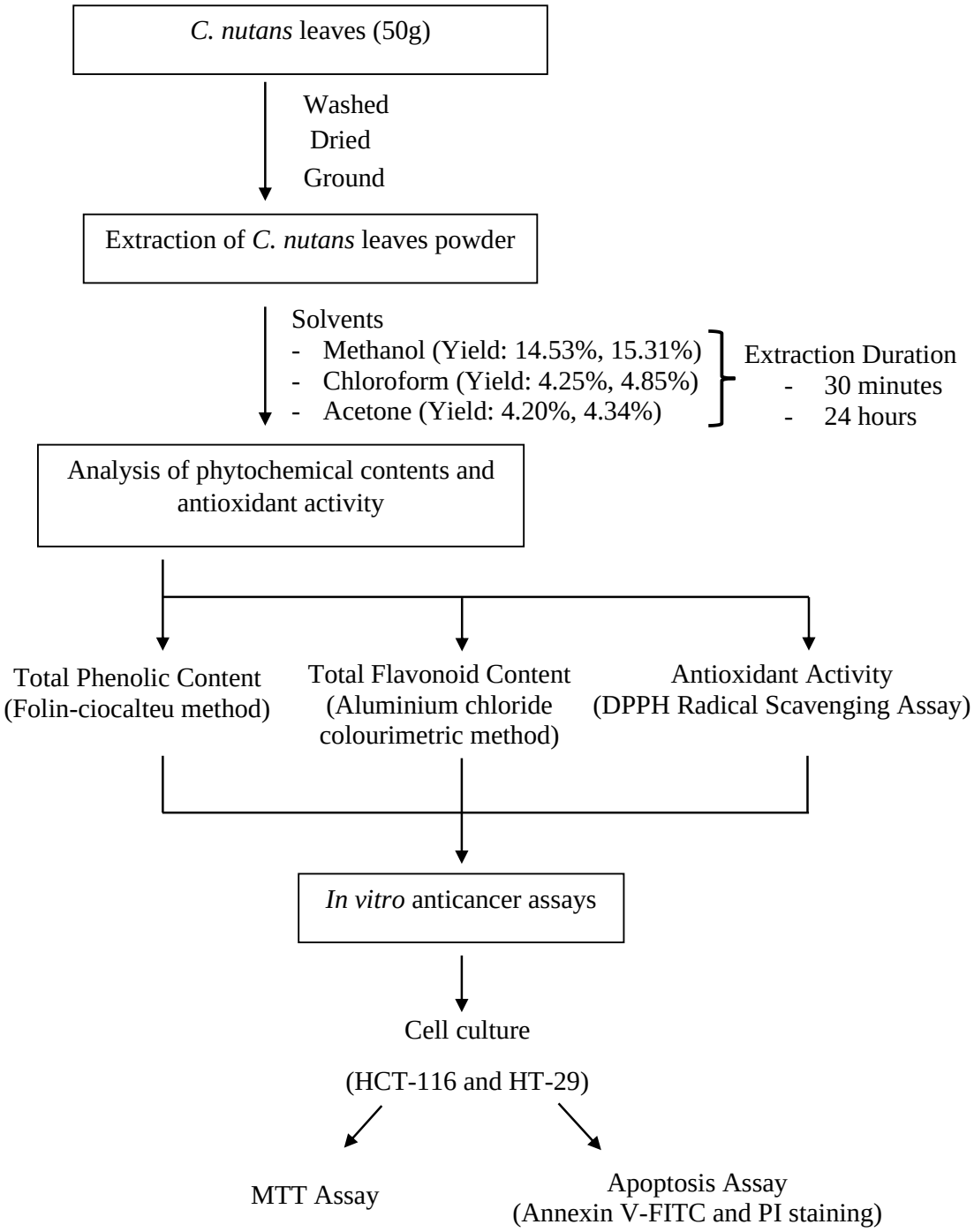


Figure 3.1: Flow chart of experimental procedures

3.2 Plant Materials

C. nutans was harvested as a whole plant from residential areas in Kuching, Sarawak, in November 2018. The plant was identified and vouched by botanist Associate Professor Dr. Wong Sin Yeng, a botanist from the Institute of Biodiversity and Environmental Conservation, UNIMAS. The leaves were plucked, washed, and dried at room temperature. The dried leaves were ground into powder form using a domestic blender (Sharp, Osaka, Japan).

3.3 Preparation of *Clinacanthus nutans* Extracts

Aliquots of 50 g powdered leaves samples were weighed and soaked in different solvents; methanol (Fisher Scientific, Vantaa, Finland), chloroform (Fisher Scientific, Vantaa, Finland), and acetone (Fisher Scientific, Vantaa, Finland), respectively at a ratio of 1:10 *C. nutans* powdered leaves to designated solvent. Magnetic stirrers (Favorit, Puchong, Malaysia) were used to stir the *C. nutans* leaves powder for 30 minutes and 24 hours. The crude extracts containing leaves residues were filtered through ashless, Grade 42 Whatman® filter paper (Whatman, Buckinghamshire, United Kingdom). The residue solvent in the filtrate (extract) was removed using a rotary evaporator (BUCHI, Flawil, Switzerland), and the extract was left to dry in a fume hood (ESCO, Singapore). The dried crude extracts were stored at 4℃ for further use. The extraction yield (%) was calculated as follows (Equation 3.1):

$$\text{Extraction yield (\%)} = \frac{\text{weight of dried crude extract}}{\text{weight of dried } C.nutans \text{ powder}} \times 100 \qquad \text{Equation 3.1}$$

3.4 Analysis of Phytochemical Content

The *C. nutans* leaves extracts were prepared using different solvent polarities, and extracted at different durations, were screened for total phenolic and total flavonoid contents.

3.4.1 Total Phenolic Content (TPC)

C. nutans extracts by different solvent polarities and extraction durations were analysed for the total phenolic content (TPC) using Folin-Ciocalteu reagent (Sigma-Aldrich, Missouri, USA) method with slight modifications from Baba and Malik (2015). Firstly, distilled water was added to 200 μL of *C. nutans* extract (1mg/mL) to make up to 3 mL (66.7 μg/mL). Then, 500 μL of Folin-Ciocalteu reagent were mixed with *C. nutans* extracts and incubated at room temperature for 3 minutes. Then, 2 mL of 20% (w/v) sodium carbonate (Sigma-Aldrich, Missouri, USA) solution was added and incubated further for an hour in the dark. A Thermo Spectronic GENESYS 20 spectrophotometer (Thermo Spectronic, Rochester, USA) was used to measure the absorbance at wavelength 765 nm. A calibration curve was plotted with serially diluted working stock of 500 μg/mL gallic acid (Sigma-Aldrich, Missouri, USA) as a standard reference. Gallic acid equivalent per dry weight of *C. nutans* extracts (mg GAE/g) was used to express the TPC of *C. nutans* extracts. All the samples for *C. nutans* extracts were conducted in triplicates. The formula was calculated as shown below (Equation 3.2):

$$\text{Total phenolic content (mg GAE/g)} = \frac{C\,V}{m} \qquad \text{Equation 3.2}$$

where ‘C’ represents the concentration determined from calibration curve (mg/mL), ‘V’ represents volume of *C. nutans* extract (mL), and ‘m’ represents mass of *C. nutans* crude extract (g).

3.4.2 Total Flavonoid Content (TFC)

The different solvents extracted *C. nutans* at different extraction durations were tested for the total flavonoid content (TFC) using the aluminium chloride (Sigma-Aldrich, Missouri, USA) colorimetric method adapted from Chan et al. (2012) with slight modifications. Firstly, 1 mL of each *C. nutans* extract at 1 mg/mL was mixed with an equal volume of 2% aluminium chloride and incubated for 10 minutes. A spectrophotometer (Thermo Spectronic, Rochester, USA) was used to determine the absorbance at the wavelength of 420 nm. Methanol (Fisher Scientific, Vantaa, Finland) was used as the blank. A calibration curve was plotted as serially diluted quercetin dihydrate (Sigma-Aldrich, Missouri, USA) at the initial concentration of 100 μg/mL was used as a standard reference. Quercetin dihydrate equivalent per dry weight of *C. nutans* extract (mg QE/g) was used to express the TFC of *C. nutans* extracts. All the samples for *C. nutans* extracts were performed in triplicates. The formula was calculated as follows (Equation 3.3):

$$\text{Total flavonoid content (mg QE/g)} = \frac{C\,V}{m} \qquad \text{Equation 3.3}$$

where ‘C’ represents concentration determined from calibration curve (mg/mL), ‘V’ represents volume of *C. nutans* extract (mL), and ‘m’ represents mass of *C. nutans* crude extract (g).

3.5 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) Free Radical Scavenging Assay

The antioxidant activity of *C. nutans* extracts was assessed using 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) (Sigma-Aldrich, Missouri, USA) radical scavenging assay as described by Herald et al. (2012) with some modifications. Firstly, 1 mL of *C. nutans* extracts from 10 μg/mL to 100 μg/mL (10 μg/mL, 20 μg/mL, 40 μg/mL, 60 μg/mL, 80 μg/mL and 100 μg/mL) was mixed with an equal volume of 100 μM DPPH. The mixture was incubated for 30 minutes in the dark. A spectrophotometer (Thermo Spectronic, Rochester, USA) was used to measure the absorbance at the wavelength of 517 nm. Antioxidant activity was expressed as scavenging ability (%) in this experiment. All the extracts and standards were carried out in triplicates and calculated with the formula below (Equation 3.4):

$$\text{DPPH Free radical scavenging activity \%} = [(A_0 - A_S) / A_0] \times 100\% \qquad \text{Equation 3.4}$$

where A_0 denotes the absorbance value of control (ethanol only) and A_S denotes the absorbance value of the sample. After scavenging ability was calculated, a calibration graph was plotted to determine the inhibition concentration (IC_{50}), the concentration that could scavenge 50% of the free radicals. Additionally, ascorbic acid (AA) (Sigma-Aldrich, Missouri, USA), which acts as a standard reference, was serially diluted from a concentration of 20 μg/mL with ethanol (Fisher Chemical, Massachusetts, USA). The antioxidant content of *C. nutans* extracts was expressed as ascorbic acid equivalent antioxidant content (AAEAC) and was calculated as follows (Equation 3.5):

$$\text{Ascorbic acid equivalent antioxidant content (AAEAC)} = \frac{\text{IC}_{50} \text{ of Ascorbic Acid}}{\text{IC}_{50} \text{ of Sample}} \times 100\% \qquad \text{Equation 3.5}$$

3.6 *In vitro* Anticancer Assays

The *C. nutans* extracts assessed anticancer properties through *in vitro* assays in this study. Two *in vitro* anticancer assays were carried out, which were 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) (Merck, Massachusetts, USA) assay and apoptosis assay by Annexin V-FITC and PI staining (BD Biosciences, New Jersey, USA). The *C. nutans* extract that exhibited higher contents of TPC, TFC, and more potent antioxidant activity was selected for the MTT assay. The *C. nutans* extracts extracted for 30 minutes or 24 hours were also selected to assess in apoptosis assay to determine its efficacy on early and late apoptosis induction of colorectal cancer cells based on the results in the MTT assay.

3.6.1 Cell Culture

Human colorectal carcinoma cell line, HCT-116 and human colorectal adenocarcinoma cell line, HT-29 kindly supplied by Institute of Medical Research, Malaysia were used in the *in vitro* anticancer assays of *C. nutans* extracts. A human epithelial cell line, NP69 (kindly supplied by Prof. Dr. Edmund from Faculty of Resource Science and Technology, UNIMAS, Malaysia) was the negative control. These normal cells were used to determine the effects of *C. nutans* extracts on the normal cell growth. NP69 cell line was derived from primary nasopharyngeal epithelial cells and commonly used in cancer studies (Chua et al., 2016). Although NP69 cell line was derived from nasopharyngeal, it was the same type of cells, epithelial cell, as HCT-116 and HT-29 cells. McCoy's 5A (modified) medium (Gibco, Waltham, USA) was used to culture colorectal cancer cell lines, HCT-116 and HT-29, with the addition of 10% fetal bovine serum (Gibco, Waltham, USA). Besides

that, keratinocyte serum-free medium (Gibco, Waltham, USA) was used for culturing normal epithelial cell line, NP69 with the addition of 25 mg bovine pituitary extract (BPE) (Gibco, Waltham, USA), 2.5 µg Human Recombinant epidermal growth factor (EGF) (Gibco, Waltham, USA), and 2% fetal bovine serum (Gibco, Waltham, USA). Before the addition of both media into the cells, they were filtered through a 0.22 µm filter membrane (Sartorius, Goettingen, Germany) to ensure sterile conditions for the cells. The cells were sub-cultured once they had reached 80% confluency, and 0.05% trypsin-EDTA (Gibco, Waltham, USA) was used to detach the cells. The desired cell number was obtained by performing cell count through trypan blue (Sigma-Aldrich, Missouri, USA) and a haemocytometer (Neubauer Haemocytometry, Pasadena, USA). The cells were incubated at 37°C in a 5% CO_2, humidified incubator (ESCO, Singapore). Prior to treatment of the human cell lines, different concentrations of chosen *C. nutans* crude extract were diluted in 0.1% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Missouri, USA). Cisplatin (kindly supplied by Prof. Dr. Edmund from Faculty of Resource Science and Technology, UNIMAS, Malaysia), a chemotherapy drug, was used as the positive control. Cisplatin is a platinum-based drugs and is widely used in cancer treatment including colorectal cancer (Pillozzi et al., 2018). Cisplatin was found to induce apoptosis by forming the platinum-DNA adducts (Huang et al., 1995; Wang and Lippard, 2005; Pillozzi et al., 2018). It was reconstituted in phosphate-buffered saline (PBS) (Sigma-Aldrich, Missouri, USA) to make a 1 mg/mL stock solution and stored at -20°C. Untreated cells with media only were used as a negative control, whereas cells with 0.1% DMSO were used as vehicle control.

3.6.2 Optimisation of Cell Number on 96-well Plates

Optimisation of cell number was carried out to determine the optimum cell number to be seeded and cultured on 96-well plates as modified from Moradi et al. (2018). The

different number of viable HCT-116, HT-29, and NP69 cells at 625 cells, 1,250 cells, 2,500 cells, 5,000 cells, 10,000 cells, and 20,000 cells respectively were seeded in 96-well plates and incubated for 24 hours. The overnight media were replaced with fresh media and the cells were left to grow for another 24 hours, 48 hours, and 72 hours, respectively. MTT assay was then conducted. A cell proliferation curve was plotted with the absorbance against cell density. The optimum cell number was obtained within the curve.

3.6.3 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was carried out, and modified method by Sheikh et al. (2017a) to assess the efficacy of *C. nutans* extracts on its ability to induce antiproliferation of HCT-116, HT-29, and NP69 respectively. The cell optimisation experiment determined the optimal cell number at 10000 cells, for all three cell lines (HCT-116, HT-29, and NP69). Therefore, 10000 viable cells from each cell line, namely, HCT-116, HT-29, and NP69 cells, were seeded in 96-well plates (Biologix, Jinan, China) for 24 hours. Methanol extracted *C. nutans* of two different extraction times, 30 minutes and 24 hours were chosen based on the phytochemical contents and DPPH radical scavenging assay results. Hence, *C. nutans* methanol extracts of two different durations, 30 minutes, and 24 hours, between 31.25 μg/mL to 1000 μg/mL (31.25 μg/mL, 62.5 μg/mL, 125 μg/mL, 250 μg/mL, 500 μg/mL, and 1000 μg/mL) were administered to the cells by replacing the overnight culture media with fresh media containing different concentrations of *C. nutans* . As a positive control, cisplatin was applied at concentrations between 0.9 μg/mL – 30 μg/mL on all three cell lines. The cells were then incubated at 37°C in a 5% CO_2, humidified incubator for three different periods (24, 48, and 72 hours). After the incubation period, 200 μL fresh culture media were added to replace the extracts and cisplatin, and another 20 μL of 5 mg/mL MTT reagent was added to the cells. Incubation for 4 hours in

the dark at 37℃ with 5% CO_2 was then performed. Formazan crystals were formed after the incubation period, and the solutions in the wells were replaced with 100 μL DMSO to solubilise the formazan crystals. A microplate reader (SpectraMax iD3 Multi- Mode Microplate Reader, Molecular Devices, San Jose, California) was used to measure the absorbance at 570 nm. A formula to calculate the cell viability was as shown below (Equation 3.6):

$$\text{Cell viability (\%)} = \frac{\text{Absorbance 570nm of sample}}{\text{Absorbance 570nm of control}} \times 100\ \% \qquad \text{Equation 3.6}$$

3.6.4 Selective Index (SI)

The SI of *C. nutans* extracts were calculated to determine the cytotoxic selectivity on normal and cancer cell lines. SI was calculated according to the formula as shown below (Equation 3.7):

$$SI = IC_{50} \text{ on normal cells} / IC_{50} \text{ on cancer cells} \qquad \text{Equation 3.7}$$

3.6.5 Determination of Early and Late Apoptosis Triggered by *Clinacanthus nutans* Extracts using Annexin V-FITC and PI Staining

To determine the efficacy of *C. nutans* extracts in inducing early and late apoptosis, the treated colorectal cancer cell lines, HCT-116 and HT-29 with Annexin V-FITC and PI staining were analysed using a fluorescent-activated cell sorter (FACS) (BD Biosciences, New Jersey, USA) cytometry. According to the MTT cell antiproliferation assay results, methanol extracted *C. nutans* with 24 hours extraction duration was chosen to be assessed in this experiment. Firstly, an adequate number of viable cells were seeded in 6-well plates

(Biologix, Jinan, China) and left in an incubator to grow for a day. After 24 hours, methanol extracted *C. nutans* (250 μg/mL, 500 μg/mL, and 750 μg/mL) and cisplatin (12 μg/mL for HCT-116 and 15 μg/mL for HT-29) were administered to HCT-116 and HT-29 cells by replacing the overnight culture media with fresh media containing different concentrations of *C. nutans* . The concentrations of methanol extracted *C. nutans* and cisplatin were chosen based on the results of IC_{50} values. In addition, two sets of cells were cultured in either a fresh medium containing 0.1% DMSO or with fresh media only. These were used to represent the vehicle control and negative control, respectively. After 24, 48, and 72 hours of treatment, the cells were washed with cold PBS twice and strained with sterile 40 μM cell strainers (Falcon, Seattle, USA) to achieve single-cell suspension and avoid cell clumping. An approximately 1×10^6 cells/mL were resuspended in 1× Annexin V Binding Buffer (BD Biosciences, New Jersey, USA) and 100 μL (1×10^5 cells) of the cell suspension were added to sterile 5-mL FACS tubes (Falcon, Seattle, USA). Then, 5 μL of Annexin V-FITC and 5 μL of PI were added to the tube. These tubes were incubated for 15 minutes in the dark before 400 μL of 1× Binding Buffer were added to each tube. These tubes of cells were then analysed by flow cytometry (BD FACSCalibur™)(BD Biosciences, New Jersey, USA).

3.7 Statistical Analysis

All experiments were conducted in triplicates. All data were expressed as mean $\pm$ standard deviation. Student's t-test via GraphPad Prism 9 was used to determine the statistical significance ($p < 0.05$) of the raw data of *in-vitro* assays to compare the significant difference between treated and untreated results.

CHAPTER 4

RESULTS

4.1 Percentage Yield

Percentage yield of *C. nutans* crude extracts were calculated, and the result is summarised as shown in **Table 4.1**. Methanol extracted *C. nutans* at 30 minutes and 24 hours resulted the highest extraction yield which were 14.53% and 15.31%, respectively. Acetone extract at 30 minutes exhibited the lowest extraction yield of 4.20%. Extraction time at 24 hours resulted higher extraction yield than 30 minutes for all types of solvent extracts.

Table 4.1: Percentage yield of *C. nutans* crude extracts

Types of Solvents	**Weight of Crude Extracts (g) ± Standard Deviation**		**Extraction Yield (%)**	
	30 minutes	**24 hours**	**30 minutes**	**24 hours**
Methanol	7.27 ± 0.16	7.65 ± 0.13	14.53	15.31
Chloroform	2.13 ± 0.06	2.43 ± 0.15	4.25	4.85
Acetone	2.10 ± 0.18	2.17 ± 0.16	4.20	4.34

4.2 Phytochemical Contents of *Clinacanthus nutans* Extracts

4.2.1 Total Phenolic Content (TPC)

Total phenolic content (TPC) of *C. nutans* extracts was assessed using Folin-Ciocalteu reagent method and expressed in the unit of gallic acid equivalent per dry weight

of *C. nutans* extracts (mg GAE/g). A calibration curve of gallic acid (**Figure 4.1**) was plotted, and it showed R^2 value of 0.9921. The acetone extracted *C. nutans* with extraction duration of 30 minutes and 24 hours resulted in the highest TPC, which were 3.06 mg GAE/g and 3.18 mg GAE/g, respectively (**Figure 4.2**). On the other hand, methanol extracts with extraction duration of 30 minutes and 24 hours showed the lowest TPC values (1.35 mg GAE/g and 1.31 mg GAE/g) among all the extracts. Chloroform extracts with extraction duration of 30 minutes and 24 hours contained 2.10 mg GAE/g and 1.94 mg GAE/g of TPC, respectively, slightly higher than methanol extracts (**Figure 4.2**).

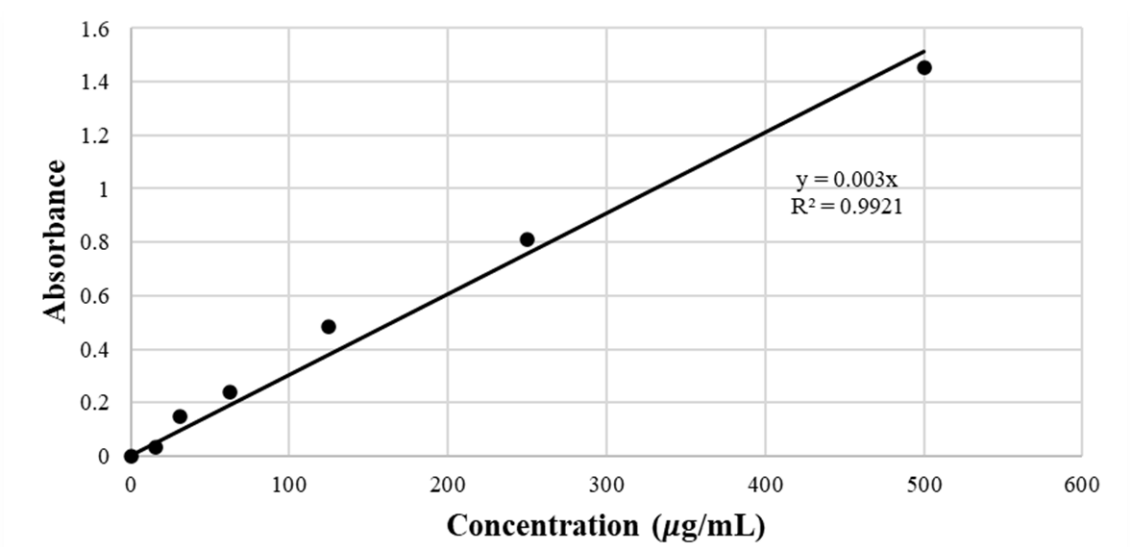
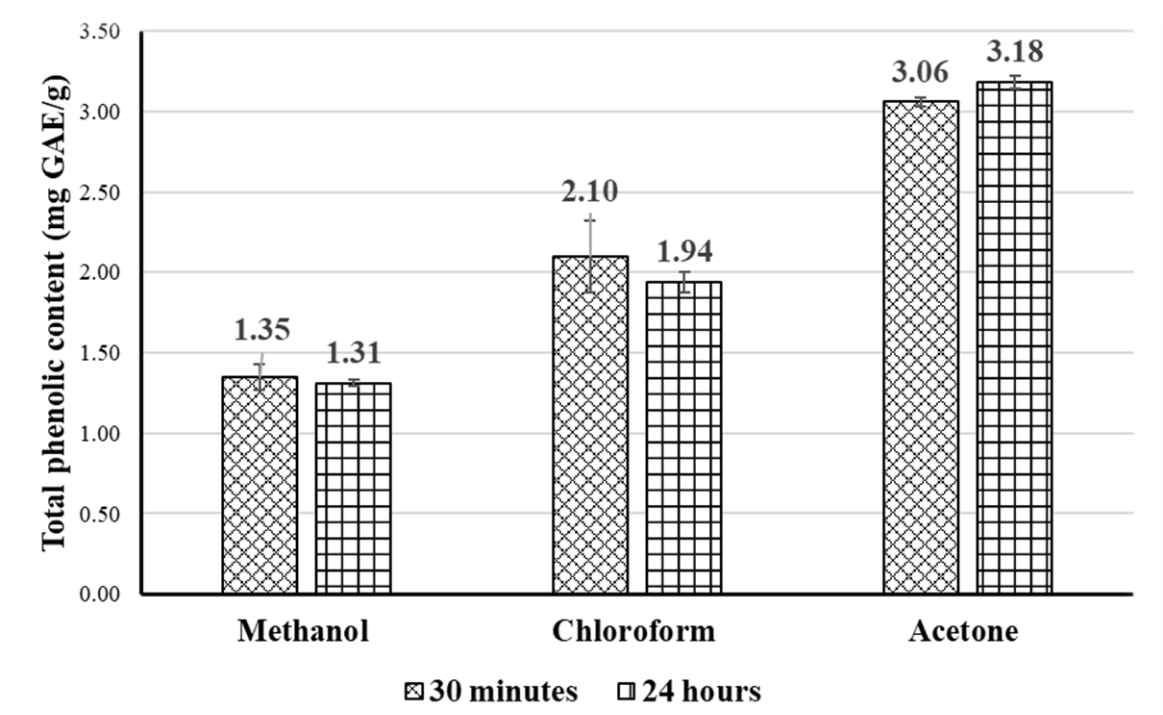


Figure 4.1: Standard calibration curve of gallic acid.



TPC was expressed in the unit of gallic acid equivalent per dry weight of *C. nutans* extracts (mg GAE/g). Results represent means ± standard deviation (n=3).

Figure 4.2: Total phenolic content of *C. nutans* extracts with different solvent polarities and extraction times.

4.2.2 Total Flavonoid Content (TFC)

Total flavonoid content of *C. nutans* extracts was determined using aluminium chloride colorimetric method. The results are expressed in the unit of quercetin dihydrate equivalent per dry weight of *C. nutans* extract (mg QE/g). A calibration curve of quercetin dihydrate (**Figure 4.3**) was plotted and the R^2 value was 0.9935. Methanol extracts with 30 minutes and 24 hours extraction times contained the highest TFC values with 0.49 mg QE/g and 0.50 mg QE/g, respectively. Chloroform extracts resulted in the lowest TFC values, with 0.06 mg QE/g for 30 minutes and 0.09 mg QE/g for 24 hours. Acetone extracts showed a slightly higher TFC value than that of chloroform extracts (0.19 mg QE/g and 0.20 mg QE/g) (**Figure 4.4**).

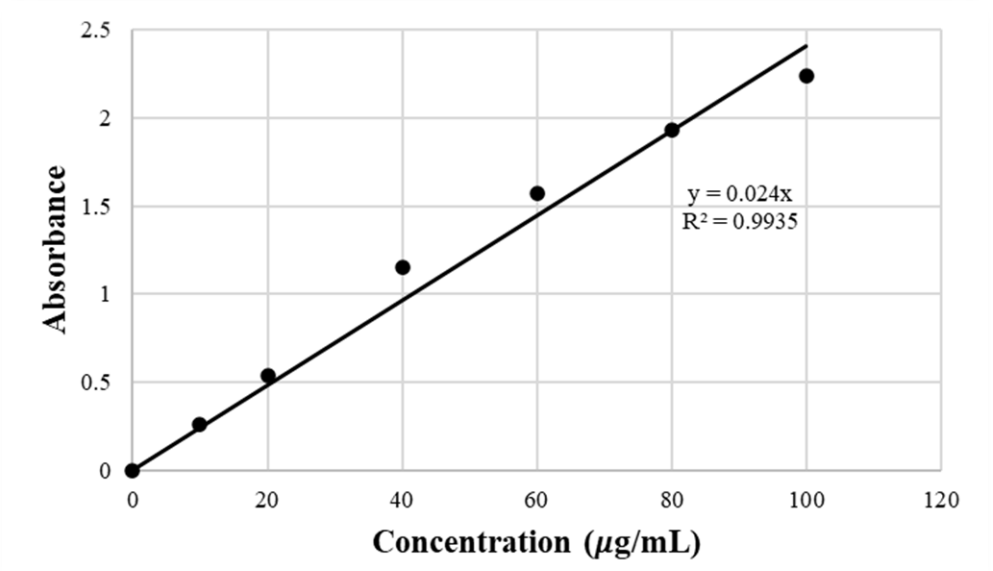
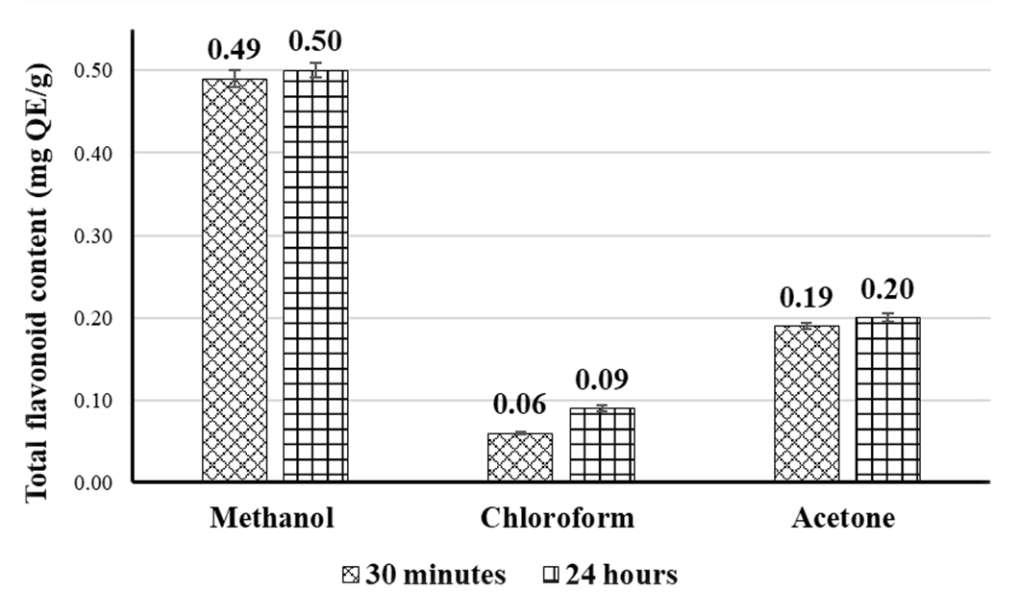


Figure 4.3: Standard calibration curve of quercetin dihydrate.



TFC was expressed in the unit of quercetin dihydrate equivalent per dry weight of *C. nutans* extract (mg QE/g). Results represent means ± standard deviation (n=3).

Figure 4.4: Total flavonoid content of *C. nutans* extracts with different solvent polarities and extraction times.

4.3 Antioxidant Activity of *Clinacanthus nutans* Extracts

The antioxidant activity of *C. nutans* extracts was screened using DPPH free radical scavenging assay. The DPPH free radical scavenging ability was expressed as IC_{50}, and the antioxidant content was expressed as ascorbic acid equivalent antioxidant content (AAEAC). The IC_{50} value of standard reference, ascorbic acid, was 5.37 ± 0.17 μg/mL. Methanol extracts resulted the strongest free radical scavenging activities with the lowest IC_{50} values at 24.25 μg/mL (30 minutes extraction duration) and 19.67 μg/mL (24 hours extraction duration). Moreover, the methanol extracts showed the highest antioxidant capacity among the other extracts, which were 22.14% for 30 minutes extraction time and 27.31% for 24 hours extraction time.

In addition, chloroform extracts showed the weakest free radical scavenging activities with the highest IC_{50} values among the other extracts. The IC_{50} values of chloroform extracts were 92.46 μg/mL (30 minutes extraction time) and 40.25 μg/mL (24 hours extraction time). Chloroform extracts also resulted the lowest antioxidant capacity of 5.81% AAEAC (30 minutes extraction time) and 13.34% AAEAC (24 hours extraction time). On the other hand, acetone extracts resulted lower IC_{50} values than chloroform extracts. The IC_{50} value of acetone extract with 30 minutes extraction time was 32.25 μg/mL whereas IC_{50} value of acetone extract with 24 hours extraction time was 23.17 μg/mL. The antioxidant capacity of acetone extracts were 16.65% AAEAC (30 minutes extraction time) and 23.18% AAEAC (24 hours extraction time) respectively (**Table 4.2**).

Table 4.2: Antioxidant activities of *C. nutans* extracts.

Types of Solvents	**IC_{50} (μg/mL)**		**AAEAC (%)**	
	30 minutes	**24 hours**	**30 minutes**	**24 hours**
Methanol	24.25 $\pm$ 0.75	19.67 $\pm$ 0.62	22.14	27.31
Chloroform	92.46 $\pm$ 9.45	40.25 $\pm$ 6.15	5.81	13.34
Acetone	32.25 $\pm$ 0.20	23.17 $\pm$ 1.55	16.65	23.18

Note: The DPPH free radical scavenging activity was expressed as IC_{50} (μg/mL), and the antioxidant content was expressed as AAEAC (%). Results represent means $\pm$ standard deviation (n=3).

4.4 *In vitro* Anticancer Activity

4.4.1 Optimisation of Cell Number on 96-well Plates

The cell proliferation curves of HCT-116, HT-29, and NP69 were plotted to optimise the cell number on 96-well plates (**Figure 4.5**). The cell number at 10,000/well was determined and cultured in each well of the 96-well plates.

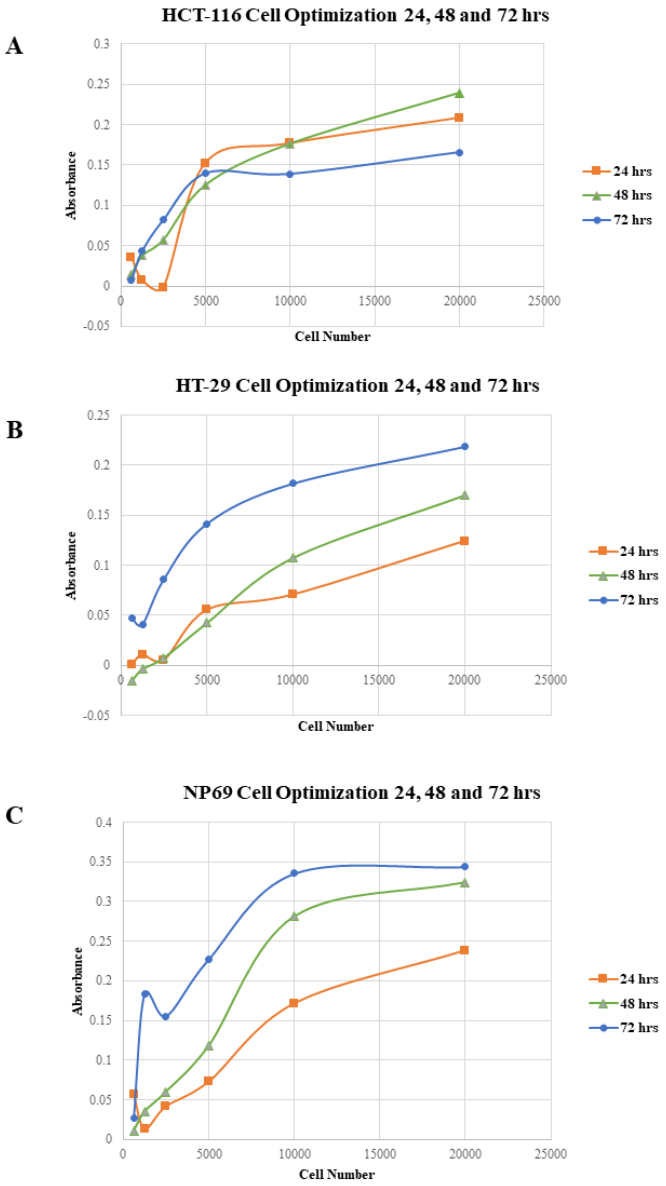


Figure 4.5: Cell proliferation curve of HCT-116 (**A**), HT-29 (**B**), and NP69 (**C**) cell lines to optimise the cell number on 96-well plates.

4.4.2 Antiproliferation Activity of *Clinacanthus nutans* Methanol Extracts against Human Colorectal Cancer Cell Lines

MTT assay was used in this study to determine the growth inhibition ability of *C. nutans* extracts against HCT-116 and HT-29 cells. Human colorectal cancer cell lines, HCT-116 and HT-29, and normal human epithelial cell line, NP69 were treated with increasing concentrations of both durations (with 30 minutes and 24 hours extraction durations) *C. nutans* methanol extracts for 24, 48, and 72 hours. The antiproliferation activity of *C. nutans* methanol extracts and cisplatin (positive control) were presented as the dose-response curve (**Figure 4.6 - 4.8**).

The cell viability of HCT-116 cells decreased from 73.35% to 57.98% when treated with 250 μg/mL of *C. nutans* methanol extract with 30 minutes extraction time from 24 to 72 hours. Moreover, 500 μg/mL of 30-minutes methanol extracted *C. nutans* decreased the cell viability of HCT-116 cells from 36.90% to 2.17% from 24 to 72 hours. The treatment of 24-hour methanol extracted *C. nutans* on HT-29 cells for 24 to 72 hours decreased cell viability from 71.89% to 40.83% and 38.14% to 12.77% at the concentration of 250 μg/mL and 500 μg/mL, respectively. The concentration that inhibited 50% of cell growth (IC_{50}) was determined from the dose-response curve, and the results are shown in **Table 4.3**.

HCT-116 cells showed a lower IC_{50} value when exposed to *C. nutans* methanol extract with 30-minute extraction than those of HT-29 cells when treated for 24 hours and 72 hours. The IC_{50} values (280.94 μg/mL and 203.54 μg/mL) of *C. nutans* methanol extracted at 30 minutes on HCT-116 cells were significantly lower than HT-29 cells (551.28 μg/mL and 226.77 μg/mL) when treated for 24 hours and 72 hours, respectively. However, the IC_{50} value (251.50 μg/mL) of *C. nutans* methanol extracted at 30 minutes was lower in HT-29 cells than HCT-116 cells (301.27 μg/mL) when treated for 48 hours.

Moreover, 24-hour *C. nutans* methanol extract exhibited a lower IC_{50} value than 30-minute extract when exposed to HT-29 cells for 24, 48, and 72 hours. Besides that, the IC_{50} values were lower when treated on HT-29 than on HCT-116 for 24, 48, and 72 hours. The IC_{50} values were 306.65 μg/mL, 231.07 μg/mL, and 217.44 μg/mL at 24, 48, and 72 hours of treatment on HT-29 cells, respectively.

As the *C. nutans* methanol extracts were treated on normal epithelial cells, NP69, the 24-hour extract resulted in higher IC_{50} values (499.67 μg/mL and 274.13 μg/mL) than 30-minute extract at 24 and 72 hours of treatment, respectively. When HCT-116, HT-29, and NP69 cells were exposed to the chemotherapy drug cisplatin, the IC_{50} values were significantly lower than *C. nutans* methanol extracts. Besides that, the selective index (SI) of *C. nutans* methanol extracts determined is shown in **Table 4.4**.

The 24-hour *C. nutans* methanol extract resulted in the highest selective index when treated on HT-29 cells for 24 hours. The SI values of 24-hour *C. nutans* methanol extract were more than one (>1) when treated on HT-29 cells at 24-, 48-, and 72-hour. In contrast, 30-minute *C. nutans* methanol extract resulted in SI values that were more than one when treated on HCT-116 cells for 24, 48, and 72 hours.

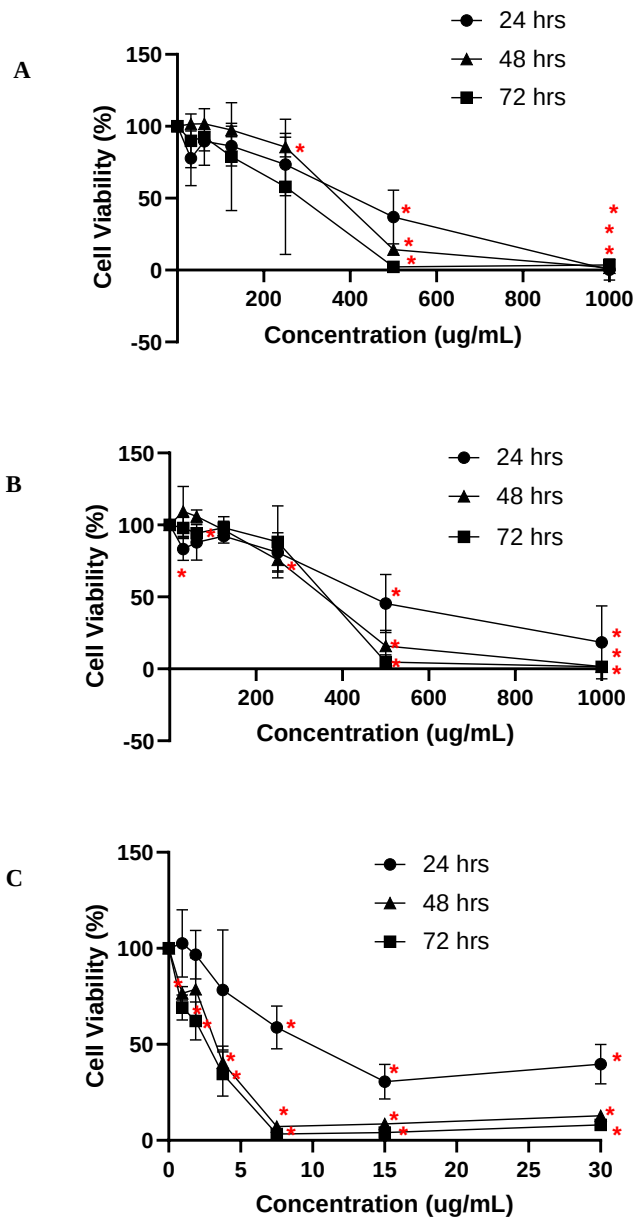


Figure 4.6: Dose-response curve of HCT-116 cell line that was exposed to methanol extracted *C. nutans* at 30 minutes (A), methanol extracted *C. nutans* at 24 hours (B), and cisplatin (C) for 24, 48, and 72 hours.

The concentrations of *C. nutans* methanol extracts were 31.25 μg/mL, 62.5 μg/mL, 125 μg/mL, 250 μg/mL, 500 μg/mL, and 1000 μg/mL whereas the concentrations of cisplatin were 0.94 μg/mL, 1.88 μg/mL, 3.75 μg/mL, 7.5 μg/mL, 15 μg/mL, and 30 μg/mL. Results represent means $\pm$ standard deviation (n=3). Asterisks (*) represents statistical significance at $p < 0.05$ as *C. nutans* methanol extracts and cisplatin were compared to vehicle control (0.1% DMSO) and untreated cells (100% cell viability), respectively.

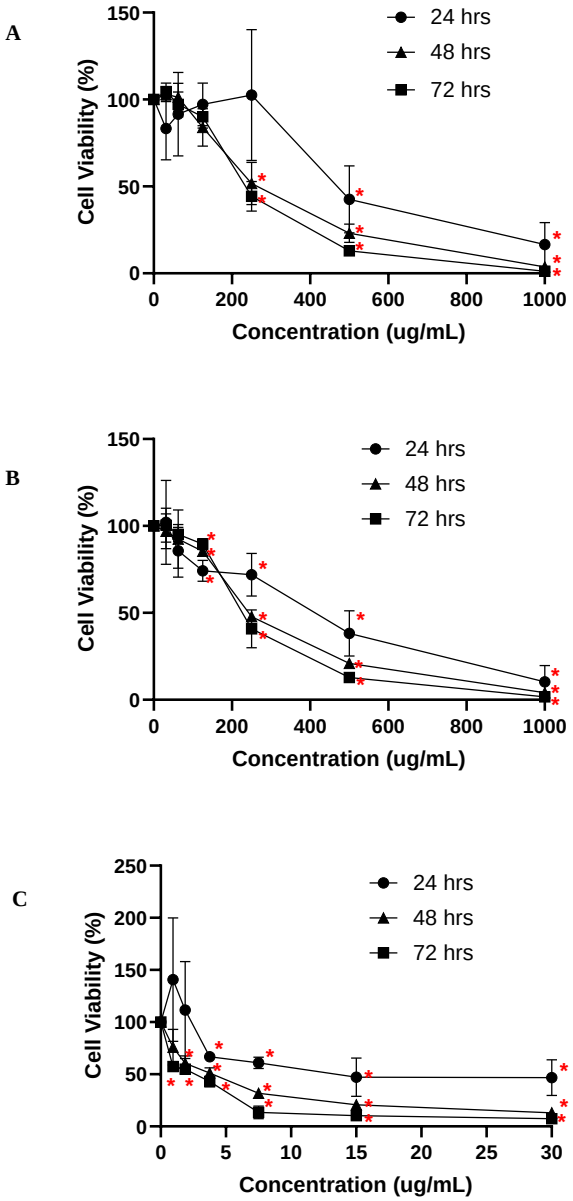


Figure 4.7: Dose-response curve of HT-29 cell line that was exposed to methanol extracted *C. nutans* at 30 minutes (A), methanol extracted *C. nutans* at 24 hours (B), and cisplatin (C) for 24, 48, and 72 hours.

The concentrations of *C. nutans* methanol extracts were 31.25 μg/mL, 62.5 μg/mL, 125 μg/mL, 250 μg/mL, 500 μg/mL, and 1000 μg/mL whereas the concentrations of cisplatin were 0.94 μg/mL, 1.88 μg/mL, 3.75 μg/mL, 7.5 μg/mL, 15 μg/mL, and 30 μg/mL. Results represent means $\pm$ standard deviation (n=3). Asterisks (*) represents statistical significance at $p < 0.05$ as *C. nutans* methanol extracts and cisplatin were compared to vehicle control (0.1% DMSO) and untreated cells (100% cell viability), respectively.

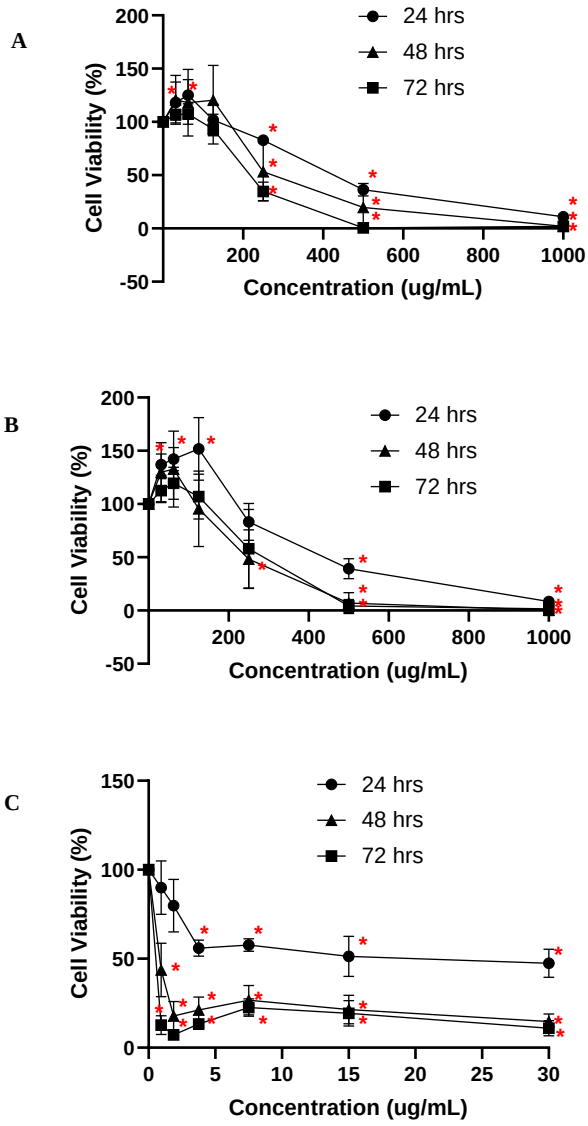


Figure 4.8: Dose-response curve of NP69 cell line that was exposed to methanol extracted *C. nutans* at 30 minutes (A), methanol extracted *C. nutans* at 24 hours (B), and cisplatin (C) for 24, 48, and 72 hours.

The concentrations of *C. nutans* methanol extracts were 31.25 μg/mL, 62.5 μg/mL, 125 μg/mL, 250 μg/mL, 500 μg/mL, and 1000 μg/mL whereas the concentrations of cisplatin were 0.94 μg/mL, 1.88 μg/mL, 3.75 μg/mL, 7.5 μg/mL, 15 μg/mL, and 30 μg/mL. Results represent means $\pm$ standard deviation (n=3). Asterisks (*) represents statistical significance at $p < 0.05$ as *C. nutans* methanol extracts and cisplatin were compared to vehicle control (0.1% DMSO) and untreated cells (100% cell viability), respectively.

Table 4.3: Anticancer activities of *C. nutans* methanol extracts and cisplatin against HCT-116, HT-29, and NP69 cell lines.

Treatment Periods	**IC_{50} (μg/mL)**		
	HCT-116	**HT-29**	**NP69**
***C. nutans* Methanol Extract (30 minutes)**			
24 hours	280.94	551.28	418.63
48 hours	301.27	251.50	311.53
72 hours	203.54	226.77	215.92
***C. nutans* Methanol Extract (24 hours)**			
24 hours	454.37	306.65	499.67
48 hours	295.15	231.07	271.62
72 hours	277.26	217.44	274.13
Cisplatin (Positive Control)			
24 hours	11.9	15.19	16.27
48 hours	3.07	3.49	< 0.94
72 hours	2.07	1.75	< 0.94

Note: The IC_{50} values were the concentrations that inhibited 50% of cell viability, obtained from the dose-response curve.

Table 4.4: Selective index of *C. nutans* methanol extracts on HCT-116 and HT-29 cells.

Treatment Periods	Selective Index (SI)	
	NP69 / HCT-116	**NP69 / HT-29**
***C. nutans* Methanol Extract (30 minutes)**		
24 hours	1.49	0.76
48 hours	1.03	1.24
72 hours	1.06	0.95
***C. nutans* Methanol Extract (24 hours)**		
24 hours	1.10	1.63
48 hours	0.92	1.18
72 hours	0.99	1.26

Note: The selective index was the ratio between IC_{50} of *C. nutans* methanol extracts on cancer cell lines and normal cell line.

4.4.3 Early and Late Apoptosis Triggered by *Clinacanthus nutans* Methanol Extract

Annexin V-FITC and PI staining method was used in this study to investigate the early and late apoptosis effects of *C. nutans* extract on HCT-116 and HT-29 cells using flow cytometry. The results of early and late apoptosis effects of *C. nutans* methanol extract on HCT-116 and HT-29 cells were shown in **Figure 4.9**, **Figure 4.10**, **Table 4.5**, and **Table 4.6**. HCT-116 and HT-29 cell lines were treated with different concentrations (250 μg/mL, 500 μg/mL, and 750 μg/mL) of 24-hour *C. nutans* methanol extract for 24, 48, and 72 hours. The untreated HCT-116 cells underwent 91.77%, 90.49%, and 87.84% of early apoptosis at

24, 48, and 72 hours. The percentage of late apoptosis of untreated HCT-116 cells were 5.94%, 4.32%, and 4.52% at 24, 48, and 72 hours, respectively. HCT-116 cells that were treated with 500 μg/mL for 24 hours resulted in 43.15% and 46.12% of early and late apoptosis, respectively. It showed a higher percentage of late apoptosis than cells treated with 12 μg/mL of cisplatin. HCT-116 cell treatment with 500 μg/mL of *C. nutans* extract demonstrated 45.83% of early apoptosis and 45.91% of late apoptosis after 48 hours. However, after 72 hours of treatment with *C. nutans* extract, the concentration of extract at 250 μg/mL showed the lowest percentage of early apoptosis at 46.34%, and the late apoptosis was 35.66%.

On the other hand, HT-29 cells resulted in a higher percentage of early apoptosis and a lower percentage of late apoptosis than HCT-116 cells. The untreated HT-29 cells resulted in 88.57%, 90.01%, and 83.39% of early apoptosis at 24, 48, and 72 hours. Besides that, 6.84%, 5.98%, and 10.29% of untreated HT-29 cells underwent late apoptosis at 24, 48, and 72 hours, respectively. HT-29 cell treatment with 250 μg/mL exhibited a lower percentage of early apoptosis and a higher percentage of late apoptosis than other concentrations. After 250 μg/mL of *C. nutans* extract treatment for 24 and 48 hours, 81.50% and 81.84% of HT-29 cells underwent early apoptosis, while 14.28% and 14.67% underwent late apoptosis. However, the percentage of early apoptosis dropped to 76.83%, and late apoptosis increased to 20.67% after 250 μg/mL of *C. nutans* extract treatment for 72 hours.

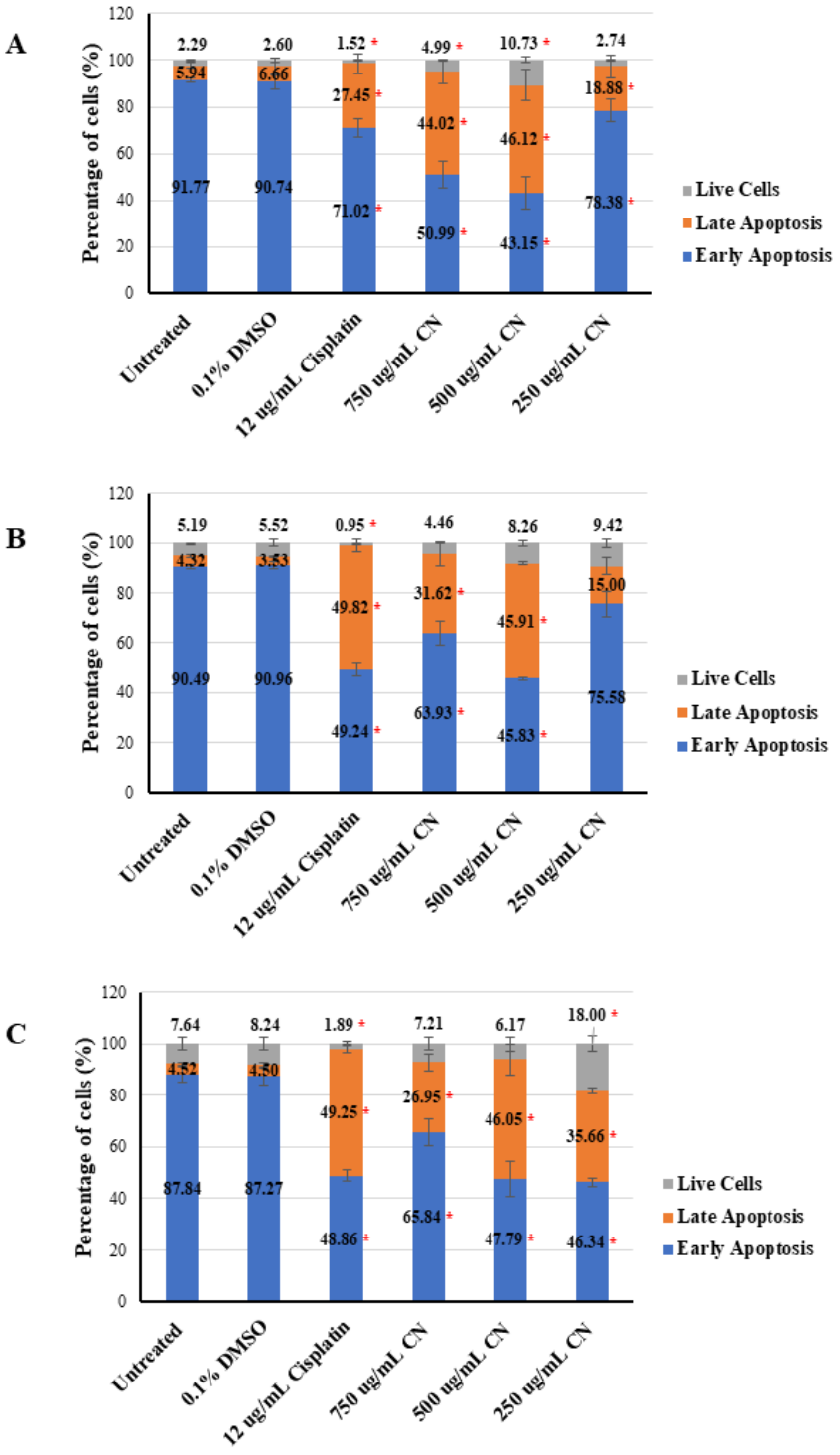


Figure 4.9: Early and late apoptosis effects of *C. nutans* methanol extract on HCT-116 at 24 (A), 48 (B), and 72 hours (C). Results represent means ± standard deviation (n=3).

Table 4.5: Early and late apoptosis effects of *C. nutans* methanol extract on HCT-116 at 24, 48, and 72 hours.

	A	**B**	**C**	**D**	**E**	**F**
24 hours						
Early Apoptosis (%)	91.77	90.74	71.02 *	50.99 *	43.15 *	78.38 *
Late Apoptosis (%)	5.94	6.66	27.45 *	44.02 *	46.12 *	18.88 *
Live cells (%)	2.29	2.60	1.52 *	4.99 *	10.73 *	2.74
48 hours						
Early Apoptosis (%)	90.49	90.96	49.24 *	63.93 *	45.83 *	75.58
Late Apoptosis (%)	4.32	3.53	49.82 *	31.62 *	45.91 *	15.00
Live cells (%)	5.19	5.52	0.95 *	4.46	8.26	9.42
72 hours						
Early Apoptosis (%)	87.84	87.27	48.86 *	65.84 *	47.79 *	46.34 *
Late Apoptosis (%)	4.52	4.50	49.25 *	26.95 *	46.05 *	35.66 *
Live cells (%)	7.64	8.24	1.89 *	7.21	6.17	18.00 *

Note: **A** = Untreated; **B** = 0.1% DMSO; **C** = 12μg/mL Cisplatin; **D** = 750μg/mL *C. nutans* ; **E** = 500μg/mL *C. nutans*; **F** = 250μg/mL *C. nutans*

HCT-116 cells were treated by different concentrations (250 μg/mL, 500 μg/mL, and 750 μg/mL) of *C. nutans* methanol extract, and 12 μg/mL of cisplatin. Untreated and treated with 0.1% DMSO represented negative control. The results were categorised as the percentage of early apoptosis, late apoptosis / necrosis, and live cells. Cell debris was included in the late apoptosis / necrosis result. Asterisks (*) represents statistical significance at $p < 0.05$ as *C. nutans* methanol extract and cisplatin were compared to vehicle control (0.1% DMSO) and untreated cells, respectively.

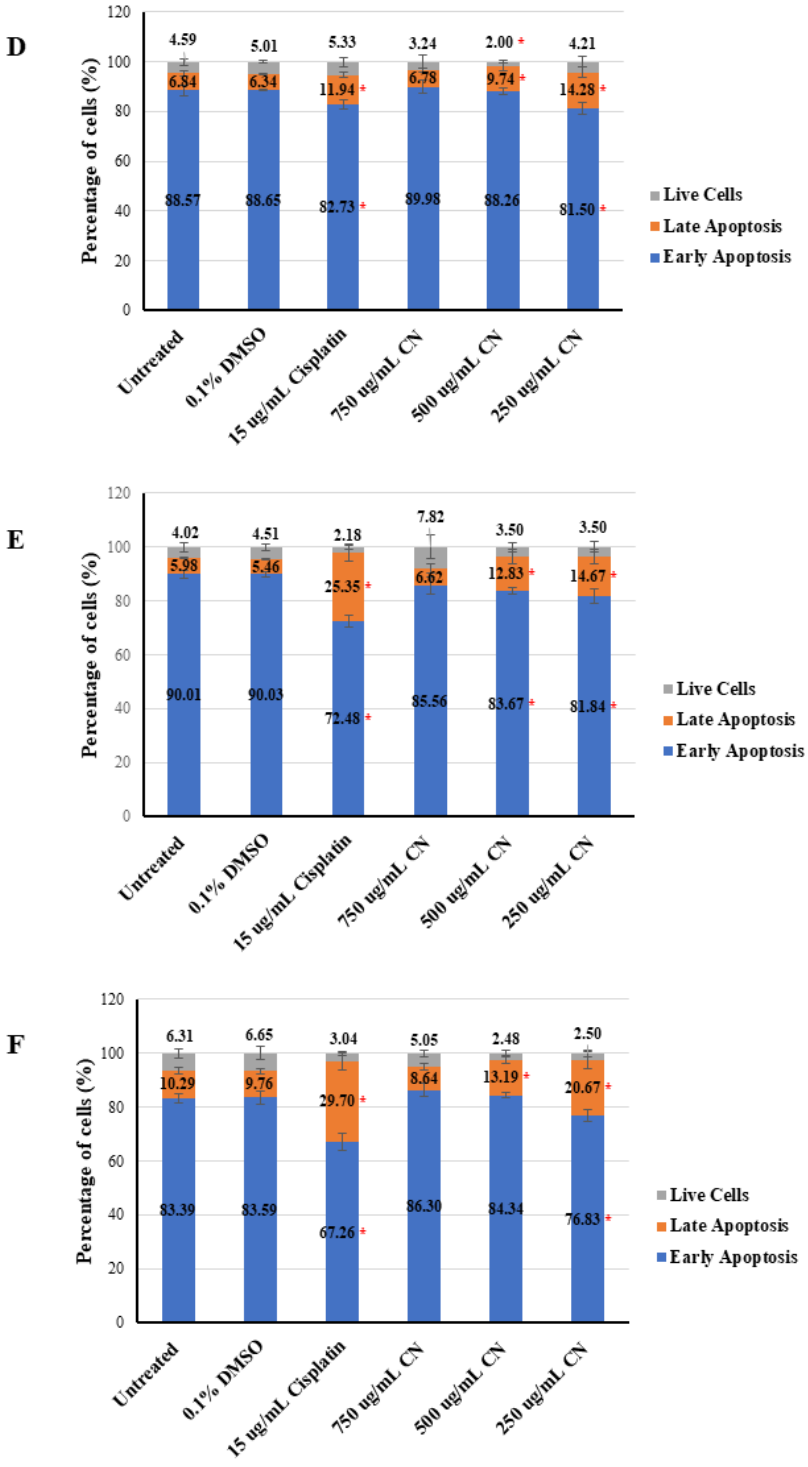


Figure 4.10: Early and late apoptosis effects of *C. nutans* methanol extract on HT-29 at 24 (D), 48 (E), and 72 hours (F). Results represent means ± standard deviation (n=3).

Table 4.6: Early and late apoptosis effects of *C. nutans* methanol extract on HT-29 at 24, 48, and 72 hours.

	A	B	C	D	E	F
24 hours						
Early Apoptosis (%)	88.57	88.65	82.73 *	89.98	88.26	81.50 *
Late Apoptosis (%)	6.84	6.34	11.94 *	6.78	9.74 *	14.28 *
Live cells (%)	4.59	5.01	5.33	3.24	2.00 *	4.21
48 hours						
Early Apoptosis (%)	90.01	90.03	72.48 *	85.56	83.67 *	81.84 *
Late Apoptosis (%)	5.98	5.46	25.35 *	6.62	12.83 *	14.67 *
Live cells (%)	4.02	4.51	2.18	7.82	3.50	3.50
72 hours						
Early Apoptosis (%)	83.39	83.59	67.26 *	86.30	84.34	76.83 *
Late Apoptosis (%)	10.29	9.76	29.70 *	8.64	13.19 *	20.67 *
Live cells (%)	6.31	6.65	3.04	5.05	2.48	2.50

Note: **A** = Untreated; **B** = 0.1% DMSO; **C** = 15μg/mL Cisplatin; **D** = 750μg/mL *C. nutans*; **E** = 500μg/mL *C. nutans*; **F** = 250μg/mL *C. nutans*

HT-29 cells were treated by different concentrations (250 μg/mL, 500 μg/mL, and 750 μg/mL) of *C. nutans* methanol extract, and 15 μg/mL of cisplatin. Untreated and treated with 0.1% DMSO represented negative control. The results were categorised as the percentage of early apoptosis, late apoptosis / necrosis, and live cells. Cell debris was included in the late apoptosis / necrosis result. Asterisks (*) represents statistical significance at $p < 0.05$ as *C. nutans* methanol extract and cisplatin were compared to vehicle control (0.1% DMSO) and untreated cells, respectively.

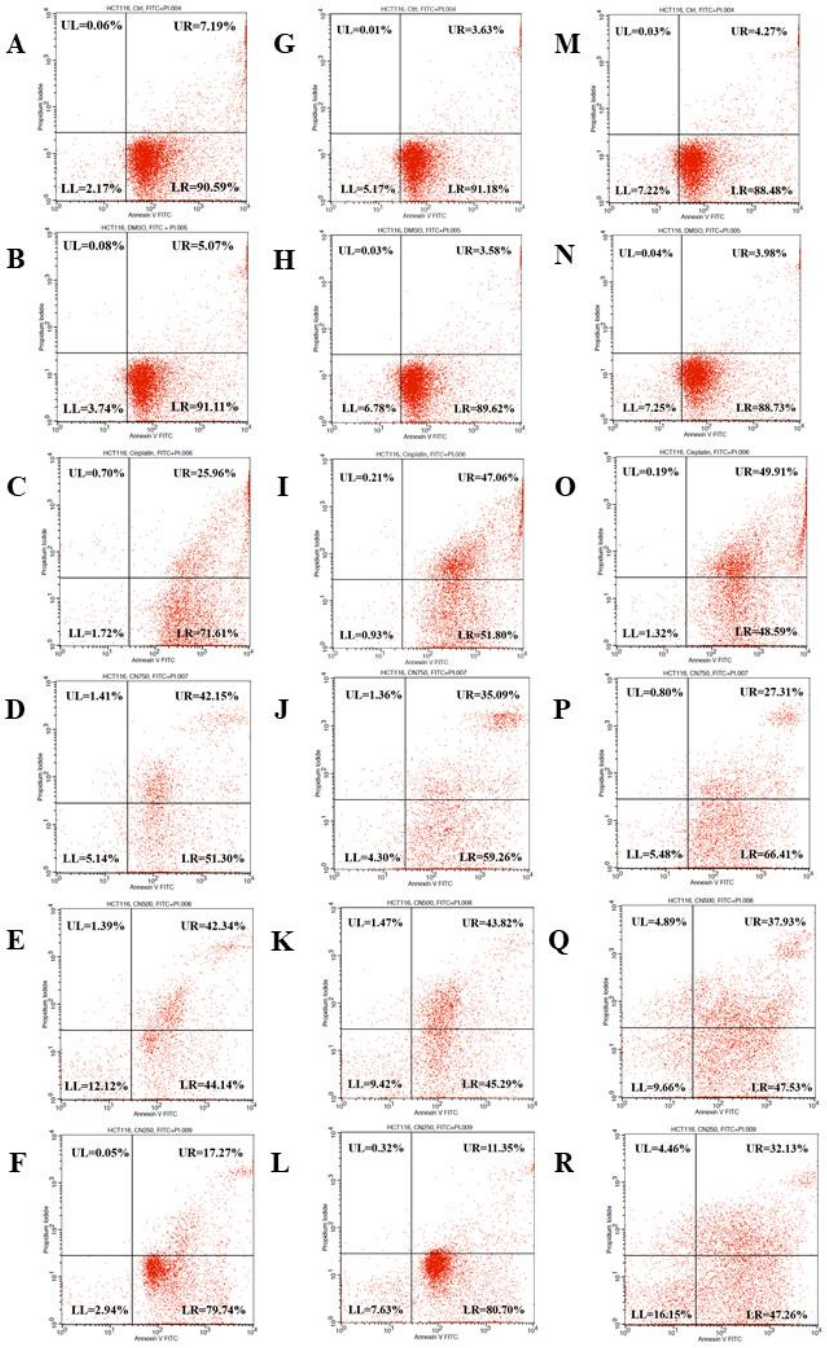


Figure 4.11: Flow cytometry analysis of apoptosis in HCT-116 cells.

HCT 16 cells were exposed to media only (untreated) (**A, G, M**), 0.1% DMSO (**B, H, N**), 12 μg/mL cisplatin (**C, I, O**), 750 μg/mL (**D, J, P**), 500 μg/mL (**E, K, Q**), and 250 μg/mL (**F, L, R**) of *C. nutans* methanol extract for 24 hours (**A, B, C, D, E, F**), 48 hours (**G, H, I, J, K, L**), and 72 hours (**M, N, O, P, Q, R**). Annexin V-FITC and propidium iodide (PI) staining method were used to determine the apoptosis effects of *C. nutans* methanol extract. Graphs with dotted plots showed lower left quadrant as live cells (both Annexin V-FITC and PI negative), lower right quadrant as early apoptosis cells (Annexin V-FITC positive and PI negative), upper right quadrant as late apoptosis cells (both Annexin V-FITC and PI positive), and upper left quadrant as necrotic cells or cell debris (PI positive only).

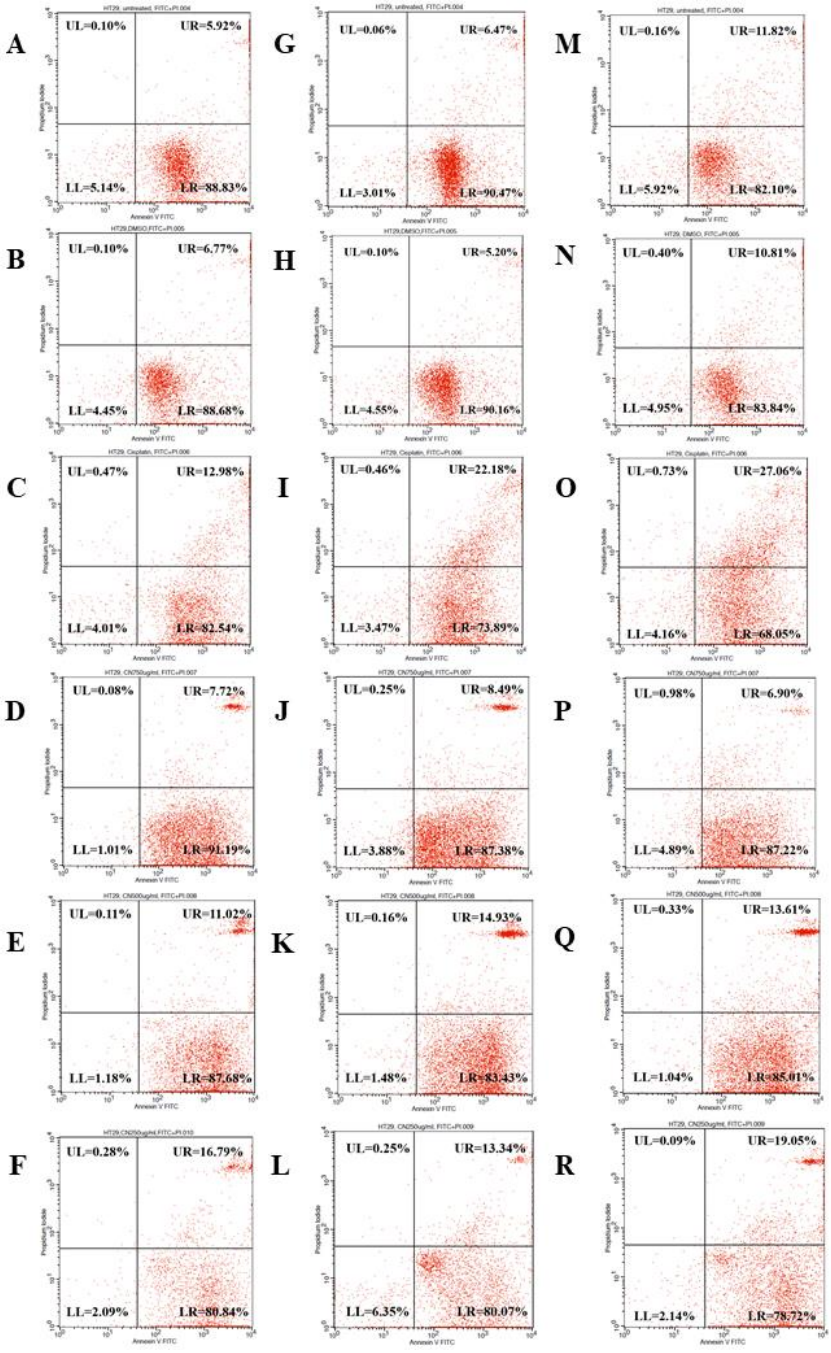


Figure 4.12: Flow cytometry analysis of apoptosis in HT-29 cells.

HT-29 cells were exposed to media only (untreated) (**A, G, M**), 0.1% DMSO (**B, H, N**), 15 μg/mL cisplatin (**C, I, O**), 750 μg/mL (**D, J, P**), 500 μg/mL (**E, K, Q**), and 250 μg/mL (**F, L, R**) of *C. nutans* methanol extract for 24 hours (**A, B, C, D, E, F**), 48 hours (**G, H, I, J, K, L**), and 72 hours (**M, N, O, P, Q, R**). Annexin V-FITC and propidium iodide (PI) staining method were used to determine the apoptosis effects of *C. nutans* methanol extract. Graphs with dotted plots showed lower left quadrant as live cells (both Annexin V-FITC and PI negative), lower right quadrant as early apoptosis cells (Annexin V-FITC positive and PI negative), upper right quadrant as late apoptosis cells (both Annexin V-FITC and PI positive), and upper left quadrant as necrotic cells or cell debris (PI positive only).

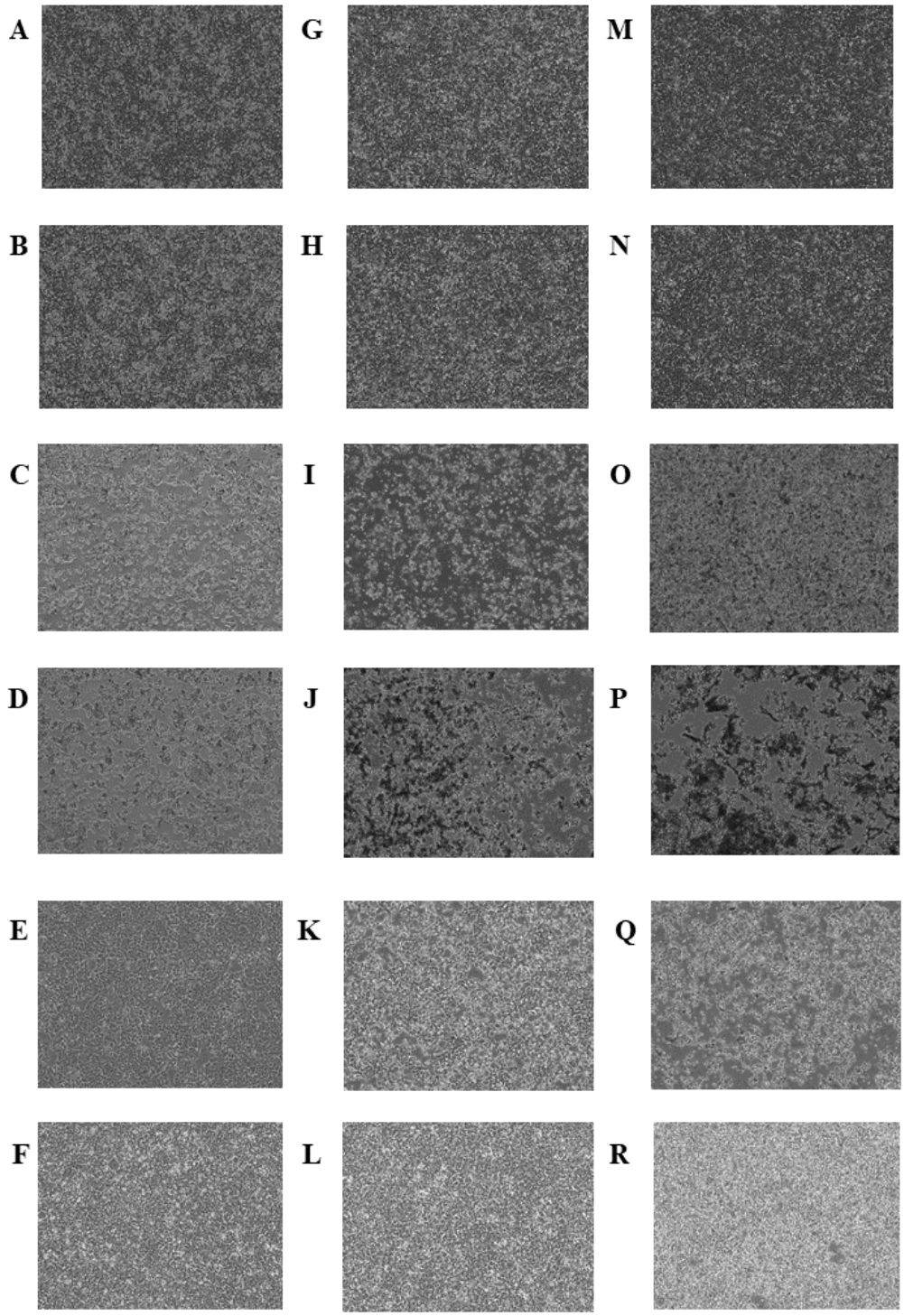


Figure 4.13: Morphology of HCT-116 cells that were exposed to media only (untreated) (A, G, M), 0.1% DMSO (B, H, N), 12 μg/mL cisplatin (C, I, O), 750 μg/mL (D, J, P), 500 μg/mL (E, K, Q), and 250 μg/mL (F, L, R) of 24-hour methanol extracted *C. nutans* for 24 hours (A, B, C, D, E, F), 48 hours (G, H, I, J, K, L), and 72 hours (M, N, O, P, Q, R) in 6-well plates under an inverted microscope.

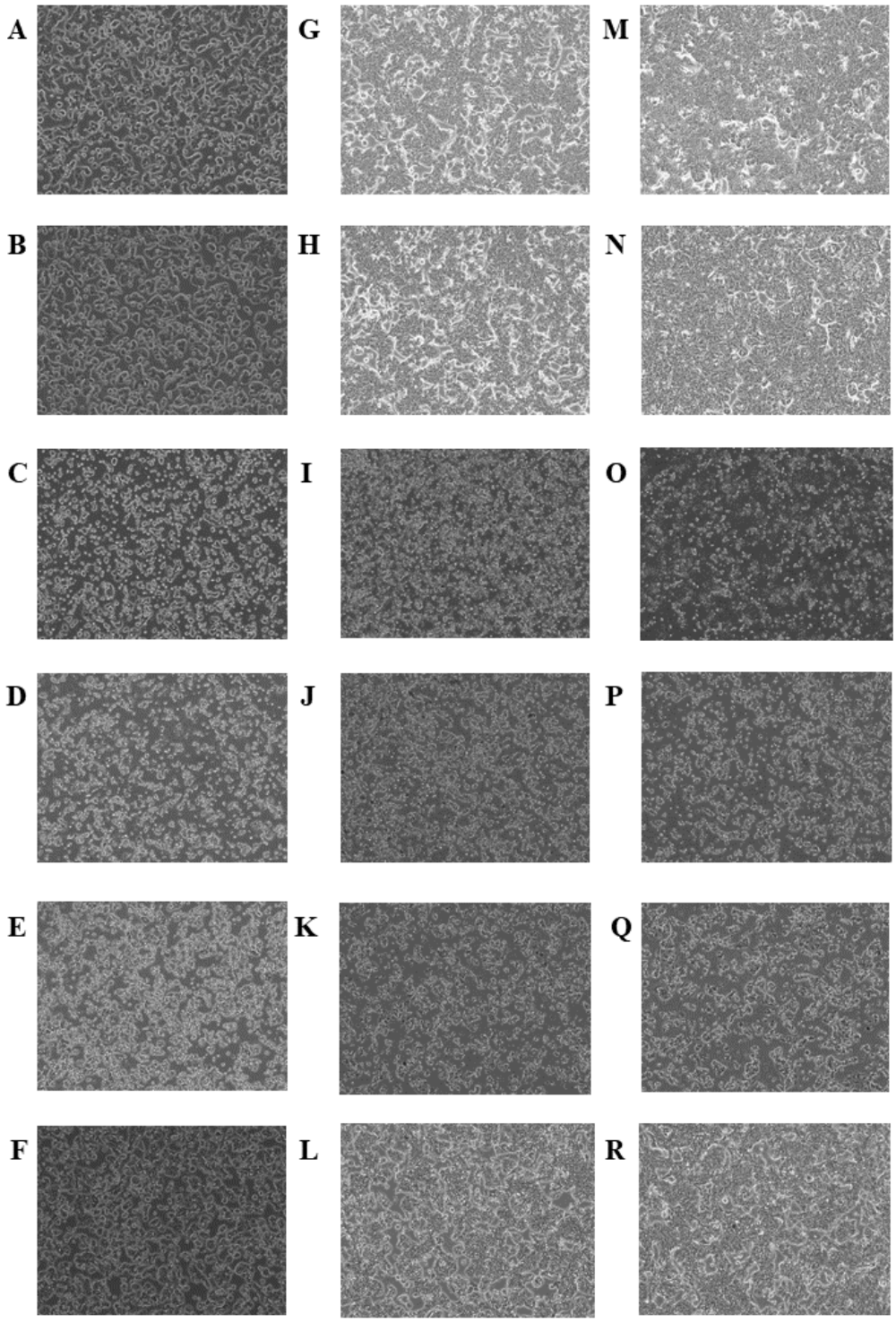


Figure 4.14: Morphology of HT-29 cells that were exposed to media only (untreated) (A, G, M), 0.1% DMSO (B, H, N), 15 μg/mL cisplatin (C, I, O), 750 μg/mL (D, J, P), 500 μg/mL (E, K, Q), and 250 μg/mL (F, L, R) of 24-hours methanol extracted *C. nutans* for 24 hours (A, B, C, D, E, F), 48 hours (G, H, I, J, K, L), and 72 hours (M, N, O, P, Q, R) in 6-well plates under an inverted microscope.

CHAPTER 5

DISCUSSION

5.1 Percentage Yield

The polarity of solvents and duration of extraction are two important parameters to consider when performing plant extraction as these could affect the phytochemical content and extraction yield. In this study, the extraction yield of *C. nutans* methanol extracts were the highest among the other solvents. In addition, methanol was found to be able to extract polyphenols and compounds with antioxidant and anticancer properties from *C. nutans* which agreed with the results of this study as the methanol extract was found to contain phenolic, flavonoids compound and possessed antioxidant and anticancer activities (Dai & Mumper, 2010; Ismail et al., 2020). The result is also supported by Zakaria et al. (2017) that *C. nutans* methanol extract exhibited a higher yield than other solvents.

On the contrary, lower polarity solvents like acetone and chloroform demonstrated a lower extraction yield than methanol, indicating that it is more favourable to extract *C. nutans* with polar solvents than non-polar solvents. The findings were also agreed with Ismail et al. (2020) that non-polar solvents like chloroform exhibited the lowest extraction yield, and water extract resulted in the highest extraction yield. Besides, this study demonstrated that the extraction duration of 24 hours resulted in a higher extraction yield than those of 30 minutes of extraction. This result is supported by another study. The extraction of *C. nutans* for 120 minutes at 60℃ resulted in the highest yield, while extraction at a high temperature for a prolonged time decreased the extraction yield (Sulaiman et al., 2017).

Thus, a longer extraction time allowed more compounds to be extracted yet at a higher temperature, the yield would be compromised (Sulaiman et al., 2017). Therefore, 24 hours of extraction time at room temperature yielded a higher amount of extract is justified.

5.2 Phytochemical Contents of *Clinacanthus nutans* Extracts

5.2.1 Total Phenolic Content (TPC)

All *C. nutans* extracts were assessed and found to contain phenolic compounds, while *C. nutans* acetone extracts contained the most phenolics compared with methanol and chloroform *C. nutans* extracts. This finding proved that semi-polar solvent (acetone) could extract more phenolic compounds than more polar (methanol) and more non-polar (chloroform) solvents. The results affirmed by Galanakis et al. (2013) that phenolic compounds were more soluble in semi-polar solvents such as acetone and alcohol than water or non-polar solvents. According to the study by Sulaiman et al. (2017), *C. nutans* leaves were extracted at a range of extraction times, from 80 minutes to 120 minutes.

The study found out that the phenolic compounds was increased as the extraction time increased to 120 minutes (Sulaiman et al., 2017). In this study, *C. nutans* leaves were extracted at two different time durations of 30-minute and 24-hour in order to compare and determine the appropriate extraction time for a more effective extract to exhibit biological activities. The phenolic content was slightly higher for 24-hour extraction than that of 30-minute extraction in acetone extract. The finding was in agreement with Sulaiman et al. (2017) where a longer duration of extraction may affect more compounds to be extracted by the solvent.

5.2.2 Total Flavonoid Content (TFC)

In this study, *C. nutans* methanol extracts were found to contain the highest level of flavonoids as compared to the counterparts of chloroform and acetone extracts. Although *C. nutans* methanol extracts accounted for the least phenolic content than chloroform and acetone, it contained the most flavonoid content. This might be due to the majority of phenolic compounds of methanol extract were flavonoids, while acetone extracts contained more other phenolic compounds than flavonoids. The findings are in accordance with Dai and Mumper (2010) that methanol could effectively extract low molecular weight phenolic compounds like flavonoids.

On the other hand, acetone was more efficient in extracting phenolic compounds with high molecular weight (Dai & Mumper, 2010). Furthermore, the flavonoid contents were slightly higher in 24-hour extraction time than that of 30-minute for all extracts. These results built on evidence that prolonged extraction time could effectively extract more flavonoids up to a particular duration before the yield plateau.

5.3 Antioxidant Activity of *Clinacanthus nutans* Extracts

In this study, *C. nutans* methanol extracts possessed the highest antioxidant activity as it required the lowest inhibition concentration to quench 50% of the DPPH free radicals (IC_{50}). This translated to the highest ascorbic acid equivalent antioxidant content (AAEAC). DPPH free radical scavenging assay is based on the reaction between antioxidants and DPPH free radicals. The antioxidants donate an electron to DPPH free radical and cause the colour to change from purple to yellow (Shahidi & Zhong, 2015). Methanol extracts exhibited the highest antioxidant activity may be due to its higher content of flavonoids. Flavonoids are

good radical scavengers as the hydroxyl group of flavonoids is highly reactive with ROS. The reaction between flavonoids and ROS causes the stabilisation and inactivation of free radicals (Panche et al., 2016).

Additionally, the antioxidant activity was higher in 24-hour extracts than in its counterpart of 30-minute extracts. The results also agree with a study conducted by Muzolf-Panek and Stuper-Szablewska (2021) in which 24-hour extraction time increased the antioxidant activity of tested herbs and spices. This result is also supported by Konan et al. (2020). The antioxidant activity increased as they increased the extraction time from 30 minutes to 2 hours through the maceration extraction method on their tested plant sample, *Palisota hirsuta* roots (Konan et al., 2020). This findings has proven that increased extraction duration is more effective in increasing the antioxidant activity potential of plant extracts.

5.4 *In vitro* Anticancer Activity of *Clinacanthus nutans* Extracts

5.4.1 Antiproliferation Activity of *Clinacanthus nutans* Methanol Extracts against Human Colorectal Cancer Cell Lines

Based on the TPC, TFC, and DPPH assays results, the methanol extracted *C. nutans* at 30-minute and 24-hour were selected to proceed into *in vitro* antiproliferation assay as methanol extracts possessed the highest extraction yield, total flavonoid content, and antioxidant activity among the extracts by other solvents. Antioxidants such as flavonoids were reported to prevent cancer initiation and promotion stages by inducing several anticancer effects such as cell cycle arrest and inhibition of mutated p53 protein (Kumar & Pandey, 2013).

Human colorectal cancer cell lines with two different cancer stages and normal human epithelial cells were used to study the antiproliferation activity of *C. nutans* methanol extracts through MTT assay in this study. HCT-116 cell line was used to represent the early stage of colorectal cancer, whereas the HT-29 cell line represented the late stage of colorectal cancer. Based on the results, 30-minute and 24-hour methanol *C. nutans* extracts exhibited antiproliferation activity against both colorectal cancer cell lines throughout the treatment periods. However, 30-minute methanol *C. nutans* extract resulted in higher antiproliferation activity in the early stage of colorectal cancer when treated at time periods of 24 and 72 hours, while higher antiproliferation activity in late stage was observed at time period of 48 hours.

Besides that, 24-hour methanol *C. nutans* extract exhibited higher antiproliferation activity in late-stage colorectal cancer at all three time periods of exposure 24, 48, and 72 hours respectively. This result was supported by Quah et al. (2017) that *C. nutans* methanol extract possessed higher antiproliferation activity on HT-29 cells with the treatment durations of 24 and 48 hours while the extraction was repeated several times and affected more beneficial phytochemical compounds were extracted (Quah et al., 2017).

On the other hand, the result contradicted the claims by Esmailli et al. (2019) that 24 hours of methanol extracted *C. nutans* exhibited the most cytotoxic with the lowest IC_{50} values to HCT-116 cells at 72 hours of treatment. This might be due to the different extraction methods used by Esmailli et al. (2019), as they extracted *C. nutans* leaves powders with methanol at a controlled range of temperature (50 to 55°C) for 24 hours. In contrast, the *C. nutans* leaves powder was extracted at room temperature for 24 hours in this study. The

slightly increased temperature might effectively extract more bioactive compounds that enhance the anticancer activity (Sulaiman et al., 2017).

Additionally, as the exposure time to the extracts increased, lower IC_{50} values were observed, indicative that *C. nutans* methanol extracts were dose- and time-dependent in inhibiting the cell proliferation. The findings are supported by other studies, although different polarity solvents and cell lines were used. For instance, the results are supported by Fong et al. (2016) that *C. nutans* methanol extract induced antiproliferation activity on D24 melanoma cells in time dependent manner. Moreover, the results were also agreed with Haron et al. (2019), where *C. nutans* extracted with polar and non-polar solvents were cytotoxic to HeLa cells in dose- and time-dependent manners. In addition, *C. nutans* dichloromethane fraction extract was found to inhibit the cell proliferation of MCF7 cells in dose- and time-dependent manners (Ismail et a., 2020). Therefore, this study provided new insight into *C. nutans* methanol extracts inhibited the proliferation of colorectal cancer cells in dose- and time-dependent manners.

Selective index (SI) values were calculated based on the ratio between IC_{50} of *C. nutans* methanol extracts on cancer cell lines (HCT-116 and HT-29) and normal cell lines (NP69). If SI value of the extract is more than one (>1), the extract is considered less toxic to normal cells and selectively against cancer cells (Segun et al., 2019; Lica et al., 2021). Thus, the higher the SI value, the safer the extract toward normal cells (Segun et al., 2019). Methanol extracted *C. nutans* at 24 hours was less toxic to normal cells and selectively inhibited HT-29 cells when incubated for 24, 48, and 72 hours, whereas methanol extracted *C. nutans* at 30 minutes incubation was less toxic to normal cells and selectively inhibited HCT-116 cells for 24-, 48-, and 72-hours incubation. The findings are in agreement with

Ismail et al. (2020) that *C. nutans* dichloromethane fraction extract was less cytotoxic to normal cells and selective to MCF7 cells, even though it used different solvents and cell lines.

5.4.2 Early and Late Apoptosis Triggered by *Clinacanthus nutans* Methanol Extract

C. nutans methanol extract that underwent 24-hour extraction was selected to assess its apoptosis effects on HCT-116 and HT-29 cell lines. It resulted in the highest extraction yield, more cytotoxic and selective to HT-29 cells at all treatment times with less toxic to normal cells than the extract that underwent 30 minutes of extraction. The concentration of *C. nutans* methanol extract at 750 μg/mL and 500 μg/mL resulted in a higher percentage of late apoptosis than the chemotherapy drug, cisplatin when exposed to HCT-116 cells for 24 hours. Thus, 500 μg/mL of *C. nutans* methanol extracts possessed similar effects as cisplatin on HCT-116 cells at 48 and 72 hours of treatment duration. The findings showed that *C. nutans* methanol extract at a 500 μg/mL concentration could exhibit similar apoptosis effects as cisplatin when administered on HCT-116 cells for 24, 48 and 72 hours.

Moreover, it demonstrated that at the concentration of 500 μg/mL, *C. nutans* methanol extract could effectively induce apoptosis in the shortest period of 24 hours on HCT-116 cells. Despite a 500 μg/mL concentration, *C. nutans* methanol extract showed cytotoxicity in normal cells based on the MTT assay result. When the concentration of the extract was lowered to 250 μg/mL and prolonged the exposure to 72 hours, the treatment of *C. nutans* methanol extract on HCT-116 shifted most of the cancer cells to late apoptosis after 72 hours of the treatment period. Haron et al. (2019) reported that 250 μg/mL of *C. nutans* dichloromethane extract caused the highest percentage of HeLa cells in the late

apoptosis stage at 48 hours of treatment time. This varied result might be due to different cell lines and solvents used, while the findings generated new insight on the apoptosis effect of *C. nutans* methanol extract on HCT-116 cells.

On the other hand, the treatment of HT-29 cells with concentrations of *C. nutans* methanol extract at 750 μg/mL and 500 μg/mL revealed similar early and late apoptosis rates, respectively, as untreated cells. This showed that both high doses were less effective in shifting the cells to late apoptosis throughout the treatment. Meanwhile, the concentration of *C. nutans* methanol extract at 250 μg/mL was effective in elevating the late apoptosis rate after treatment on HT-29 cells for 72 hours compared to untreated cells.

Orientin, a type of flavonoid found in *C. nutans*, induced apoptosis on HT-29 cells in a dose-dependent manner as HT-29 cells underwent early to late apoptosis when the concentration increased in 24 hours incubation (Huang et al., 2015; Thangaraj et al., 2019). This might explain the apoptotic effect of *C. nutans* methanol extract on HT-29 cells. Hence, 250 μg/mL of *C. nutans* methanol extract was ideal for treating HCT-116 and HT-29 cells while causing no harm to normal cells. Besides that, the 24-hour methanol extracted *C. nutans* resulted higher antiproliferation activity against HT-29 cells than HCT-116 cells based on the MTT assay. However, HCT-116 cells exhibited a higher percentage of late apoptosis than HT-29 cells when treated with higher doses of *C. nutans* methanol extract. This might be due to most of HCT-116 cells had progressed to late apoptosis while HT-29 cells remained at early apoptosis stage. The findings build on the new evidence that a high dosage of *C. nutans* methanol extract may induce other anticancer pathways instead of apoptosis in HT-29 cells.

The Annexin V-FITC and PI staining method could not distinguish between late apoptosis and necrosis. Therefore, others should further study the identification of the mechanism. Since the apoptosis assay results showed a high percentage of HCT-116 cells underwent late apoptosis throughout the treatment periods, *C. nutans* methanol extract could be more effective in causing late apoptosis in HCT-116 cells than HT-29 cells. The findings were the first to report that *C. nutans* methanol extract could inhibit both HCT-116 and HT-29 cells growth through early and late apoptosis in a dose- and time-dependent manner.

5.5 Chapter Summary

In short, *C. nutans* methanol extracts and 24 hours of extraction duration yielded higher amounts of extracts. *C. nutans* acetone extracts contained the highest total phenolic content, whereas *C. nutans* methanol extracts contained the highest total flavonoid content. *C. nutans* methanol extracts also exhibited the most potent antioxidant activity as they contained of high total flavonoid content, which was an excellent free radical scavenger. The extraction duration at 24 hours exhibited higher total phenolic contents in acetone extract while higher total flavonoid contents and antioxidants activity in all extracts. These findings showed that 24 hours of extraction duration could extract more phytochemical contents. In addition, 24-hour *C. nutans* methanol extract exhibited higher antiproliferation activity in HT-29 cells than in HCT-116 cells. It resulted in a lower IC_{50} values with less toxicity to normal cells throughout the treatment periods (24, 48, and 72 hours).

Moreover, the apoptosis assay showed that high doses (750 μg/mL and 500 μg/mL) of *C. nutans* methanol extract at 24 hours extraction duration was less effective in inducing late apoptosis in HT-29 cells. On the contrary, the high doses increased the percentage of

late apoptosis in HCT-116 cells throughout the treatment periods. However, high doses of *C. nutans* methanol extract might be harmful to normal cells as the concentrations were higher than the IC_{50} values of NP69 cells. Meanwhile, a low dose (250 μg/mL) of *C. nutans* methanol extract at 24 hours extraction duration effectively increased the percentage of late apoptosis in both HCT-116 and HT-29 cells when treated for 72 hours.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

The present study assessed the phytochemical contents, antioxidant activity, and *in vitro* anticancer properties of *C. nutans* leaf extracts. *C. nutans* acetone extracts were found to have a higher total phenolic content than that of methanol and chloroform extracts. In addition, *C. nutans* methanol extracts accorded the highest total flavonoid content and the strongest antioxidant activity among the other extracts. Hence, *C. nutans* methanol extracts were selected to proceed to the *in vitro* study. In dose- and time-dependent manners, these extracts corroborated the antiproliferative activity against the HCT-116 and HT-29 cells. Phenolics and flavonoids were detected in both 30-minute and 24-hour extractions. These extracts exhibited varying antioxidant and antiproliferation activities. The findings suggested that 24-hour was an optimal duration to extract *C. nutans* as it produced a higher yield and extracted more flavonoid content and antioxidant activity than that of 30-minute extracts.

Furthermore, methanol extracted *C. nutans* at 24-hour duration showed lower IC_{50} values on HT-29 cells and less toxic to normal cells than those of 30-minute extracts. Additionally, methanol extracted *C. nutans* at 24 hours induced early and late apoptosis in HCT-116 and HT-29 cells. A low dose of *C. nutans* methanol extract at 250 μg/mL effectively increased the percentage of late apoptosis in HCT-116 and HT-29 cells after 72 hours of treatment.

In conclusion, the findings suggested that *C. nutans* possessed anticancer properties against colorectal cancer cell lines, HCT-116 and HT-29 through the apoptosis pathway. The

findings provided new perspective for the future study to prove *C. nutans* as an alternative treatment against early and late stages colorectal cancer. Hence, further studies should be carried out to assess the endpoint for optimal extraction duration in relation to the yield, identify the bioactive compounds and delineate the detailed mechanisms behind these and other anticancer properties.

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APPENDICES

Appendix 1

Journal Publications

1. **Phung, J. H. Y.,** Fong, I. L., Khong, H. Y., & Ban, W. K. (2021). *In vitro* antioxidant and anticancer activities of *Clinacanthus nutans* extracts. *Science, Engineering and Health Studies*, *15*, 21050014. https://li01.tci-thaijo.org/index.php/sehs/article/view/248287
2. Ban, W. K., Fong, I. L., Khong, H. Y., & **Phung, J. H. Y.** (2022). Wound healing, antimicrobial and antioxidant properties of *Clinacanthus nutans* (Burm. f.) *Lindau* and *Strobilanthes crispus* (L.) *Blume* extracts. *Molecules*, *27*(5), 1722. https://www.mdpi.com/1420-3049/27/5/1722
3. **Phung, J. H. Y.**, Fong, I. L., Khong, H. Y., & Ban, W. K. (2020). *In-vitro* study of antioxidant activity and anticancer properties of *Clinacanthus nutans* extracts. *In Proceeding of International Conference on Innovative Sciences and Technologies for Research and Education (InnoSTRE) Special Edition*, Universiti Teknologi MARA Sarawak branch, Samarahan Campus, 25-26 November 2020, p18-19. (ISBN no: 978-967-0828-42-8)

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In vitro antioxidant and anticancer activities of *Clinacanthus nutans* extracts

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Phung, J. H. Y., Fong, I. L., Khong, H. Y., and Ban, W. K. (2021). In vitro antioxidant and anticancer activities of clinacanthus nutans extracts. Science, Engineering and Health Studies, 15, 21050014.

ABSTRACT

Clinacanthus nutans (CN), from the Acanthaceae family is a medicinal plant widely used in Thailand and Malaysia. CN is commonly used as a treatment of inflammation, cancer, and herpes virus infection. This study aimed to determine the antioxidant activity and anticancer properties of CN leaves extracts on human colorectal cancer cell lines, HCT 116 and HT-29. In this study, CN leaves powders were extracted in methanol, chloroform, and acetone at different durations. The crude extracts were assessed for total phenolic content (TPC), total flavonoid contents (TFC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay. The extract using acetone showed with the highest TPC. The highest total flavonoid content was the methanol extract. The DPPH radical scavenging activity, IC_{50} of methanol extract was 19.67 µg/mL and exhibited the most efficacious antioxidant property among the others. The 24-hour methanol extract showed the most promising results on MTT assay. Therefore, CN methanol extract is a promising candidate to proceed in other anticancer studies such as cell cycle arrest and apoptosis assays.

Keywords: *Clinacanthus nutans*; colorectal cancer; anticancer; antioxidants; phenolic; flavonoid

1. INTRODUCTION

Cancer causes the second highest mortality in the world. In 2018, it was estimated to result in 9.6 million deaths (WHO, 2019). Colorectal cancer is the third most fatal cancer and the fourth most diagnosed cancer worldwide (Rawla et al., 2019). According to the Malaysian national cancer registry report 2012-2016, colorectal cancer was the most common cancer in male and the second most common in female (Azizah et al., 2019). It is possible to prevent colorectal cancer progression if early polyps were detected. Nonetheless, early detection of polyps is not routinely performed unless the patient has underlying hereditary history of colorectal cancer. This is commonly treated with surgery and in the advanced stage, a single chemotherapeutic drug, 5-fluorouracil (5-FU) would be routinely used or combined with adjuvants like avastin and oxaliplatin (Sheikh et al., 2017a; Cutsem et al., 2015). Undetected polyps usually lead to formation of adenomas and carcinomas before the late stage of metastasis leading to high mortality risk (Sheikh et al., 2017a).

Many studies have reported that increased levels of intracellular free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) triggered cancer formation (Yong et al., 2013). ROS and RNS are normally found in the human body as they are by-products of metabolism in normal cells (Yong et al., 2013). These ROS and RNS damage the normal cells, especially DNA, resulting in genomic instability and induce cancer initiation (Yong et al., 2013). Many medicinal plants have been reported to

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Wound Healing, Antimicrobial and Antioxidant Properties of *Clinacanthus nutans* (Burm.f.) *Lindau* and *Strobilanthes crispus* (L.) *Blume* Extracts

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Abstract: *Clinacanthus nutans* is known to be an anticancer and antiviral agent, and *Strobilanthes crispus* has proven to be an antidiuretic and antidiabetic agent. However, there is a high possibility that these plants possess multiple beneficial properties, such as antimicrobial and wound healing properties. This study aims to assess the wound healing, antioxidant, and antimicrobial properties of *Clinacanthus nutans* and *Strobilanthes crispus*. The *Clinacanthus nutans* and *Strobilanthes crispus* leaves were dried, ground, and extracted with ethanol, acetone, and chloroform through cold maceration. In a modified scratch assay with co-incubation of skin fibroblast and Methicillin-resistant *Staphylococcus aureus*, *Clinacanthus nutans* and *Strobilanthes crispus* extracts were assessed for their wound healing potential, and the antimicrobial activities of *Clinacanthus nutans* and *Strobilanthes crispus* extracts were performed on a panel of Gram-positive and Gram-negative bacteria on Mueller–Hinton agar based on a disc diffusion assay. To assess for antioxidant potential, 2,2-diphenyl-1-picrylhydrazyl (DPPH), total phenolic and total flavonoid assays were conducted. In the modified scratch assay, *Clinacanthus nutans* extracts aided in the wound healing activity while in the presence of MRSA, and *Strobilanthes crispus* extracts were superior in antimicrobial and wound healing activities. In addition, *Strobilanthes crispus* extracts were superior to *Clinacanthus nutans* extracts against *Pseudomonas aeruginosa* on Mueller–Hinton agar. Acetone-extracted *Clinacanthus nutans* contained the highest level of antioxidant in comparison with other *Clinacanthus nutans* extracts.

Keywords: *Clinacanthus nutans*; *Strobilanthes crispus*; wound healing; methicillin-resistant *Staphylococcus aureus*; *Pseudomonas aeruginosa*; scratch assay; antimicrobial; antioxidant

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1. Introduction

Wound healing, a skin repairing process, is one of the most complicated processes in the human body. To close an open wound, multiple cells and stages are intrinsically involved, including haemostasis, inflammation, angiogenesis, growth, re-epithelialization, and re-modelling. However, harmful substances or opportunistic microorganisms may invade the body and hamper this process.

According to [1], commensals, such as *Propionibacterium acnes*, *Streptococcus pyogenes*, and *Staphylococcus aureus*, can be easily isolated on the surface of the skin, whereas opportunists *Enterococcus* spp. and *Pseudomonas aeruginosa* can be isolated in the absence of healing and in the presence of persistent inflammation, eventually leading to chronic wounds. The existence of methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most challenging issues when dealing with chronic wounds, as colonization and replication of MRSA on the wound further delays the wound healing process [2]. With the discovery of antibiotics, bacterial infections were alleviated but at a dire cost with the emergence of

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