CHARACTERISATION OF STANDARDISED FRACTION FROM *Clinacanthus nutans* (SF1) AND ITS ANTI TUMOR EFFECTS ON IN VITRO AND XENOGRAFT MODEL

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by

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS

WHO	World Health Organization
HPV	Human Papilloma Virus
DNA	deoxyribonucleic acid
NCSM	National Cancer Society Malaysia
m	meter
HSV	herpes simplex virus
NMR	nuclear magnetic response
HPLC	High Performance Liquid Chromatography
FTIR	Fourier Trans Infra-Red
GCMS	Gas Chromatography Mass Spectrometry
LCMS	Liquid Chromatography Mass Spectrometry
LCMS/QTOF	Liquid Chromatography Mass Spectrometry/Time of Flight
NO	Nitric Oxide
A549	lung cancer cells
CNE1	nasopharyngeal cancer cells
HepG2	liver cancer cells
D24	melanoma cells
CN30	30% *Clinacanthus nutans* ethanol extract
SF1	standardized fraction 1
DMSO	dimethyl sulfoxide
BSA	bovine serum albumin
DMEM	Dulbecco's Modified Eagle Medium
RPMI 1640	Roswell Park Memorial Institute Medium 1640
v/v	volume per volume

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
EDTA	Ethylenediaminetetraacetic acid
OD	optical density
PBS	phosphate buffered saline
KOH	potassium hydroxide
NaCl	sodium chloride
NaOH	sodium hydroxide
IgG1	antibody Immunoglobulin G type 1
IgG2b	antibody Immunoglobulin G type 2b
FACS	florescent activated cell sorting
PI	propidium iodide
TLC	thin layer chromatography
Bax	apoptosis regulator protein
Bcl-2	apoptosis deregulator protein
p53	tumour protein
SiHa	cervical squamous carcinoma cell
HeLa	cervical adenocarcinoma cell
NIH	fibroblast normal cell
AST	aspartate aminotransferase
ALT	alanine aminotransferase
nu / nu	nude mouse strain
BALB/c	albino, laboratory-bred strain mouse
BW	body weight
g	gram
ml	milliliter

%	percent
^{o}C	degree Celsius
M	molar
ppm	parts per million
nm	nanometer
LC	liquid chromatography
MS	mass spectrophotometer
HPV 16	human papilloma virus type 16
HPV 18	human papilloma virus type 18
NIH/3T3	fibroblast cell
ATCC	American Type Cell Collection
FBS	Fetal Bovine Serum
µm	micrometer
µl	microliter
hr	hour
Rf	retention factor
rpm	rotation per minute
CO_2	carbon dioxide
IC_{50}	concentration that inhibit half of cell population
PE	phycoerythrin
IVC	individual ventilated cages
SPF	specific pathogen free
IMR	Institute Medical for Research
RTV	relative tumour volume
V_t	tumour volume at day n

V_o	tumour volume at day 0
TC (%)	relative tumour growth ratio
T_{RTV}	test group's RTV
C_{RTV}	common negative control group's RTV
DPX	Dibutylphthalate Polystyrene Xylene
HRP	horse radish peroxidase
DAB	3,3′-Diaminobenzidine
ANOVA	analysis of variance
S.D	standard deviation
CV	central vein
CCV	congested central vein
H&E	Hematoxylin & Eosin
TIC	Total Ion Current
TCC	Total Compound Chromatogram
METLIN	Metabolite and Chemical Entity Database / MassHunter Qualitative Analysis B.05.00 software
FITC	Fluorescein isothiocyanate

PENCIRIAN FRAKSI PIAWAI *Clinacanthus nutans* (SF1) DAN KESAN ANTI TUMOR TERHADAP MODEL IN VITRO DAN XENOGRAF

ABSTRAK

Kanser serviks adalah penyebab utama kematian wanita di dunia dan ketiga di Malaysia. Kini, tumbuhan perubatan menjadi rawatan alternatif yang popular kerana kesan sampingan rawatan moden dan mudah didapati. *Clinacanthus nutans* (*C. nutans)* telah dikenal pasti dan dipercayai oleh penduduk tempatan sebagai alternatif kepada rawatan kanser. Menurut kajian awal, satu fraksi dari *C. nutans* telah menunjukkan perencatan yang aktif terhadap kanser serviks manusia, in vitro. Oleh itu, kajian ini dijalankan untuk mengenal pasti sebatian antikanser aktif dari *C. nutans* dan menentukan kesan penindasan tumor bersama mekanisme untuk menilai potensi *C. nutans* sebagai rawatan alternatif untuk kanser serviks. Mulanya, pengoptimuman fraksinasi berpandu bioassai telah dijalankan. Semua fraksi *C. nutans* yang terkumpul diperiksa untuk aktiviti sitotoksik ke atas sel kanser servik manusia HeLa dan SiHa dan sel normal, NIH, oleh Assai MTT. Fraksi piawai yang paling berpotensi, SF1 dicirikan dan dikenal pasti oleh Fourier Transform Infrared (FTIR), Spektrometer Jisim Kromatografi Liquid (LCMS) dan analisa fitokimia kuantitatif. Mekanisme antikanser SF1 dinilai oleh analisa sitometri aliran untuk perkembangan kitaran sel, mod kematian sel dan ekspresi protein menggunakan pewarnaan berganda Annexin-V/propidium iodida dan antibodi khusus berkonjugat; Bax, Bcl-2, p53 dan cytochrome C. Penyiasatan lanjut terhadap kesan penindasan tumor dijalankan menggunakan tikus nude xenograf sebagai model subjek hidup. Tikus telah diinduksikan dengan sel SiHa. Apabila isipadu tumor mencapai 100 mm^3, rawatan SF1 diberikan sekali sehari secara suntikan intraperitoneal, selama 28 hari. Kemudian, tumor dan hati diambil untuk analisis; pewarnaan hematoxylin dan eosin

(H&E) dan imunohistokimia (IHC) menggunakan caspase-3. Darah dikumpulkan untuk penilaian tahap aspartat aminotransferase (AST) dan alanine aminotransferase (ALT). Penemuan ini mencadangkan bahawa konstituen utama SF1 dikenal pasti sebagai sebatian alkaloid dengan amina sebagai kumpulan berfungsi. Rawatan SF1 menunjukkan sitotoksiksiti yang lebih baik dengan perencatan pertumbuhan terbaik terhadap SiHa sel (IC_{50} = 9.98±1.24 µg/ml) berbanding HeLa sel (IC_{50} = 81.21±1.17 µg/ml) dan menunjukkan sitoselektif dengan tiada IC_{50} dikesan ke atas NIH. SF1 mengaruh apoptosis awal dalam proliferasi sel SiHa dengan merencat sel di titik kawalan G1/S. Peningkatan p53 diikuti dengan peningkatan pro-apoptosis Bax dan penurunan anti-apoptosis Bcl-2 serta peningkatan tahap cytochrome C selepas rawatan dengan SF1 juga dibuktikan. Hasil kajian juga menunjukkan bahawa isipadu tumor dalam tikus SF1 (60.18±2.17 mm^3) yang dirawat berkurangan berbanding dengan kawalan negatif (139.16±12.97 mm^3). Selepas rawatan SF1, 43.74% nisbah pertumbuhan tumor relatif (T/C) dan 0.64 ± 0.03 daripada isipadu tumor relatif (RTV) telah dikira. SF1 menunjukkan kadar perencatan yang baik dengan lebih daripada 50% tumor ditindas. Tahap ALT dan AST dalam tikus SF1 yang dirawat kekal dalam julat normal berbanding kumpulan sisplatin yang menunjukkan kesan toksik. Analisa H&E menunjukkan tiada ketoksikan yang abnormal pada hati dan pengurangan mitosis pada tumor apabila dirawat dengan SF1. Analisa IHC mengesahkan peningkatan ekspresi pengantara penting bagi apoptosis, caspase-3, dalam tikus yang dirawat SF1. Kesimpulannya, SF1 menunjukkan aktiviti antikanser dengan mengaruh apoptosis melalui penahanan kitaran sel di titik kawalan G1/S melalui tapak jalan p53 diperantara mitokondria. Kesan penindasan tumor SF1 ditunjukkan pada perkembangan kanser serviks manusia xenograft dalam tikus nude. Oleh itu, penemuan ini menyediakan data potensi SF1 sebagai ubat terapeutik pada masa depan untuk rawatan kanser serviks.

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ABSTRACT

Cervical cancer is a leading cause of death in women, worldwide, and third in Malaysia. Nowadays, medicinal plants become an alternative treatment due to side effects of conventional treatment and easily available. C*linacanthus nutans* has been locally recognized and was claimed for cancer treatment. From our pilot study, a fraction of *C. nutans* shown a potent inhibition on human cervical cancer cells, in vitro. Therefore, this study was conducted to characterize active anticancer compounds from *C. nutans* and determine tumor suppression effect with its mechanism in order to evaluate the potential of *C. nutans* as an alternative treatment for cervical cancer. Initially, optimization methods of bioassay-guided fractionation was carried out. All collected fractions of *C. nutans* were examined for cytotoxic activity towards human cervical cancer cell lines, HeLa and SiHa, and normal cell line, NIH by MTT Assay. The most potent standardized fraction, SF1 was characterised and identified by Fourier Transform Infrared (FTIR), Liquid Chromatography Mass Spectrometer (LCMS) and quantitative phytochemical analysis. The anticancer mechanism of SF1 was evaluated by flowcytometric analysis for cell cycle progression, mode of cell death and protein expression using Annexin-V/propidium iodide double staining and specific antibody conjugated fluorescent dye; Bax, Bcl-2, p53 and cytochrome C. Further investigation of SF1 on tumor suppression effect was conducted using xenografted nude mice as a model of living subject. The nude mice were subcutaneously inoculated with SiHa cells. Tumor volume and body weight were recorded at 3 days intervals. When tumor volume reached 100 mm^3, SF1 was

administered once daily via intraperitoneal injection for 28 days. Tumor and liver were surgically removed and fixed for hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) using caspase-3. Blood was collected by cardiac puncture for assessment of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) level. The findings suggested that the major constituent of SF1 was identified as alkaloid with functional group, amines. SF1 exhibited better cytotoxicity with best growth inhibition against SiHa (IC_{50} = 9.98±1.24 µg/ml) compared to HeLa (IC_{50} = 81.21±1.17 µg/ml) and showed cytoselectivity with no IC_{50} detected on NIH cells. SF1 induced early apoptosis in SiHa cells with arrested cell cycle at G1/S checkpoint. Up-regulation of p53 followed by increasing of pro-apoptotic Bax and decreasing of anti-apoptotic Bcl-2 as well as increment of cytochrome C levels upon treatment with SF1 were also shown. The results showed that tumor volume (60.18±2.17 mm^3) in SF1-treated mice were reduced compared to negative control (139.16±12.97 mm^3). Upon SF1 treatment, 43.74± 2.27% of relative tumor growth ratio (T/C) and 0.64±0.03 of relative tumor volume (RTV) were calculated. SF1 showed a good inhibition rate with more than 50% of tumor were suppressed. ALT and AST level in SF1-treated mice were remained in normal ranges compared to cisplatin group indicating no sign of toxicity effects. The H&E analysis revealed no abnormal toxicity condition on liver and reduced number of mitosis on tumor upon treatment with SF1. The IHC analysis confirmed an increased expression of a crucial mediators of apoptosis, caspase-3, in SF1-treated mice. In conclusion, SF1 demonstrated anticancer activity by inducing apoptosis through arrested G1/S cell cycle checkpoint via p53 mediated mitochondrial pathway. The tumor suppression effect of SF1 was demonstrated on the growth of xenografted human cervical cancer in nude mice. Thus, these findings provided a data for a potential of SF1 as future therapeutic drug for cervical cancer treatment.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Malaysia is among developing countries that going through rapid transformation of social life and economic changes. This situation has led to improvement of lifestyles that usually occur to other industrialised countries. As consequences, cancer diseases rises as one of the major health threats related to quality of life in this world. Habli *et al.* (2017) stated that, rise of prevalence in cancer has captured attention of researchers who have been determined to find and develop better and effective treatments.

For the past decades ago, cervical cancer has been the most common types of gynecological cancer malignancies worldwide (Zhen *et al.*, 2016). It is the third most common cancer among females in Malaysia, after breast and colorectal cancer (Bruni *et al.,* 2018; Zaridah, 2014; Othman, 2003). The current treatment of cervical cancer are surgical removal of certain reproductive organs and radiotherapy with the help of chemotherapy. Those treatments has remain first option to treat cervical cancer. However, these approaches are limited by resistance, toxicity to surrounding healthy cells in body and expensive operation.

Currently, many anticancer drugs has been developed using medicinal plants in both traditional and modern medicine (He *et al.*, 2017; Ghasemzadeh *et al.*, 2014). The use of natural sources in cancer treatment have been applied extensively by many Malaysian old folks to improve survivorship. The medicinal plants have been an asset to Malaysian due to richness of our herbal resources in the forests (Ch'ng *et al.*, 2016).

World Health Organization (WHO) reported that in developing countries such as Malaysia, 85% of people utilised traditional medicines derived from medicinal plants for their primary health care (World Health Organization, 2017). This positive trend could be due to resistance towards commercial anticancer drugs and existence of certain side effects of current cancer treatment. In fact, medicinal plants constitutes largest and valuable sources of natural products that have been proven potent against various disease including cancer (Habli *et al.*, 2017; Alam *et al.*, 2016). Therefore, new and safe drugs that originated from medicinal plants are necessary in order to improve standard treatments, in vitro and in vivo.

1.2 Rationale of study

With the current trend, it is important to extensively develop a cure for cervical cancer from common medicinal plants that have been locally utilised. This is due to many cancer patients believed the effect of medicinal plants that can improve survivorship and offer better lifestyle upon diagnosed with the disease. In this study, *C. nutans* has been used based on traditional claim from our old folks in combating cancer and easily available (Alam *et al.,* 2016; P'ng *et al.,* 2013; Hariana, 2013).

Based on our pilot study, one of the active fraction from *C. nutans* namely F11 was able to inhibit proliferation of cervical cancer cells, HeLa (Roslan *et al.*, 2018). Initially, bioassay guided fractionation was carried out using column chromatography and several fractions were collected. All fractions were tested for cytotoxicity test by MTT Assay. F11 was found as the most potent fraction from the elution of ethyl acetate : hexane (1 : 1) by inhibiting the growth of HeLa cells at G1 phase. This indicate that the cell division has stop and cell death was induced via apoptosis as reported in the study. Hence,

the aim of the present study were to further characterize F11 and its anticancer mechanism, in vitro and in vivo, in order to evaluate the potential of *C. nutans* as an alternative treatment for cervical cancer.

The findings of this study could have provide proper guidance and support the traditional knowledge of local people in using *C. nutans* for cervical cancer treatment. It is also provide new knowledge about the bioactive compound from local plant that have medicinal properties of anticancer. With increasing concern of cancer chemoprevention from herbal medicinal plant, the utilization of local medicinal plant in Malaysia such as *C. nutans* might have beneficial effect towards cancer treatment.

1.2.1 Hypothesis of study

This current study was conducted to serve as a fundamental basis to further govern the characterisation of SF1, an active anticancer fraction derived from *C. nutans* and to study its tumor suppression effect. The anticancer mechanism will be investigated in order to evaluate the potential of SF1 as an alternative treatment for cervical cancer. At the end of this study, the findings in between biological activities, in vitro apoptosis study, in vivo tumor suppression effects and characterisation of *C. nutans,* specifically SF1, with anticancer properties will be elucidated. Thus, the anticancer mechanism of SF1 and its effectiveness will provide updated knowledge and information towards cancer treatment. In future, if it is proven to be effective and potent towards cervical cancer treatment, a new potential alternative drug for anticancer could be further formulated and developed for human beneficial.

1.3 Objectives of study

1.3.1 General Objective

To characterize active anticancer compounds from *C. nutans* and determine the tumor suppression effect with its anticancer mechanism in order to evaluate the potential of *C. nutans* as an alternative potential treatment for cervical cancer

1.3.2 Specific Objectives

1) To characterize the active fraction extracted from the leaves of *C. nutans* which responsible for anticancer activity using Fourier Transform Infra-Red (FTIR), Liquid Chromatography Mass Spectrophotometry (LCMS) and quantitative phytochemical analysis
2) To determine the cytotoxic activity of active fraction from the leaves of *C. nutans* towards human cervical carcinoma by comparing with non-cancerous cell lines using MTT Assay
3) To investigate the mechanism of anticancer effect of active fraction from the leaves of *C. nutans* using flowcytometry, with observation of cell cycle profile, measurement of mode of cell death and detection of apoptotic protein, in vitro.
4) To further investigate the anti tumor effect of active fraction from the leaves of *C. nutans* using xenograft model, with observation of tumor suppression effect, biochemical profile and histopathological studies.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

In 2017, the International Agency Research on Cancer (IARC), the specialised cancer agency of WHO reported more than 14 million new cancer cases and almost 10 million cancer-related deaths in its online database, GLOBOCAN. More than half of all cancer cases and cancer deaths in 2015 occurred in developing countries. Tremendous efforts have been put into developing a cure for this disease, but the number of cancer patients remain higher and increases as new cases arises annually. This percentage is predicted to be increase by 2025 (International Agency for Research on Cancer, 2018). Experts have anticipated that the main cause of this disease has been due to rapid uncontrollable multiplication and spreading of abnormal cells. This phenomenon is caused by mutation that constantly emerge in the genome of cancer cells during cells division (Habli *et al.*, 2017).

2.2 Cervical cancer

The cervix is a cylindrical structure composed of stroma and epithelium, located at the lowermost part of the uterus. WHO reported that cancer of the cervix uteri is the fourth most common cancer among women with 284,823 women being diagnosed with cervical cancer and 144,434 dying from the disease worldwide, annually (Bruni *et al.*, 2018). In Asia, this type of cancer ranks the third most frequent cancer among women (Bruni *et al*., 2018). An estimated population of 1673.2 million women aged 15 years and older who are at risk of developing cervical cancer in Asia. The National Cancer Society

of Malaysia (NCSM) reported that cervical cancer is the third most common cancer among women and the fourth leading cause of death in women aged between 15 and 44 years old, in Malaysia (Zaridah, 2014; Indramalar, 2015). In Malaysia, although screening and immunisation programmes have been widely implemented, the mortality rate due to cervical cancer is still higher than United Kingdom's (Zaridah, 2014).

Cervical cancer is usually characterised by a premalignant condition known as cervical intraepithelial neoplasia (Tsao *et al.*, 2004). Among the major factors contributing to cervical cancer is human papillomavirus (HPV) infection such as HPV 16, 18, 31, 33, 35, 45, 52 and 58. However, almost 70% of the cancers worldwide comprise squamous cell carcinoma, HPV 16 and followed by adenocarcinoma, HPV 18, worldwide (Bruni *et al.*, 2018; Zaridah, 2014; Choudari *et al.*, 2013). Due to this prevalence scenario, two types of cervical cancer cell lines which are HeLa (HPV 18) and SiHa (HPV 16), were used throughout this study. Figure 2.1 showed simplified illustrations on genomic difference between HPV 16 and HPV 18. The prevalence of HPV increases with lesion severity of 41% - 67% high-grade cervical lesions and 16% - 32% low-grade cervical lesions (Bhatla *et al.*, 2018). The majority of HPV infections resolve spontaneously and do not cause symptoms or diseases. However, Zaridah (2014) reported that persistent infection with specific types of HPV, most frequently types 16 and 18, usually leads to precancerous lesions. Cervical cancer is a potentially preventable disease. However, certain kinds of lesion may develop into cervical cancer if no further treatment or precaution is taken.

The genome of HPV is a circular double-stranded DNA molecule of approximately 8000 base pairs (Saito and Kiyono, 2007). Bruni *et al.* (2018) reported that there is an increasing evidence linking HPV DNA with cancers of the anus, vulva,

vagina and penis, although the information is limited compared to cervical cancer. The HPV viral oncogenes, E6 and E7 have been shown to be the main contributors to the development of HPV-induced cervical cancer and increased expression. This happened probably due to integration of the viral DNA in the host cell genome which has been detected in invasive cancers and a subset of high-grade lesions (Saito and Kiyono, 2007). Both E6 and E7 HPV oncogenes interact and inhibit the activities of tumor suppressors by inactivate the action of tumor suppressor p53 and/or retinoblastoma protein (pRB) as shown in Figure 2.2 which adapted from Burd (2003).

Generally, anal cancers are predominantly squamous cell carcinoma, adenocarcinoma and basaloid carcinomas, while vulvar cancers represent the majority of the vulvar lesions which occur more often in older women and rarely associated with HPV (Bruni *et al.*, 2018). Most vaginal cancers are squamous cell carcinoma, which is generally attributable to HPV, followed by clear cell adenocarcinomas and melanoma. In most cases, metastatic cervical cancer usually has been misclassified as vaginal cancer (Bruni *et al.*, 2018; Bhatla *et al.*, 2018).

With this alarming situation, there is an urgent need to prevent the increment of cervical cancer case by developing potent and economical approaches for early detection and treatment of cervical cancer.

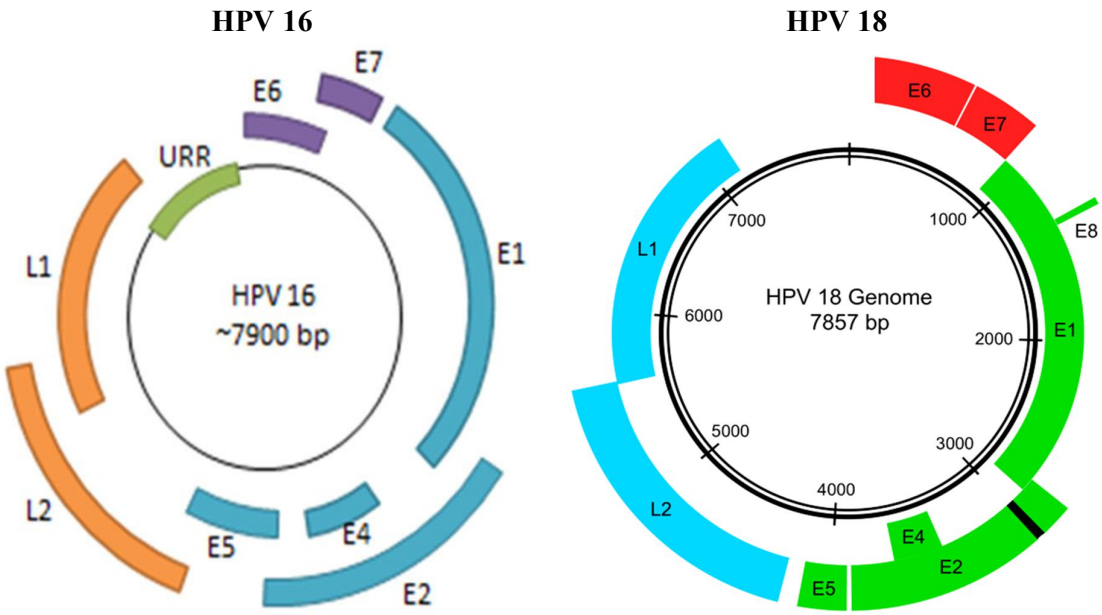

Figure 2.1 Simplified illustrations on genomic difference between HPV 16 and HPV 18 where URR : regulation of virus gene expression & virus replication; L1 : major capsid protein; L2 : minor capsid protein E1 : DNA replication; E2 : replication and transcription; E4 : viral release; E5 : immune evasion; E6 : binds p53; E7 : binds pRB (Adapted from Shanmugasundaram, S. & You, J. (2017) and Chakravorty, A. & Sugden, B. (2018)).

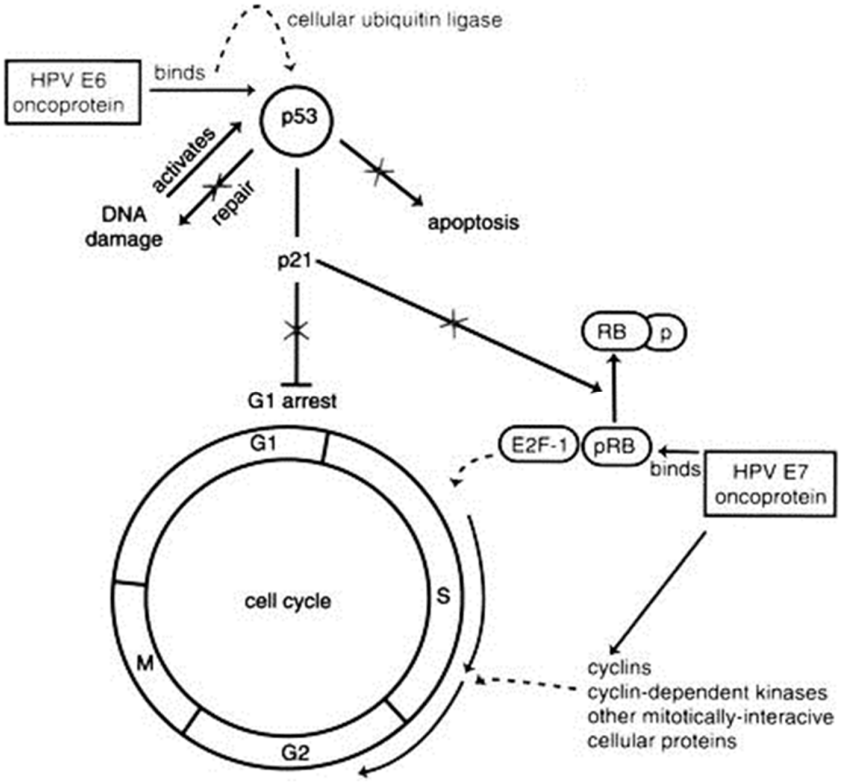

Figure 2.2 Pathogenesis of oncogenic HPV where HPV E6 and E7 genes encode multifunctional proteins that bind primarily to cellular p53 and pRB proteins, disrupt their functions, and alter cell cycle regulatory pathways, leading to cellular transformation (Burd, 2003)

2.2.1 Treatment of cervical cancer

Generally, the treatment of cervical cancer involves multidisciplinary strategies. Various factor should be taken into consideration before any treatment is started. The locality of the cancer, categories of the cancer and not forgotten stages of the cancer including status of metastasis. The cervical cancer is usually treated with combination of surgery, radiotherapy and chemotherapy. The ultimate aim for any alternative treatment that targets cervical cancer cells is specifically to reduce unnecessary side effects and increase the efficiency of cancerous cell killing without affecting any surrounding healthy tissues. Since the treatment of cervical cancer involves continuous strategies, the cost for prolonged treatment is a major concern among patients and the government. Annually, it costs more than RM 300 million to manage cervical cancer from the prevention steps until handling the invasive cases (Zaridah, 2014). Apart from that, precautionary measures such as HPV vaccination before infected with cervical cancer should be taken into consideration among women regardless of age.

The most common treatment for cervical cancer is surgery, which is more preferred for early stage of cervical cancer (American Cancer Society, 2016). Nevertheless, cancer cells tend to attack healthy tissue nearby and potentially spread to distant, thus becoming metastatic. Most women with cervical cancer opt for uterus removal, thus unable to conceive after surgery (National Cancer Institute, 2017).

Other than that, radiotherapy is another option to treat cervical cancer. This treatment provides equal good results in terms of local control and survival for long term effect. Usually, in order to avoid the risk of recurrence, the option for radiation therapy comes after the surgical removal of tumor, known as adjuvant radiation (Bhatla *et al.*,

2018). The disadvantages of this method are prolonged overexposure to the radiation beam and its effect on non – cancerous tissues. Low doses should be considered to avoid damage to healthy tissue but the accomplishment for cancer treatment would be delayed.

Apart from that, chemotherapy is also among the popular choice to battle cervical cancer. Generally, it is a treatment that uses cytotoxic anticancer drug. However, it does not distinguish between cancerous and healthy fast-growing cells in the body, such as hair and blood cells (Sayyad *et al.*, 2009). The use of chemotherapy drugs are controversial. It is mainly related to drug resistance where the immune system of patients has been compromised with chemotherapy routine treatment (Yip *et al.*, 2014). The current chemotherapy routine is unable to target specifically to cancer cells. The chemotherapy drugs kill both normal and cancerous cells due to the route of drug administration, which delivers the drugs to the whole body (Bhatla *et al.*, 2018).

The most extensively used for chemotherapeutic anticancer drug is cisplatin, the first metal antitumor drug (Sayyad *et al.*, 2009; Bhatla *et al.*, 2018). A combination of cisplatin and radiotherapy restores p53 function and enhances the radiosensitivity of HPV 16-positive SiHa cells (Huang *et al.*, 2004). However, the significant role played by cisplatin is limited by its undesirable effect towards healthy cells and burdens patients with diarrhoea, fatigue and compromised immune system (Zhen *et al.*, 2016). Eventually, the growth of cancerous cervical cells is weakened throughout treatment period with cisplatin, but the cells become resistant to the continuous treatment. This leads to possible reoccurrence.

Based on above situation, it is recommended to utilise chemopreventive agents either from natural sources, synthetic or biochemical agents to delay or prevent

progression of cancerous cell (Tsao *et al.*, 2004). Chemopreventive agents originating from natural sources are believed to be more effective and relatively non-toxic towards suppressing HPV-positive cancer cells with lesser side effects compared to biochemical treatments (Zhen *et al.,* 2016; Yaacob *et al.*, 2015).

2.3 Mechanism of cell death

Cytotoxic effect can be defined as inhibition of cancerous cell proliferation induced by certain agents that cause cell death. Mode of cell death can be implemented through apoptosis, necrosis, or aging (Elmore, 2007).

2.3.1 Necrosis

Necrosis is a toxic process where the cells enter a passive situation, uncontrolled, unregulated and energy-independent mode of death. Traditionally, necrosis is considered the primary form of accidental cell death caused by inflammation. In necrotic cell death, external factors such as severe environmental perturbations and harsh physicochemical stimuli including mechanical factors, abrupt changes in temperature, osmotic pressure, excessively high concentration of chemical toxins and injury could damage the cell irreversibly, causing necrotic cell death (Su *et al.*, 2016). This situation causes uncontrolled release of inflammatory cellular contents, thus resulting in changes of cell morphology. Cell swelling, formation of cytoplasmic vacuoles, formation of blebbed cytoplasm, and condensed swollen mitochondria are among typical morphological changes that occur during necrosis (Kerr *et al.*, 1972; Elmore 2007; Zakaria *et al.*, 2009). There is an influx of water and ions after the cell and its organelles swell and rupture. Due to this, necrotic cell death is usually related to induced and triggered inflammatory

cascade, which is not desirable in treating cancer. Most of anticancer studies involving traditional medicinal plants prefer drug treatment that elicit less necrotic effect to avoid unwanted side effects (Su *et al.*, 2016; He *et al.*, 2017; Zakaria *et al.*, 2017)

2.3.2 Apoptosis

Apoptosis is a type of active programmed cell death that plays a vital role in our body, including regulation of homeostasis. The apoptosis term was introduced by Kerr *et al.* (1972). In apoptosis, the cells shows morphological changes such as chromatin condensation, cytoplasmic cell shrinkage, fragmented DNA and apoptotic bodies. The mechanisms of apoptosis pathway involves a cascade of molecular events. Kumar and Clarks (2016) reported that treatment of cancer with chemotherapy works by triggering apoptotic pathways in the body. In apoptosis, there are two signaling pathways known as extrinsic and intrinsic pathways. In the extrinsic pathway, the damage of cell death involved extracellular effect (He *et al.*, 2017) and is activated by death receptors of the tumor necrosis factor (TNF) receptor superfamily.

In the intrinsic pathway which also or known as mitochondrial pathways, there are intracellular damage such as DNA damage. This pathway is activated by signal transduction involving mitochondria and Bcl-2 protein family. He *et al.,* (2017) stated that the ratio of Bax to Bcl-2 is an important indicator to assess apoptotic levels which high ratio of Bax/Bcl-2 presents apoptosis that induced by various factors including internal and external environmental factors. Bcl-2 inhibits the release of cytochrome C from mitochondria, which directly inactivates caspase-3 thus arrests apoptosis. Interestingly, Bax acts in an opposite manner by facilitating the release of cytochrome C from mitochondria and triggering molecular cascade of caspase, thus promoting apoptosis.

p53 is an anti-oncogene which is most highly involved in cancer. The p53 participates in the regulation of cell cycle and later repair the damaged DNA. The cell cycle consists of four phases namely G1 (gap phase), S (DNA synthesis), G2 (gap phase) and M (mitosis). At G1 phase, transcription of cell cycle control genes and synthesizes protein begins while conducting series of checks before DNA synthesis. During S phase of cell cycle, DNA synthesis occurs and entire genome of cell is replicated whereas a gap phase G2/M allows newly replicated DNA continues to grow and prepare to enter M phase where cell divides its copied DNA into two daughter cells (Haron *et al.,* 2019; Habli *et al.,* 2017). The cell cycle system consists of several checkpoints that pause during each phase transitions to assess cellular conditions are proper for further growth and division. If the DNA damaged cannot be repaired, p53 acts by arrest cell cycle before entering mitosis and then induced apoptosis event in cancerous cells.

Another hallmark of both apoptosis pathways are the release of cytochrome c and activation of several caspases such as caspase -9, -8 and caspase -3. The key biochemical event involved in the induction of apoptosis is the activation of caspase-3, which is mediated through proteolytic cleavage of procaspase-3 via upstream caspases; caspase 7/9 or caspase 8 (Kumar and Clarks, 2016). The activity of the caspase-3, which is an effect caspase involved in both intrinsic and extrinsic pathways of apoptosis, is expected to be altered in drug-induced cytotoxicity of cancer cells. Therefore, drugs that restore the apoptotic pathways have the potential to be effective treatment for tumors (Roslan *et al.*, 2018; Yaacob *et al.*, 2015). An ideal therapeutic goal for cancer treatment is to trigger selective apoptosis in tumor cells. Most of the antitumor drugs kill the cancer cells by stimulating the apoptotic pathway (He *et al.*, 2017; Yaacob *et al.*, 2015). Since targeting apoptosis is a good alternative strategy for cancer prevention and treatment, the potential

of *C. nutans* as an anticancer agent towards cervical cancer in this current study should not be wasted. Table 2.1 represented the differences between apoptosis and necrosis.

Table 2.1 Differences between apoptosis and necrosis (Kumar and Clarks, 2016; Su *et al.*, 2016; He *et al.*, 2017).

APOPTOSIS	NECROSIS
Active process	Passive process
Regulated	Unregulated
Programmed cell death	Accidental cell death
Controlled cell death	Uncontrolled cell death
Caused by intrinsic cellular response	Caused by severe environmental changes
Detachment and engulfed by phagocytes	Recruitment of inflammatory cells
Non – random DNA fragmentation	Random DNA degradation
Blebbing of plasma membrane	Loss of plasma membrane integrity
Formation of apoptotic bodies	Leakage of cellular contents
Increase in cell volume	Shrinkage in cell volume

2.4 Traditional medicinal plant

2.4.1 Background

Medicinal plants are the basis of most traditional medicines. Local communities have a way of life that is interconnected with their surrounding environments. Their traditional knowledge including knowledge of species, ecological interactions and other environmental phenomena are obtained through observation, practice and adaptation. From here, there is widespread recognition of traditional medicinal plants such as the Tongkat Ali (*Eurycoma longifolia*), Hempedu Bumi (*Andrographis paniculata)* and others as well (National Policy on Biological Diversity, 2016). Hence, the traditional claim and belief in complementary medicine derived from medicinal plants should be explored and commercialised in order to curb cancer. In addition, the resistance of cancer cells towards commonly used treatment urges the discovery of novel components based on the routine from our old folks who used medicinal plants (Habli *et al.*, 2017). Recently, this situation has been supported as scientific research on medicinal plants which is becoming more significant with several numbers of bioactive compounds being shown to exhibit variety of biological activities in vitro and in vivo. In cancer treatment itself, the majority of studies focus on the induced cytotoxicity from well-known plant-derived drugs such as vinblastine, topotecan, taxol, vincristine and vinflunine that are used clinically, worldwide (Habli *et al.,* 2017; Liu *et al.,* 2017; Nassan, 2015;). For example, the vincristine which is derived from the leaves of the plant *Catharanthus roseus* has prominent antimicrotubule effects on the cells of metastatic urothelial carcinoma, able to treat hepatocellular carcinoma and breast cancer (Holmsten *et al.,* 2016; Huang *et al.,* 2015). However, there are also dilemma when these plant-derived drugs exhibited a

certain level of cytotoxic reactions in patients such as neurotoxicity and abdominal problems (Habli *et al.,* 2017; Kampan *et al.,* 2015; Musa *et al.,* 2013)

2.4.2 Safety

The common reason for refusal of conventional treatment to treat cancer including doubts about its safety. Cancer patients believe that alternative remedies such as medicinal plants are free from harmful effects despite no proper scientific analysis have been performed (Sayyad *et al.*, 2009; Yaacob *et al.*, 2015; Bruni *et al.*, 2018). Certain medicinal plants are fragile, easily contaminated and degraded. Thus, to maintain their safety, quality and efficacy, they should be properly handled during preparation (Ibrahim, 2004). The lack of scientific information including authentication of plant material, its active principle, efficacy of dosage and mechanism of action of the plants may lead to harmful effects on our bodies to the point of death (Khoo *et al.*, 2018). Therefore, actual knowledge regarding properties of the plants, its safety and important information related to the suitable dosage should be established. Hence, there is an urgent need to fulfill and ameliorate the safety knowledge of medicinal plants with formal scientific documentation.

2.5 Cervical cancer and traditional medicinal plant

Since our old folks, the trend showed that more than 60% of the currently used anticancer drugs are derived from natural sources such as plants, marine organisms and microorganisms (Alam *et al.*, 2016; Choudari *et al.*, 2013). Many previous studies have suggested the potential of medicinal plant as anticancer agents (Roslan *et al.*, 2018; Yaacob *et al.*, 2015; Ma *et al.*, 2012). To date, there are more than hundreds of drugs

originated from medicinal plants that have been approved and commercialised for cervical cancer treatment, such as vinblastine, vincristine and paclitaxel (Habli *et al*., 2017; Safarzadeh *et al.*, 2014).

The use of traditional medicinal plant has become a trend in combating cervical cancer. For example, fisetin, a naturally occurring flavonoid, exhibited anticancer effect and induced apoptosis in HeLa cell lines, both in vitro and in vivo (Ying *et al.*, 2012). Chloroform extract of *C. nutans* leaves has also been proven to inhibit the growth of HeLa cells (Yong *et al.*, 2013). Curcumin, a natural compound originating from *Curcuma longa* was able to counteract the proliferative response of estradiol and induced apoptosis in cervical cancer cell, C33A (Singh and Singh, 2011). Interestingly, an aqueous extract of *Ficus religiosa* induces cell cycle arrest in SiHa cells and induced apoptosis in HeLa cells (Choudari *et al.*, 2013). Recently, He *et al.* (2017) reported that liquiritin, a major constituents derived from *Glycyrrhiza radix* exhibited suppressive effects on the growth of SiHa cells in vitro by activating caspase-3 and on xenograft mice in vivo.

2.5.1 *Clinacanthus nutans*

In Malaysia, one of the renowned medicinal plants is *C. nutans* which belongs to family of Acanthaceae. This plant is a native herb in tropical regions of Southeast Asia. Acanthaceae is one of the largest families of medicinal plants that have been utilised as effective traditional medicines. *C. nutans* is recognised by a few different vernacular names (Table 2.2), such as *belalai gajah* in Malaysia and Brunei (Zulkipli *et al.*, 2017; Huang *et al.*, 2015).

Table 2.2 Vernacular names for *Clinacanthus nutans*

Vernacular names	Language	Country	Reference
Belalai gajah	Malay	Malaysia, Brunei	P'ng *et al.*, 2013
Sabah Snake Grass	English	Malaysia, Brunei	Arullappan *et al.*, 2014
Dandang Gendis	Indonesian	Indonesia	Hariana, 2013
You Dun Cao	Mandarin	China	Ying, 2013
Phaya Yo	Thai	Thailand	Quattrocchi, 2012

This plant is a perennial herb with a rambling shrub that grows in nearly every habitat such as open or dense forests, scrublands, valleys and wet fields in tropical areas (Zulkipli *et al.*, 2017; Alam *et al.,* 2016; Yong *et al*., 2013). The stems are cylindrical, yellow when dry, densely striate and subglabrous. The shrub can grow up to 1 – 3 m tall and has branches (Zulkipli *et al.*, 2017). The flower possessed dark red colour with a green base, however due to the leaves of *C. nutans* are commercially sold as beneficial health products, the plant is often harvested earlier before it can flower. Thus, *C. nutans* flowers are rare to be found.

2.5.1 (a) The use of *C. nutans* in folk medicine

C. nutans has been used as a traditional medicine in China and Southeast Asia, mainly in Indonesia, Thailand and Malaysia. This plant has been appointed as medicinal herbs to be used in hospitals under the primary healthcare program in Thailand (Khoo *et al.*, 2018). The old folks from Malaysia and Thailand utilised this plant widely for the treatment of skin rashes, insect and snake bites, herpes simplex virus (HSV) and varicella-zoster virus lesions, mental tension, diabetes and rheumatoid arthritis (Tu *et al.*, 2014).

Some studies reported that *C. nutans* leaves are consumed as juices, tea and fresh drinks (Fazil *et al.*, 2016; Alam *et al.*, 2016). Some old folks also eat the raw leaves of *C. nutans*. It is a common scenario in the local community that this plant is utilised in other forms such as tinctures, elixirs, poultices, powders and other formulations for chronic disease management or health-boosting purposes (Khoo *et al.*, 2018). The leaves of *C. nutans* have gained its popularity as a supplement and medicine to treat various diseases especially cancer. There are traditional claims that the decoction of *C. nutans* leaves can

cure cancer. However, its side effect is unknown. The healing effect has not been clinically tested in laboratory animals although the usage of this plant among the public is still growing (Asyura *et al.*, 2016).

2.5.1 (b) Phytochemistry of *C.nutans*

Many previous studies have shown that the aerial parts of plants such as leaves and stem contain a high amount of bioactive compound with anticancer properties including flavonoids, phenolic compounds, alkaloids and terpenoids, and these compounds can be extracted using specific organic solvents (Fazil *et al.*, 2016; P'ng *et al.*, 2013). Wanikiat *et al.* (2008) reported that the major phytochemical constituents of *C. nutans* are stigmasterol, β-sitosterol, lupeol, betulin, six known C-glycosyl flavones, vitexin, isovitexin, schaftoside, isomollupentin, 7-O-β-gluco pyranoside, orientin, isoorientin, two glycoglycerolipids, a mixture of nine cerebrosides, five sulfur-containing glucosides and a monoacylmonogalactosylglycerol.

Sakdarat *et al.* (2008) extracted *C. nutans* with chloroform and chromatographically separated different varieties of chlorophyll a and chlorophyll b with a total of eight other compounds were isolated. However, only two compounds, trigalactosyl and digalactosyl diglyceride showed a good anti-herpes simplex virus (HSV) activity. Later, Sakdarat *et al.* (2009) isolated three more chlorophyll derivatives that were characterised by nuclear magnetic resonance (NMR) and found out that these compounds have anti-HSV activity.

More phytochemicals named eicosane, 1-nonadecene, heptadecane, dibutylphthalate, n-tetracosanol-1, heneicosane, behenic alcohol, 1-heptacosanol,

nonadecyl heptafluorobutyrate, heneicosane, eicosayl trifluoroacetate, heptadecane and few more compounds with 1,2 – benzenedicarboxylic acid, mono are the major chemical constituents identified present in *C. nutans* using Gas Chromatography Mass Spectrohotometry (GCMS), with relative peak area more than 28.6% (Yong *et al.*, 2013). Other researchers have reported the presence of flavone C-glycosides as active constituents in *C. nutans* extracts by High Performance Liquid Chromatography (HPLC) analysis which the treatment led to a significant and dose-dependent reduction in tumor size and weight (Huang *et al.*, 2015).

2.5.1 (c) Biological activities of *C. nutans*

C. nutans extract has been proved to possess various biological activities. For example, the aqueous extract of *C. nutans* showed a potential antiangiogenic agent. The extract successfully suppressed endothelial cell (HUVEC) proliferation and migration by abolished the sprouting of vessels in aortic rings (Ng *et al.*, 2018). Foong and colleagues (2017) reported that *C. nutans* aqueous extract possessed antiallergic property by inhibiting the early phase of IgE-mediated mast cell degranulation. *C. nutans* also exhibited antiviral activity towards HSV (Kunsorn *et al.*, 2013). Polyherbal formulation of *C. nutans* with another traditional medicine also showed good wound healing activity where the ethyl acetate fraction of the formulation had the fastest wound healing activity with 89.28% inhibition (Aslam *et al.*, 2016). The methanol extract of *C. nutans* also influenced peripherally and centrally mediated antinociceptive activity via the modulation of the opioid/NO-mediated (Rahim *et al.,* 2016).

The antiproliferative and anticancer activities of *C. nutans* have also been widely studied. For example, the chloroform extract of *C. nutans* is capable of free radical

scavenging and displayed significant proliferative effects on various cancer cells such as HeLa, HepG2, IMR32, K562, LS-174T, NCL-H23 and SNU-1 cells (Yong *et al.*, 2013). Ng *et al.* (2017) showed that hexane and chloroform extracts of *C. nutans* have antiproliferative effect against A549 lung cancer cells, CNE1 nasopharygeal cancer cells and HepG2 liver cancer cells. The aqueous extract of *C. nutans* displayed potent antiangiogenic properties and has emerged as one of strategies to combat cancer (Ng *et al.*, 2018). Treatment with *C. nutans* methanol extract demonstrated apoptosis induction in D24 melanoma cells (Fong *et al.*, 2016). Roslan *et al.* (2018) proved that an active fraction from *C. nutans* is able to inhibit proliferation of HeLa cells by arresting at G1/S phase. A previous study reported that roots of *C. nutans* ethyl acetate extract promotes apoptosis induction on cervical cancer cells, HeLa via mitochondrial dependent manner (Teoh *et al*., 2016). There is limited study on the potential of *C. nutans* as an anticancer agent in animal models. Huang *et al*. (2015) reported that main components of *C. nutans* known as CN30, has a significant inhibitory effect on tumor volume and induced apoptosis in hepatoma cells.

Apart from being known as an anticancer agent, *C. nutans* has also been traditionally claimed to have antioxidant effects. The ethanol extract of *C. nutans* has the highest total phenolics content and total tannins content, followed by ethyl acetate, dichloromethane and hexane extracts, respectively (Sulaiman *et al.*, 2015), the ethanol extract exhibited highest antioxidant activities. Total phenolics content was high in a fraction of *C. nutans* ethyl acetate which is responsible for attenuation of oxidative stress (Sarega *et al.*, 2016). Le *et al.* (2017) also suggested that phytosterols isolated from the hexane extract of *C. nutans* exhibits immunomodulatory effects. Through LCM
S analysis, flavonoids was identified in a polyherbal formulation that contained *C. nutans* which performed a potent role in wound healing (Aslam *et al.*, 2016). UHPLC analysis