ANTICANCER EFFECTS OF *Clinacanthus nutans* CRUDE EXTRACTS IN CERVICAL CANCER CELLS

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To my beloved mother and father.

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ABSTRACT

Cervical cancer has one of the highest death recorded percentages among women in the world especially in less developed countries. Moreover, the presence of cancer stem cells in cervical cancer tumours is believed to cause resistance to conventional therapies. Previous studies have shown that *C. nutans* extracts possess antioxidant and anti-proliferative effects towards several cervical cancer cell lines including HeLa cells. This study is focused at examining the anticancer effects of ethanolic *C. nutans* leave extract on cervical cancer cell viability, proliferation and its ability to induce apoptosis in cervical cancer cells. In order to achieve these objectives, the MTS, CyQuant and active caspase 3/7 assays as well as immunofluorescence microscopy analysis were conducted respectively on Hela cells which represent an invasive form of cervical cancer. Treatment of *C. nutans* leave extract at concentrations of 0-50 µg/mL showed a significant reduction in cell viability and proliferation in a dose dependent manner on the Hela cells. An IC_{50} of 40 µg/mL and 20 µg/mL *C. nutans* were observed respectively in inducing reduction in cell viability and proliferation. Furthermore, Hela cells treated with *C. nutans* resulted in a reduced expression level of CD133 cervical cancer stem cell maker compared to the untreated cells. A further reduction in cell proliferation compared to cell viability in Hela cells and reduced CD133 expression may suggest that *C. nutans* may specifically target cervical cancer stem cells. Additionally, an increased caspase-3/7 activity in Hela cells treated with *C. nutans* indicated that the plant extract induced apoptosis. Apoptosis was also demonstrated through change in Hela cell morphology and formation of apoptotic bodies upon *C. nutans*. Findings of this study suggest that *C. nutans* is a potential anticancer agent which can be used in adjuvant chemotherapy treatment of cervical cancer.

ABSTRAK

Kanser servik merupakan salah satu kanser yang mencatatkan peratusan kadar kematian yang tinggi dalam kalangan wanita terutama di negara yang kurang membangun. Selain itu, kehadiran stem sel kanser dalam ketumbuhan kanser servik, dipercayai telah menjadi halangan kepada terapi konvensional. Kajian terdahulu telah menunjukkan bahawa ekstrak tumbuhan ini mempunyai kesan antioksida dan anti-proliferatif terhadap sel kanser servik termasuk sel HeLa. Kajian ini telah menyasarkan untuk mengkaji kesan antikanser ekstrak etanol *C. nutans* seperti kadar sel hidup dan proliferatif disamping kemampuan untuk mencetuskan apoptosis terhadap sel kanser servik. Bagi mencapai kesemua objektif kajian ini, kaedah MTS, CyQuant, caspase 3/7 yang aktif dan mikroskop immunofluoresen telah dianalisis terhadap sel HeLa yang juga mewakili bentuk invasif kepada sel kanser servik. Rawatan ekstrak daun *C. nutans* pada kepekatan antara 0-50 µg/mL telah menunjukkan pengurangan sel hidup dan proliferatif yang ketara terhadap sel HeLa. IC_{50} *C. nutans* pada kepekatan 40 µg/mL dan 20 µg/mL, tiap-tiap satunya telah dilihat mencetuskan pengurangan sel hidup dan proliferatif. Tambahan pula, sel HeLa yang dirawat dengan *C. nutans* telah menunjukkan pengurangan kadar pengekspresan CD133 penanda stem sel kanser servik berbanding sel yang tidak dirawat. Hasil kajian ini mencadangkan bahawa *C. nutans* mampu mensasarkan stem sel kanser servik. Disamping itu, peningkatan aktiviti caspase-3/7 dalam sel HeLa yang dirawat dengan *C. nutans* menunjukkan ekstrak tumbuhan ini mampu mencetuskan apoptosis. Kesan apoptosis juga ditunjukkan melalui morfologi sel HeLa dan pembentukan badan apoptotic kesan daripada rawatan *C. nutans*. Kajian ini telah membuktikan bahawa *C. nutans* berpotensi untuk menjadi ejen yang membantu rawatan kanser servik.

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LIST OF ABBREVIATIONS

IARC	-	International Agency for Research on Cancer
WHO	-	The World Health Organization
ICO	-	Institute Catala d'Oncologia
HPV	-	Human Papillomavirus
C. nutans	-	*Clinacanthus nutans*
HeLa	-	Henrietta Lacks cell line
MTS	-	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
DNA	-	Deoxyribonucleic acid
IC_{50}	-	Half maximal inhibitory concentration
ASR	-	Age-Standardised Rate
RB	-	Retinoblastoma
TP53	-	Tumour protein 53
DMEM	-	Dulbacco's Modified Eagle Medium
FBS	-	Fetal Bovine Serum
CO_2	-	Carbon dioxide
CSCs	-	Cancer stem cells
Pap	-	Papanicolaou
STAT3	-	Signal transducer and activator of transcription 3
ROS	-	Reactive oxygen species
HBSS	-	Hank's Balanced Salt Solution
ATP	-	Adenosine triphosphate
SEM	-	Standard error of the mean
PS	-	Phosphotidylserine
MOM	-	Mitochondrial outer membrane
NF-κB	-	Nuclear factor kappa B

PARP	-	Poly (ADP-ribose) polymerase
FADD	-	Fas-associated death domain
DISC	-	Death-inducing signalling complex
PROM 1	-	Prominin 1
SP	-	Side population
FACS	-	Fluorescence-activated cell sorting
NSP	-	Non-SP
CCSC	-	Cervical cancer stem cell

LIST OF SYMBOLS

Gy	-	Gray (unit), SI unit of absorbed radiation
μg/mL	-	Microgram/Milliliter
μL	-	Microliter
nm	-	Nanometre
rpm	-	Revolutions per minute
h	-	Hours
nM	-	Nanomolar

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

One of the main leading causes of morbidity in the world is cancer with reported estimation of 8.2 million deaths and 14 million new cases in 2012 (Ferlay *et al*., 2015). Additionally, this number is predicted to increase by 70% over the next 20 years (Stewart *et al*., 2014). According to the Global Cancer Statistics in 2012, cervical cancer is the third leading cancer that causes deaths in less developed countries and number fourth in the worldwide for the most common cause of cancer among females (Torre *et al*., 2015). It is estimated that about 230, 200 cervical cancer cases took place in developing countries (Hun *et al*., 2015). In addition, the International Agency for Research on Cancer (IARC) has claimed that in 2012 alone, a woman was dying every four minutes due to cervical cancer in Asian Pacific countries including Malaysia (Farooqui *et al*., 2013). In 2010, a summary report by WHO/ICO (Institut Catala d'Oncologia) stated that 631 people die among the 2126 Malaysian women who were diagnosed with cervical cancer annually (Muhamad *et al*., 2015). All of these facts had proven that cervical cancer is one of the most deadly diseases among women.

Cervical cancer commonly occurs through abnormal cell growth lining the cervix area (Sharma *et al*., 2017). In general, the cervix area is considered as the lower part of the uterus up to the end of the vagina. There are several causes of cervical cancer including human papillomavirus (HPV) infection, smoking, having sex with many partners and consume of birth control pills (Jemal *et al*., 2011; Torre *et al*., 2015). Among them, the most reported cases were caused by the infection of HPV with 99% detection in cervical tumour (Hun *et al*., 2015). Particularly, HPV have several subtypes and there are two oncogenic subtypes that lead to the cervical cancers that are HPV types 16 and 18 (Colombo *et al*., 2012). Many studies have been done to develop vaccines that could prevent from high-risk types of HPV infection such as HPV types 16 and 18 (Yeung *et al*., 2011).

Some of the popular treatments used to treat cervical cancer are surgery, radiation, chemotherapy and some cases used combination of chemotherapy and radiation (Varatharajan *et al*., 2012). In addition to surgery and radiation, the used of cytotoxic chemotherapeutic drugs to treat cervical cancer also become one of the popular method to treat late stage cancer. Though it becomes one of the main cancer treatment methods, the used of conventional chemotherapeutic drugs usually resulted to the severe side effects toward patients as well as multidrug resistance (Pratheeshkumar *et al*., 2012; Fong *et al*., 2016). Other than conventional treatments, alternative treatment by using the combination of conventional therapeutic drugs with medicinal plants is very popular lately among modern medicinal practitioner. Moreover, most of the clinically established medicines were originally made from natural based product (Ravishankar *et al*., 2013). Several studies had reported the anticancer effects of medicinal plants toward several cancer cells (Fong *et al*., 2016).

At present, there are rapid and extensive studies to develop therapeutic drugs that can be used in chemotherapy treatment to treat cervical cancer (Bruni *et al*., 2014). Natural source based therapeutic drug are getting much attention in research for its low-toxicity effect toward non-cancerous cells which may reduce the side effects of the treatment (Yong *et al*., 2013). Thus, most of the recent researches are

focusing to discover novel bioactive compound from medicinal plants which are one of the main ingredients used in natural-based therapeutic drugs (Fong *et al*., 2016). One of the medical plants that have been identified as an anticancer agent is *Clinacanthus nutans* (*C. nutans*) (Danmin *et al*., 2015). *C. nutans* is a medicinal herb with proven anticancer, antiviral, anti-inflammatory and antioxidant properties has become an important plant of research in the recent years (Ruhaiyem *et al*., 2015). Most of the phytochemical constituents extracted and isolated from leaves, roots, stems, barks, flowers and bulbs of the plant had demonstrated anticancer properties (Fong *et al*., 2016). In addition, *C. nutans* leaf extracts has been proven for its bioactive compounds which act as potential antioxidants as well as cytotoxic and antimicrobial agents (Sangeetha *et al*., 2014).

Several bioactive components that have been isolated from *C. nutans* are flavonoids, terpenoids, glycoglycerolipids, C-glycosyl flavones and sulphur containing glucosides (Ruhaiyem *et al*., 2015). Whilst the main compound isolated from crude ethanol *C. nutans* leaf extract is flavonoids which consists of catechin, quercetin, kaempferol and luteolin (Ghasemzadeh *et al*., 2014). This polyphenolic compounds have been reported possessed a wide spectrum of pharmacological effects such as anti-cancer activities. Previous studies revealed that this plant secondary metabolite mediated in the cancer induction and progression through regulation of various enzymes and receptors during signal transduction pathways associated to apoptosis, differentiation, inflammation, proliferation, metastasis and angiogenesis (Ravishankar *et al*., 2013).

Some previous and recent studies have been done separately to determine *C. nutans* cytotoxicity and anti-proliferative effects toward several cancer cells (Yong *et al*., 2013; Fong *et al*., 2016). Cell proliferation and apoptosis can be considered as one of the main mechanisms in cancer prevention (Fazil *et al*., 2016). Apoptosis or cell cycle arrest induction might be one of the mechanisms for HeLa cell growth inhibition caused by *C. nutans*. Unfortunately, the complete mechanism of action of *C. nutans* as an anticancer agent toward HeLa cells has not yet studied. Therefore,

this study approaches to identify the possible mechanism of action of *C. nutans* extract in cervical cancer cells which may aid in the treatment of cervical cancer.

Three main assays were used in this study to determine the cytotoxic effect, proliferative activity and mechanism of *C. nutans* toward HeLa cells. The first assay used was metabolic-based assay where the number of viable cells detected is based on the reduction of MTS tetrazolium salt into formazan product (Wang *et al*., 2010). Next, the second assay used was DNA-based assay where the proliferative activity of HeLa cells was demonstrated by the binding of fluorescent dye to the nucleic acid of the cells. This cellular DNA-based method has been proven to be slightly more sensitive than metabolic activity assay as the number of viable cells is determined by the highly regulated cellular DNA in the cells (Jones *et al*., 2001; Fazil *et al*., 2016). Cellular DNA is highly regulated in living cells and this will allow the binding of fluorescent dye which then can be detected through fluorescent microplate reader with suitable excitation wavelength (Wang *et al*., 2010). The last assay used in this study was apoptotic assay. This assay was done to determine the mechanism of *C. nutans* toward HeLa cells. Only the half maximal inhibitory concentration (IC_{50}) was used for this assay. Overall, this study presented the cytotoxicity and anti-proliferative effect of *C. nutans* together with its anticancer mechanism toward HeLa cells.

1.2 Problem Statement

The survival rate of cervical cancer for less developed regions such as Eastern and Middle Africa still at the lowest percentage compare to the more developed regions (Hun *et al*., 2015). Additionally, cervical cancer related vaccine such as cervarix is too costly for low and middle income patients to afford (Fagot *et al*., 2011; Kemp *et al*., 2011; Siegel *et al*., 2012). Therefore, in order to reduce drug cost for this cancer treatment, many local traditional medicinal plants with potential

anticancer properties are being identified and studied. Despite all available conventional treatments used to treat cervical cancer, detrimental effects toward patients due to acquired resistance to drugs or treatments as well as unspecific cytotoxicity inducing drugs have urged medical practitioner to shift their focus towards alternative treatment by using non-toxic plant-derived therapeutic drugs (Filipa Brito *et al*., 2015; Alam *et al*., 2016). *C. nutans* has been known as a traditional medicinal herb that used to treat various kinds of diseases including cancer (Yong *et al*., 2013). Its antiviral, antioxidant and anti-proliferative effects has been proved and mainly caused by the effect of its bioactive compounds such as flavonoids and phytosterols (Sak, 2014; Ghasemzadeh *et al*., 2014). Although *C. nutans* has been tested in several cancer cells and types of solvent, but previous studies had showed that there was only one type of solvent that have IC_{50} less than 20 μg/mL which is the allowed dosed fixed by the National Cancer Institute (NCI) for potential anticancer drug. The cytotoxic effect of this herb towards cervical cancer cell line such as HeLa has made it as a potential chemopreventive agent in cervical cancer treatment (Yong *et al*., 2013). Thus in this current study, we wish to see the cytotoxicity effect of *C. nutans* particularly in ethanolic extract toward HeLa cells.

1.3 Objectives of the Study

Following are the objectives of this study:

1) To study the cytotoxic effects of *C. nutans* crude extract in cervical cancer cells.
2) To investigate the anti-proliferative effects of *C. nutans* crude extract in cervical cancer cells.
3) To determine the ability of *C. nutans* crude extract to induce apoptosis in cervical cancer cells.

1.4 Scope of the Study

The scope of this study is to elucidate the anticancer effect of *C. nutans* towards cervical cancer cells by studying its cytotoxic and anti-proliferative effects as well as its ability to induced apoptosis. The first two objectives of this study were focused on the identification of the cytotoxic and anti-proliferative effects of *C. nutans* crude extract in HeLa cells line. Then the apoptotic assay was done by comparing *C. nutans* treated HeLa cells with control (untreated) HeLa cells and Taxol treated HeLa cells as positive control. Overall, this study is aimed at evaluating the effectiveness of *C. nutans* in cervical cancer cells treatment for the development of cancer therapeutic drug.

1.5 Significance of the Study

The unspecific cytotoxicity and severe side effects caused by conventional drugs in chemotherapy treatment of cervical cancer has changed the interest of medical practitioner to identify a potential therapeutic agent from natural-based source. Several previous studies proved that natural-based drug has reduced side effect and non-toxic to the non-cancerous cells (Yong *et al*., 2013; Alam *et al*., 2016). This study evaluates the potential of *Clinacanthus nutans* as an anticancer agent in the cancer cervical treatment. The used of plant-based therapeutic drugs as alternative treatment to treat cervical cancer not only cost effective but also cause no harm to patients in comparison to the current conventional treatments. Thus, it is important to study for the cytotoxicity of this medicinal plant toward HeLa cervical cancer cell line before it can be used in a clinical treatment. Furthermore, this study also identified the mechanism of *C. nutans* crude extracts toward HeLa cells. The determination of *C. nutans* anticancer effect will give an insight to medical practitioner on how its work to halt the development of HeLa cells in cervical cancer.

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