PROTEOMIC ANALYSES OF *Streblus asper* ROOT EXTRACT ON CERVICAL CANCER

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PROTEOMIC ANALYSES OF *Streblus asper* ROOT EXTRACT ON CERVICAL CANCER

by

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LIST OF SYMBOLS

%	Percentage
ºC	Degree Celsius
g	Gram
g/ml	Gram per milliliter
mg/ml	Milligram per milliliter
ml	Milliliter
µg	Microgram
µg/ml	Microgram per milliliter
µl	Microliter
nm	Nanometer

LIST OF ABBREVIATIONS

1DE	1 dimensional electrophoresis
AJCC	American Joint Committee on Cancer
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANXA	Annexin
AST	Aspartate aminotransferase
BSA	Bovine serum albumin
BT	Brachytherapy
CCD	Charge coupled device
CD	Cluster of differentiation
CID	Collision induced fragmentation
DAB	3,3′-Diaminobenzidine
DAVID	Database for annotation, visualization and integrated discovery
DDI	Distilled deionized
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EBI	European bioinformatics institute
EBRT	External beam radiotherapy
ECD	Electron capture dissociation
EDTA	Ethylenediamine tetraacetic acid
ESI	Electrospray ionisation
ETD	Electron transfer dissociation
FBS	Fetal bovine serum
FDR	False discovery rate
FIGO	International federation of gynaecology and obstetrics
FT-ICR	Fourier transform ion cyclotron resonance
G6PD	Glucose-6-phosphate dehydrogenase
GO	Gene ontology
HBeAg	Hepatitis B e antigen
HBsAg	Hepatitis B surface antigen

HBV	Hepatitis B virus
HCD	High-energy collision dissociation
HDI	Human development index
H&E	Haematoxylin and eosin
HIV	Human immunodeficiency virus
HOS	Osteosarcoma
HPO	Human phenotype ontology
HPV	Human papillomavirus
HSP	Heat shock protein
HUPO	Human proteome organization
IARC	International agency for research on cancer
IC_{50}	Half maximal inhibitory concentration
IVC	Individually ventilated cage
LCMS	Liquid chromatography mass spectrometry
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NIH	National institute of health
OECD	Organization of economic corporation and development
OTMS	Orbitrap mass spectrometry
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PSM	Peptide-spectrum matches
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RIPA	Radioimmunoprecipitation
SCC	Squamous cell carcinoma
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulphate
TFA	Trifluoroacetic acid
TIC	Total ion chromatograms
TNF	Tumour necrosis factor
USM	Universiti Sains Malaysia
WHO	World health organization

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ANALISA PROTEOMIK TERHADAP EKSTRAK AKAR *Streblus asper* PADA KANSER SERVIKS

ABSTRAK

Barah pangkal rahim adalah barah ketiga yang paling banyak berlaku di kalangan wanita. Meskipun rawatan barah telah maju, namun masih terdapat sebanyak 569,847 kes baru dan 311,365 kematian berkaitan barah pangkal rahim yang dicatatkan di seluruh dunia pada tahun 2018. Pilihan rawatan semasa seperti radioterapi dan kemoterapi banyak memberi kesan sampingan terhadap pesakit dan ini menghalang keberkesanan rawatan. Sebaliknya, rawatan menggunakan bahan semulajadi didapati mampu memberi kesan yang lebih baik. Oleh itu, kajian ini dilakukan untuk menyiasat potensi ekstrak akar *Streblus asper* untuk dijadikan prospek alternatif terhadap barah pangkal rahim. Kebolehan ekstrak akar *Streblus asper* untuk merencatkan pertumbuhan barah ini dikaji melalui pendekatan *in vitro* dan *in vivo*. Eksperimen *in vitro* bermula dengan penentuan nilai IC_{50} ekstrak akar *Streblus asper* melalui ujian biru Alamar, diikuti dengan pemeriksaan morfologi sel-sel HeLa. Penglibatan proses apoptosis dalam mekanisma rawatan adalah ditentukan menggunakan pewarnaan Aneksin V dan pengiraan kuantiti menggunakan mesin *flow cytometri*. Eksperimen *in vivo* pula dibuat menggunakan model xenograf haiwan. Kumpulan tikus SCID yang mengandungi tumor dirawat melalui pemberian ekstrak akar *Streblus asper* secara oral selama 2 minggu sebelum proses bedah siasat dilakukan. Seterusnya, ujian TUNEL dilakukan untuk mengenalpasti penglibatan proses apoptosis dalam mekanisma rawatan. Bagi analisis proteomik, teknik *microarray* protin digunakan untuk mengenalpasti marker apoptosis yang memainkan peranan penting dalam mekanisma rawatan. Selain itu, analisis LC-MS juga dibuat

untuk mengenalpasti protin yang sangat terekspresi yang menyumbang kepada mekanisma rawatan. Akhir sekali, isi kandungan ekstrak akar *Streblus asper* didedahkan untuk mengenalpasti kompaun terbesar yang mungkin bertanggungjawab dalam perencatan barah pangkal rahim secara *in vitro* dan *in vivo*. Keputusan kajian ini adalah termasuk nilai IC_{50} yang didapati pada kadar 0.25mg/ml, selepas 72 jam rawatan. Analisis mesin *flow cytometri* mengesahkan bahawa ekstrak ini menyebabkan proses apoptosis dalam sel-sel HeLa yang dirawat teraktif berbanding sel-sel HeLa yang tidak dirawat. Pengaktifan proses apoptosis berlaku melalui peningkatan ekspresi protin seperti TNF alfa, TNF beta, TRAIL R1, and TRAIL R2. Analisa proteomik seterusnya mendedahkan bahawa terdapat beberapa protin lain yang berkemungkinan penting dalam mekanisma rawatan. Ini termasuk galektin 1, protin heat shock jenis 10, demsidin, keratin 9, tropomiosin 4 , myristoylated alanine-rich C-kinase, protin tumor D52, protin riseptor folat alfa, dan parathimosin. Sama seperti dapatan *in vitro*, perencatan tisu tumor didapati berkait rapat dengan proses apoptosis, melalui kenaikan ekspresi kaspes 8 dan penurunan ekspresi bcl-w, cIAP2, and XIAP. Protin seperti aneksin A2, protin 14-3-3, transgelin 2, galektin 1, keratin 18, protin heat shock jenis 70, protin regulasi glukosa 78, gelsolin, enoles alfa, cofilin 1, vimentin, dan kalretikulin telah dikenalpasti memainkan peranan yang tinggi dalam mekanisma rawatan secara *in vivo*. Analisa terakhir menggunakan mesin GC-MS mendapati bahawa terdapat 13 bahan kandungan dalam ekstrak akar *Streblus asper,* dengan myo-inositol dikesan sebagai kandungan tertinggi dalam ekstrak. Menariknya disini, myo-insitol yang dijumpai ini telah dibuktikan oleh banyak laporan sebelumnya bahawa ianya mampu merencatkan barah. Sebagai rumusan, kami mencadangkan bahawa myo-inositol yang didapati dari ekstrak akar *Streblus asper* berkemungkinan besar menjadi penyumbang utama yang bertanggungjawab dalam mengawal ekspresi

semua protin-protin yang dijumpai dalam kajian ini, sebagai salah satu cara untuk merencatkan barah pangkal rahim secara *in vitro* dan *in vivo*. Dapatan daripada kajian ini memberi gambaran awal yang membuktikan potensi sebenar ekstrak akar *Streblus asper* dan kandungan bahan-bahannya untuk menjadi prospek alternatif bagi rawatan barah pangkal rahim.

PROTEOMIC ANALYSES OF *Streblus asper* ROOT EXTRACT ON CERVICAL CANCER

ABSTRACT

Cervical cancer is the third most prevalent cancer in women. Despite recent advances in cancer treatment, there were 569,847 incidents and 311,365 deaths worldwide in 2018. The current treatment options such as radiotherapy and chemotherapy have been typically found to cause adverse side effects that hinder the treatment efficacy. In contrast, natural products have been found to provide better treatment efficacy. Therefore, this study was conducted to explore the potential of one natural product known as *Streblus asper* root extract to be an alternative prospect to the current treatment practise. The anticancer effect of *Streblus asper* root extract was evaluated using *in vitro* and *in vivo* approaches. The *in vitro* experiment begins with the determination of IC_{50} value of *Streblus asper* root extract using Alamar blue assay, followed by HeLa cells morphological assessment. The involvement of apoptosis in the treatment mechanism was determined using Annexin V staining and quantitation by flow cytometry. *In vivo* experiment on the other hand, was conducted using animal xenograft model. The treated group of tumour bearing SCID mice was given *Streblus asper* root extract via oral gavage for 2 weeks before postmortem was conducted. Following this, TUNEL assay was performed to identify the involvement of apoptosis in the treatment mechanism. As for proteomic analyses, protein microarray was conducted to identify apoptotic markers that play a significant role in the treatment mechanism. Besides, LC-MS analysis was also carried out to identify highly expressed proteins that contribute to the treatment mechanism. Lastly, the content of *Streblus asper* root extract was revealed using GC-MS analysis to identify the major compound

that may be responsible in the inhibition of cervical cancer *in vitro* and *in vivo*. The results of the study include IC_{50} value which was found to be at 0.25 mg/ml, after 72 hours of treatment. The flow cytometry analysis confirmed that the extract causes apoptosis induction on HeLa cells in comparison to the untreated HeLa cells. The apoptosis induction occurs via the upregulation of TNF alpha, TNF beta, TRAIL R1, and TRAIL R2. Subsequent proteomic analyses revealed highly regulated proteins that may be of importance in the treatment mechanism. These include galectin 1, heat shock protein 10, dermcidin, keratin 9, tropomyosin 4, myristoylated alanine-rich C-kinase, tumour protein D52, folate receptor alpha, and parathymosin. Similar to *in vitro* findings, the inhibition of cervical tumour tissue was discovered to be associated with apoptosis, through the upregulation of caspase 8 and downregulation of bcl-w, cIAP2, and XIAP. Proteins such as annexin A2, 14-3-3 protein, transgelin 2, galectin 1, keratin 18, heat shock protein 70, glucose regulated protein 78, gelsolin, alpha enolase, cofilin 1, vimentin, and calreticulin were identified to be highly regulated in the *in vivo* treatment mechanism. Final evaluation by GC-MS revealed 13 phytochemical compounds in the *Streblus asper* root extract with myo-inositol identified as the major one. Interestingly, myo-inositol has been proved by many previous reports to exhibit anticancer ability. In conclusion, we suggest that the myo-inositol derived from *Streblus asper* root extract could be the potential compound that regulates all the identified proteins found in the study as a way to inhibit cervical cancer *in vitro* and *in vivo*. These findings provide preliminary evidence that prove the potential of the *Streblus asper* root extract and its major compound to be an alternative prospect for cervical cancer treatment.

CHAPTER 1

INTRODUCTION

1.1 Research background

Cancer is an abnormal growth of cells caused by multistep process involving modification and mutation to genes that regulate normal cellular function including cell growth control processes (Klaunig, et al., 2010). Cervical cancer which occur at the transformational zone of cervix develops through steps such as *Human Papillomavirus* (HPV) infection, HPV persistence, progression to dysplasia, and invasion (Bedell et al., 2020). With an estimation of 569,847 incidences and 311,365 deaths in 2018 worldwide, cervical cancer was ranked third most prevalent cancer in women (Bruni et al., 2018). Low socio-economic status, smoking cigarette, early sexual activity, multiple sexual partners, and multiple childbirths experiences have been determined to be risk factors for cervical cancer (Kashyap et al., 2019). Nevertheless, the primary risk factor for cervical carcinogenesis is mostly HPV type 16 and 18 infection. HPV 16 and 18 were also strongly associated with most of the invasive cases (Ahmed et al., 2017; Wang et al., 2020).

The preventive measure that has been practised in accordance to this primary risk factor is vaccination. HPV vaccination has been reported to contribute to a 46% risk reduction for histologically confirmed high grade cervical abnormalities, and 34% reduction for other cervical abnormalities among young women (Crowe et al., 2014). Despite its success rate, HPV vaccination is still not widely practised, and a study from O' Leary reported that the significant barriers that limit its usage in high-risk adolescent groups include lack of parents' knowledge about its safety, unawareness of parents on the requirement for multiple doses, and low perceived risk of HPV infection

(O'Leary et al., 2018). Another preventive measure for cervical cancer is screening of precancerous lesions through Pap smear test. Pap smear has been used increasingly in developing countries as a screening tool for early detection. As a consequence, it has been reported that this has contributed to a reduction of mortality rates from cervical cancer in women above 30 year old (Vicus et al., 2014). However, this pre-cancerous screening is practised only by certain groups of the society who possess adequate knowledge and awareness about the importance of early detection (Ashtarian, et al., 2017). As elucidated in a study, prevention against cervical cancer, especially in developing countries, would unlikely be effective until necessary steps are taken to address all the barriers and obstacles identified (Akinlotan et al., 2017; Lee & Lee, 2017). Women diagnosed with cervical cancer are typically given three treatment options. These include surgery, radiotherapy, and chemotherapy. Although chemotherapy can prolong the survival rate of cancer patients, ovarian dysfunction as a major side effect of this treatment, is unavoidable (Overbeek et al., 2017). Radiotherapy on the other hand can result in early and late side effects. Early side effects include mucositis, redness and peeling of skin, hair loss, nausea, diarrhoea, pneumonitis, and vomiting, while late side effects include nerve damage, fibrosis, myelitis, telangiectasia, tissue necrosis, and stricture (Evans & Staffurth, 2018). Therefore, to avoid these adverse side effects, using natural products with better efficacy against cervical cancer is an attractive alternative.

Over the years, it has been determined that natural products offer various medicinal values. These include anti-proliferative activity against cervical cancer. Extracts from *Rubus occidentalis* (Zhang et al., 2011), *Angelica Sinensis* (Cao et al., 2010), *Bryophyllum pinnata* (Mahata et al., 2012), *Pleurotus tuberregium* (Maness et al., 2011), *Cinnamomum cassia* (Koppikar et al., 2010), *Berberis spp.* (Mahata et al.,

2011), *Limoniastrum guyonianum* (Krifa et al., 2013), and *Phyllanthus emblica Linn* (Mahata et al., 2013) have been proven to exert this effect. However, there is another plant which possesses medicinal value but is underexplored, *Streblus asper*. *Streblus asper*, from the family of *Moracea,* is a plant that mostly grows in tropical countries, including Sri Lanka, Malaysia, Thailand, the Philippines, and India. (Rastogi, et al., 2007). It is known as "*Kesinai*" in Malaysia. From root to leaf, *Streblus asper* extract and its constituents have been traditionally used to treat a diversity of maladies (Seeni, et al., 2012). It can be used as antifilarial (Chatterjee et al., 1992), antifungal (Taweechaisupapong et al., 2005, 2006), anti inflammatory (Sripanidkulchai et al., 2009), antimicrobial (Taweechaisupapong, et al., 2000), antiviral (Chen et al., 2012; Li et al., 2012, 2013), antioxidant and antihyperglycemic (Kumar et al., 2012). In addition, past reports discovered that *Streblus asper* extract possesses anticancer potential on human cancers such as osteosarcoma (HOS cells) and tongue carcinoma (SCC-15 cells) (Seeni et al., 2012). According to Seeni et al. (2012), the root extract of *Streblus asper* is capable of causing cytotoxic effects and apoptosis on HOS cells and SCC-15 cells. In relation to this, it is hypothesized that the root extract of *Streblus asper* possesses similar anticancer potential on cervical cancer. Using *in vitro* and *in vivo* study models, the analyses mainly consist of validation on apoptosis involvement and identification of differentially expressed set of proteins through comparative proteomic analysis between untreated and treated samples. The differences in protein profiles warrant further investigation including the identification of a major compound in the *Streblus asper* root extract that is likely to cause inhibition of cervical cancer.

1.2 General Objective

This study aims to examine the anticancer potential of *Streblus asper* root extract on cervical cancer using *in vitro* and *in vivo* models.

1.3 Specific Objective

1.3.1 Objective 1

To determine the apoptosis involvement in the treatment process of *Streblus asper* root extract on human cervix adenocarcinoma cell line (HeLa).

1.3.2 Objective 2

To identify differentially expressed protein in the human cervix adenocarcinoma (HeLa) cell line treated with *Streblus asper* root extract.

1.3.3 Objective 3

To determine the apoptosis involvement in the treatment process of *Streblus asper* root extract on cervical cancer tumour xenograft.

1.3.4 Objective 4

To identify differentially expressed protein in the tumour tissues treated with *Streblus asper* root extract.

1.3.5 Objective 5

To identify the major phytochemical compound in the *Streblus asper* root extract that may be responsible for cervical cancer inhibition *in vitro* and *in vivo*.

1.4 Hypothesis

Streblus asper root extract demonstrates anticancer potential on cervical cancer *in vitro* and *in vivo.*

CHAPTER 2

LITERATURE REVIEW

2.1 Cervical cancer

2.1.1 Pathophysiology

Cervix is a cylinder-shaped neck of tissue that is located at the end passage of vagina. As shown in Figure 2.1, it consists of two major parts; ectocervix and endocervix. The ectocervix is the most inferior portion of the cervix, while endocervix is an inward extended portion of the cervix (Prendiville & Sankaranarayanan, 2017). The ectocervix consists of a circular opening area at its centre known as external os. This external os of the cervix connects the cervix to the vaginal canal. The internal os on the other hand, is an area that joins the vaginal canal to the uterus (Evbuomwan, & Chowdhury, 2021)

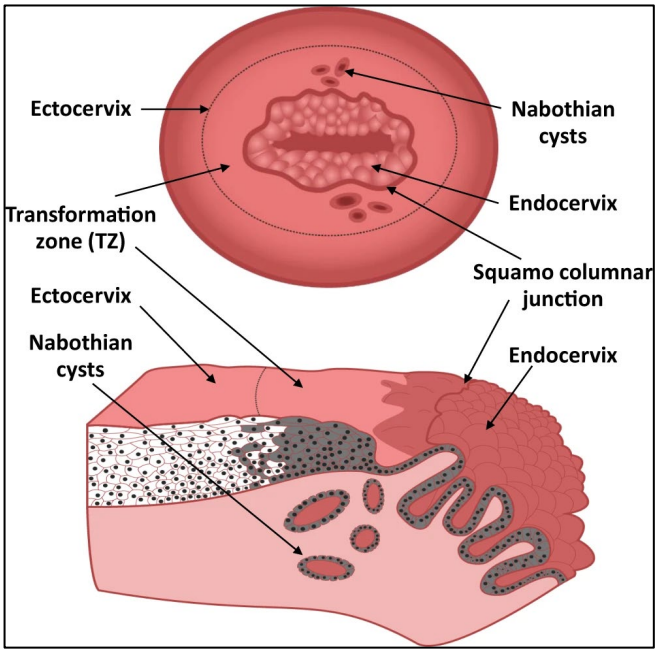

Figure 2.1: Illustration of the horizontal section of the uterus (Deng et al., 2018)

Cervical cancer is generally referred to as an uncontrolled proliferation of cells at the cervix. This abnormal cell growth can spread to other parts of the body. There are two major histopathologic types of cervical cancer; squamous cell carcinoma and adenocarcinoma. Squamous cell carcinoma belongs to the autonomous cell growth at the transformation zone of the cervix, known as the squamocolumnar junction. Adenocarcinoma on the other hand, is an abnormal growth of columnar epithelium (Prendiville & Sankaranarayanan, 2017). Squamous cell carcinoma constitutes approximately 90% of the cervical cancer cases while the remaining 10% are adenocarcinoma cases (World Health Organisation, 2015)

2.1.2 Incidences

In 2018, about 18.1 million new cancer cases were recorded worldwide. From those, 3.2% were cervical cancer, making up a total of 569,847 cases. Out of the 9.6 million deaths caused by cancer, cervical cancer constituted about 311,365 deaths. This represents 3.3% of all cancer-related deaths globally. The severity of cervical cancer makes it the most frequently diagnosed cancer in 28 countries as shown in Figure 2.2. The incidence and mortality related to cervical cancer is second, after breast cancer in lower/medium Human Development Index (HDI) setting. (Bray, et al., 2018). Malaysia reported that cervical cancer is the third leading cancer among females after breast cancer (48.9%) and colon cancer (11%) (Bruni, et al., 2018)

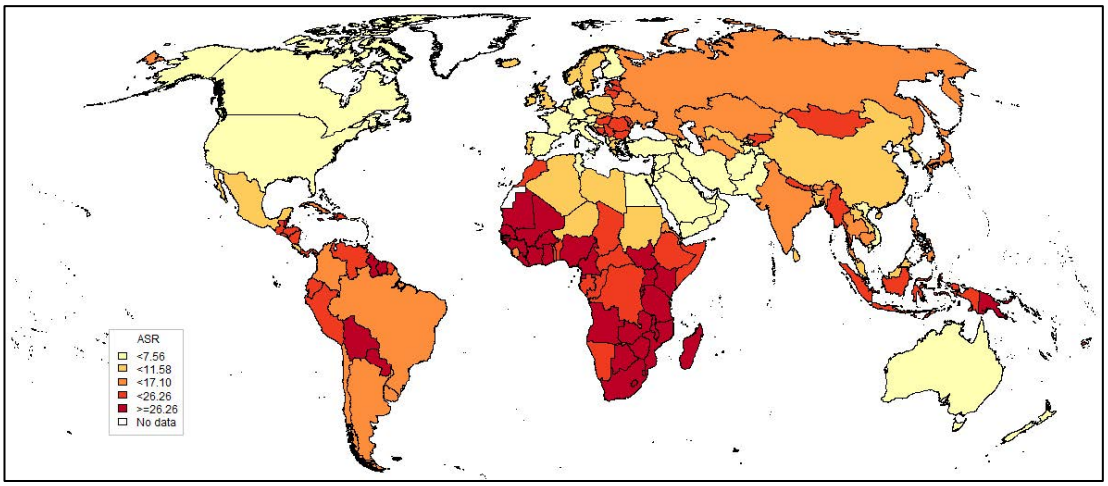

Figure 2.2: The distribution of cancer cases affecting females worldwide (Bruni, et al., 2018)

2.1.3 Symptoms

Most of the women with cervical cancer typically experience symptoms like fatigue, vaginal discharge, lower abdominal pain, intermenstrual bleeding, postmenopausal bleeding, weight loss, postcoital bleeding, and dysuria, and these symptoms eventually influenced them to seek for consultation (van Schalkwyk et al., 2008; Kim. et al., 2015). Newly diagnosed patients at an advanced stage, patients undergoing palliative chemotherapy, or those with recurrence, have been determined to experience other symptoms like nausea, depression, anxiety, drowsiness, dyspnoea, and anorexia (Kim et al., 2015).

2.1.4 Risk factors

Cervical cancer is associated with numerous risk factors which encompass tobacco use, *Human Papillomavirus* (HPV) infection, continuing usage of hormonal contraceptive pills, and *Chlamydia* infection. Among the risk factors listed, HPV infection is responsible for up to 95% of all cervical malignancies (Rizzo & Feldman,

2018), and has also been reported as the aetiological agent to cervical carcinogenesis (de Freitas, et al., 2014; Mittal & Banks, 2017). Out of the 280 types of HPV, 200 of them can potentially infect humans. From these, the HPVs can be divided into two groups, the low-risk group that is frequently associated with benign genital warts, and the high-risk group that causes cervical cancer (Cubie, 2013). According to the International Agency for Research on Cancer (IARC) updates (Bouvard et al., 2009), the low-risk group includes HPV 6, 11, 34, 40, 42, 43, 44, 53, 54, 61, 66, 70, 69/71, and 74 (Adebamowo et al., 2017; Chikandiwa et al., 2018), whereas the high-risk group consists of HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59. Among the latter group, HPV type 16 and 18 are most commonly identified in invasive cervical cancer cases (Ramakrishnan, et al., 2015). Despite cervical malignancies mostly caused by HPV infections, numerous studies have also discovered that smoking also accounts for cervical malignancies and tobacco use has been found to be positively correlated with cervical cancer (Siokos et al., 2019; Sugawara et al., 2019). Smokers were more likely to contract cervical cancer compared to non-smokers (Sugawara et al., 2019). *Chlamydia trachomatis* infection, a sexually transmitted disease, has been found to be another risk factor associated with cervical cancer. Past studies have shown that *Chlamydia trachomatis* infection is a cofactor for HPV infection, causing cervical intraepithelial lesion development (Tavares et al., 2014). Cervical intraepithelial lesion is a type of precancerous dysplasia that could potentially lead to cervical malignancy if left untreated. The potential synergistic effect between HPV infection and *Chlamydia trachomatis* infection was also found to lead to cytological abnormalities of the cervix (Nonato et al., 2016). Studies have also found that long-term use of hormonal contraceptives increases the risk of cervical cancer (Khatun et al., 2018). Women who have been using hormonal contraceptive for more than 5 years are more

prone to getting cervical cancer compared to women who have used these contraceptives less than 5 years (Khatun et al., 2018). The risk factors like HPV and *Chlamydia trachomatis* infection as well as prolonged exposure to oral contraceptive have been found to increase the risk of developing high grade squamous intraepithelial lesion (HSIL) and invasive cervical carcinoma (Khieu et al., 2020; Mahgoub & Ibrahim, 2018). HSIL cytoabnormalities include moderate and severe dysplasia (Mukhopadhyay et al., 2013). Invasive carcinoma on the other hand, is always preceded by HSIL and therefore, many characteristics possessed by HSIL can also be found in invasive carcinoma. These include the presence of smaller cells with high nuclear to cytoplasmic ratio, hyperchromatic nuclei, and fine to coarsely granular chromatin (Khieu et al., 2020). Distinctive characteristic that differ invasive carcinoma from HSIL is the presence of tumour diathesis in the background (Mukhopadhyay et al., 2013). This is the only characteristic that differentiate invasive carcinoma fom HSIL and therefore, as reported, invasive carcinoma in certain cases mistakenly interpreted as HSIL and can be found in 3.5% of the HSIL cases (Mukhopadhyay et al., 2013). Nevertheless, all of the cytopathological features that characterize HSIL and invasive carcinoma can be effectively identified by Pap smear (Bal et al., 2012).

2.2 Challenges in cervical cancer prevention

2.2.1 HPV vaccination

The fact that high-risk HPV infection is accountable for most cervical cancer cases led to the establishment of the HPV vaccination program as part of the standard preventative measure for people. The vaccines that are widely used across the globe include Gardasil™, Gardasil 9™ and Cervarix™. Gardasil™ is a quadrivalent vaccine

that offers protection against HPV infection type 6, 11, 16 and 18 (Bharadwaj et al., 2014). HPV type 6 and 11 are frequently associated with genital warts. Gardasil 9™ on the other hand is a 9-valent vaccine designed for HPV infection type 6, 11, 16, 18, 31, 33, 45, 52 and 58 (Cuzick, 2015). Cervarix™ is a type of bivalent vaccine that offers protection against HPV type 16 and 18 (Bharadwaj et al., 2014). All these vaccines were proven to have good immunogenicity against precancerous cervical intraepithelial lesion (CIN) (Lehtinen et al., 2012; Munoz et al., 2010), and were able to protect up to 70% of the vaccinated population (Schiller, et al., 2012). Based on the World Health Organization's (WHO) recommendation, girls below 15-years-old are advised to obtain two doses of vaccine over the span of 6 to 12 months, while those aged over 15 are required to receive three doses over 6 months (Gilca et al., 2018; World Health Organization, 2017). The compliance towards the dose requirement is imperative as it reflects the vaccine efficacy. As of today, there are approximately 70 countries in the world that make HPV vaccination as part of their national immunization program (Sankaranarayanan et al., 2018). The introduction of the HPV vaccination programme has shown a remarkable positive effect globally. Following the HPV immunization program in nine countries such as the USA, Australia, England, Scotland, New Zealand, Sweden, Denmark, Canada, and Germany, the incidence rate of HPV type 16 and 18 infection reduced significantly by 68% (Drolet et al., 2015). Despite the vaccination success rate, a large percentage of the population in the world still suffer from cervical cancer, because there are limitations to the implementation of HPV vaccination. These include the high cost of HPV vaccines regimen, and limited knowledge among parents on the HPV side effects (Holman et al., 2014). The price of HPV vaccination can be costly and vary between USD1.49 to USD18.94 per individual (Levin, et al., 2014). This significantly hampers the efforts to widen the coverage of

HPV vaccination, especially in low- and middle-income countries (Laurent, et al., 2018). Besides that, past studies also reported that due to the fear of possible adverse effects, parents with low knowledge are less likely to have their daughters vaccinated (Bonanni et al., 2018; Nickel et al., 2017). Based on these findings, cost and parents' knowledge are important factors to the success of HPV vaccination.

2.2.2 Pap smear

Similar to all cancers, cervical cancer can be controlled and treated if detected at an early stage. To achieve that goal, Pap smear is a likely solution. Pap smear is a screening tool that provides early detection for precancerous changes of the cervix. These changes are known as Cervical Intraepithelial Neoplasia (CIN). They are graded according to their severity starting from CIN 1 followed by CIN 2, and CIN 3. As recommended by the World Health Organisation (WHO), pap smear screening is ideally conducted on women between 30 to 49-years-old (World Health Organization, 2013). Pap smear screening has been proven to play a protective role against cervical cancer mortality for women above the age of 30 (Vicus et al., 2014), and has contributed to a 4% reduction in cervical cancer mortality (Yoshida, et al., 2018). Despite pap smear screening success in providing early diagnosis for cervical cancer, there are significant barriers that hinder the effectiveness of the pap smear implementation. Priaulx, et al. (2018) identified six significant challenges to effective cervical screening including lack of knowledge, failure in identification of an eligible population, difficulties in access that lead to demotivation of participation, weaknesses in screening programme operation, insufficient monitoring and following-up with non-responders, and inadequacy of systematic monitoring of treatment outcome. A study by Maar et al., (2013) in a developed country emphasized similar

findings in Canada where they had different barriers such as insufficiency of appropriate health care providers, weak recall-based screening system, geographical difficulties, poor transportation system, lack of health awareness, socioeconomic disparities, compliance issues, and cross ethnic trust issues. However, the obstacles to effective cervical screening seem to be more critical in low- and middle-income countries. This is because these countries have to deal with difficulties like widespread poverty, poor healthcare facilities, health illiteracy, unsupportive political situations and so forth (Catarino, 2015). Considering the presence of these boundaries, necessary steps should be taken to address all the barriers effectively in order to produce an improving outcome. Cooperation from all members of society, including individuals and policy makers, is also needed to ensure the success of this programme.

2.3 Weaknesses in current treatment options

2.3.1 Radiotherapy

Normally, patients with cervical cancer are given three treatment options; radiotherapy, chemotherapy, and surgery. Radiotherapy or radiation therapy is a method of applying high-energy X-rays, using a linear accelerator, on a localised area that will affect cancer growth. Cancer cells that are exposed to this high-energy beam will result in an irreversible deoxyribonucleic acid (DNA) double helix damage, causing the cells to undergo programmed cell death (Smith & Prewett, 2017). In some cases, radiotherapy is used to control local large tumours of cervical malignancy, using a combination of external beam radiotherapy (EBRT) and brachytherapy (BT) (Tornero-López & Guirado, 2018). Radiotherapy is capable of causing complete remission. Studies documented that this achievement subsequently resulted in better

patient prognosis, with an average 5-year overall survival (Hanna et al., 2018). The patients survivability following radiotherapy improved by 51% with a 10-year overall survival rate (Kim et al., 2018). However, the accessibility to radiotherapy, especially in third world countries is low compared to first world nations. It is documented that among the 139 low- and middle-income countries, only four of them were able to meet their people's need, while 55 countries did not even have radiotherapy facilities, and the remaining 80 countries could provide the treatment to an average of 36.7% of the patients (Datta, et al., 2014). In a different study, from a total of 39 European countries, they were able to provide up to 74.3% of their peoples' requirements for radiation therapy facilities and human resources (Datta, et al., 2016). This clearly indicates the vast disparity in accessibility between countries most likely hampers the effort to reduce the mortality rate of cervical malignancy. On top of that, radiation therapy for cervical cancer often employs the use of high intensity X-ray beams at the pelvic area, consequently causing urologic complications. The urologic complications include radiation cystitis, lower urinary tract dysfunction, stricture disease, fistula formation, and the development of a second primary cancer (Lobo et al., 2018). Radiotherapy can also have early and late side effects. Patients with early or acute side effects normally experience erythema, desquamation, hair loss, mucositis, diarrhoea, pneumonitis, marrow ablation, nausea, and vomiting. While late or chronic side effects often result in fibrosis, necrosis, nerve damage, myelitis, telangiectasia, and stricture (Evans & Staffurth, 2018). These unavoidable adverse effects of radiation therapy are also causes of its major drawbacks.

2.3.2 Chemotherapy

Chemotherapy, another cervical cancer treatment option, involves the use of cytotoxic drugs to induce apoptosis in cancer cells. There are over 41 cytotoxic drugs, classified based on two criteria; its biochemical properties and its cell cycle effects. Chemotherapy is normally prescribed prior-to or after the primary cancer has been removed. If the regimen is initiated before the definitive treatment takes place, then it is referred to as a neoadjuvant, while if it is initiated after, the chemotherapy is regarded as an adjuvant. These decisions are made based on several characteristics like tumour sizes, cancer stages, biological features, patient's wishes, patient's age, comorbidities, and performance status (Bhosle & Hall, 2009). Depending on the justification of the oncologist, chemotherapy in certain cases is initiated concurrently with radiotherapy. However, the most imperative part of executing chemotherapy or chemoradiation therapy regimen is monitoring the patient status. This is because chemotherapy often goes hand-in-hand with toxicities. As described earlier, chemotherapy was shown to increase the risk of ovarian dysfunction in the elderly at the time of treatment (Overbeek et al., 2017). Antiangiogenic agents which are commonly used in treating gynaecological malignancy, are synonymous with various adverse events like hypertension, left ventricular dysfunction and congestive heart failure, acute vascular event, and bleeding tendencies (Gunderson, et al., 2018). Bevacizumab, one of the antiangiogenic agents, causes diarrhoea, perforations, and fistulae in gynaecologic cancer patients (Burger, et al., 2007; Cannistra et al., 2007). Bevacizumab was also reported to cause renal toxicities like proteinuria, whereas cetuximab and panitumumab cause electrolyte imbalances (Abbas, et al., 2015). Chemotherapy also results in acute kidney injury, worsening of pre-existing kidney failure, and thrombotic microangiopathy (Porta, et al., 2015). In addition to that, the

combination of chemotherapy and radiation therapy often lead to haematological and gastrointestinal toxicities (de Azevedo et al., 2017). Most of the patients who experienced these toxicities presented with grade I and II, while 44% of them presented with grade III and IV. Taken together, the negative consequences of chemotherapy and chemoradiation therapy are unavoidable. Therefore, an alternative approach against cervical cancer is worth investigating.

2.4 Alternative approach to cervical cancer

In the past ten years, natural products have been widely accepted as potential substitutes to existing preventive and treatment strategies for cancers. This paradigm shift has resulted in many natural products and its derivative compound being documented to significantly suppress cervical cancer growth. Zhang et al. (2011) proved that black raspberry crude extract can significantly inhibit cervical cancer cell lines such as HeLa, SiHa, and C-33A. Similar inhibitory ability has been seen in many other plants, including *Kaffir lime* leaf extract (Tunjung, et al., 2015), *Artocarpus artilis* pulp extract (Jamil, et al., 2018), *Bryophyllum pinnata* leaf extract (Mahata et al., 2012), *Pleurotus tuberregium* extract (Maness et al., 2011), *Thymus kotschyanus* extract (Doosti,et al., 2018), and *Coryllus avellana* (Gallego et al., 2017). *Scutellaria discolor* extract is able to exert inhibitory activity on breast carcinoma, hepatocellular carcinoma, epithelial carcinoma, lung adenocarcinoma, prostate carcinoma, cervical epidermoid carcinoma, and cervical carcinoma, with maximal inhibition recorded on cervical carcinoma (Laishram et al., 2015).

The inhibition of cervical cancer is normally evaluated based on its responses on related pathways such as proliferation, apoptosis, cell cycle, and metastasis. For instance, *Mimusops elengi,* which is also known as Spanish cherry, is able to trigger

the apoptosis mechanism on SiHa, a cervical cancer cell line (Ganesh, et al., 2014). A more recent study documented the ability of *Garcinia indica* extract to inhibit cell proliferation, invasion, migration, cell cycle progression and induced programmed cell death on cervical malignancy (Zhao et al., 2018). Koppikar and co-researchers (2010) on the other hand concluded that cinnamon extract exerts anti cervical cancer effect through apoptosis by causing loss of mitochondrial membrane potential. *Primula vulgaris* extract was found to suppress cervical cancer immortality through cell cycle arrest (Demir, et al. 2018). *Pinus massoniana* bark extract is capable of inhibiting cervical cancer through the metastatic pathway. (Wu, et al. 2011).

With regard to our scope of interest, in this study, our candidate of choice is an underexplored plant known as *Streblus asper*. *Streblus asper,* from the *Moraceae* family, is a tree that grows indigenously in tropical countries like Sri Lanka, Malaysia, Thailand, the Philippines, and India (Rastogi et al., 2006). Known as "Kesinai" in Malaysia, the *Streblus asper* tree, which has many branches, is approximately 4 to 15 metres high. The leaves are slightly oval in the front and are about 4 to 12 centimetres long, mildly coarse on both sides, and slightly tapers at the tip as shown in the Figure 2.3. Traditionally, almost all parts of *Streblus asper* tree which include root, stem, bark, leaves, latex, fruit, and seed, have been useful for various ailments. Based on the review from Rastogi et al. (2006), root was traditionally used to counter snake venom, epilepsy and obesity. The stem on the other hand was known to provide soothing effects against toothache. Its stem bark and seeds were used for treating gastrointestinal problems, like dysentery and diarrhoea. Its leaves and fruit extracts were suitable for eye irritation. Its latex was used for reducing swelling of the cheek.

Figure 2.3: *Streblus asper* Lour tree and its leaves

Many studies have been conducted to scientifically prove the ethnomedicinal values of *Streblus asper*. Verma and co-researchers (2016) discovered the potential of *Streblus asper* extract as an effective anticonvulsant agent, using an *in vivo* model. Chen et al. (2012) in contrast, studied the effects of different parts of *Streblus asper* tree on the production of *hepatitis B surface* antigen (HBsAg) and *hepatitis Be* antigen (HBeAg), where heartwood, barks, and roots were found to significantly inhibit the HBsAg and HBeAg production. The ethanolic extract of *Streblus asper* was found significantly inhibited the paw oedema of rats due to carrageenan injection (Sripanidkulchai, et al, 2009). Nie et al., (2016), proved the ability of *Streblus asper* to inhibit the growth of five bacteria including *Saccharomyces cerevisiae, Bacillus subtilis, Pseudomonas aeruginosa, Escherichia coli*, and *Staphylococcus aureus*. Besides, *Streblus asper* was also seen able to reduce the number of *Streptococcus mutans* colonies, which are normally associated with caries and plague in the oral cavity (Taweechaisupapong et al., 2000). *Streblus asper* leaf extract on the other hand was discovered able to inhibit biofilm formation caused by subgingival pathogens like

Porphyromonas gingivalis and *Aggregatibacter actinomycetemcomitans* (Taweechaisupapong, et al., 2014). According to Singsai et al. (2015), the anti-Parkinson effects of *Streblus asper* leaf extract improved motor and cognitive function deficits caused by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in MPTP-treated C57BL/6 mice. The positive effect of *Streblus asper* was also observed on fungal adherence. Taweechaisupapong and co-researchers (2006), pointed out that the pretreatment of *Streblus asper* on acrylic surface successfully inhibited the adherence of *Candida albicans* onto the surface. Furthermore, the leaf and bark extract of *Streblus asper* were also reported to have antioxidant capabilities which are useful in reducing the formation of reactive oxygen species (ROS), known to be destructive to cells (Kumar et al., 2015; Prasansuklab, et al., 2018). The extract also exerts neuroprotective effects against glutamate-induced cell death (Prasansuklab, et al., 2017). *Streblus asper* has also been proved to show positive inhibitory potential on human cancers, such as osteosarcoma and tongue carcinoma (Seeni, et al., 2012). The root extract of the plant was seen to cause cytotoxic effects and apoptosis on both osteosarcoma (HOS) and tongue carcinoma (SCC-15) cell lines, leading to significant inhibition of the cell's growth. Taking into consideration the anticancer potential of *Streblus asper* root extract on these types of human cancers, it is hypothesized that similar potential of the root extract can be observed on cervical cancer.

2.5 Apoptosis and cervical cancer

Apoptosis is a systematic process of cell death in which the cell itself participates actively in the process. Apoptosis takes place throughout the development and aging process of the cell. It occurs as a balanced mechanism to maintain the cell population in tissues, and the functional role of the body system. Cells that undergo

apoptosis normally have specific morphological characteristics such as cell shrinkage, pyknosis, membrane blebbing, followed by cell fragmentation and phagocytosis of apoptotic bodies by macrophages (Kerr et al., 1972). These morphological disturbances have similar characteristics across cell types and species (Wong, 2011), providing a perfect clue on apoptosis involvement before confirmatory assay is conducted. Apoptosis is important in preserving and protecting the body system from diseases. It serves to eradicate any abnormal cells including highly proliferative cells like cancer (Pfeffer & Singh, 2018). The apoptotic process is initiated under a variety of circumstances. The activation can occur via two different pathways known as extrinsic and intrinsic.

The extrinsic pathway of apoptosis is also known as the death receptor pathway. This pathway begins with the activation of the death domain resulting from the binding of receptors from the tumour necrosis factor (TNF) family which include FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5 (Elmore, 2007). The binding of TNF-α and TNFR1 allow the recruitment of receptor interacting protein (RIP) and Fas-associated protein with death domain (FADD) to form a complex with adaptor protein tumour necrosis factor receptor type 1-associated death domain protein (TRADD). This subsequently leads to the formation of a death-inducing signaling cascade (DISC), resulting in the activation of auto-catalytic events of procaspase-8 to caspase-8. The activation of caspase-8 initiates the caspase execution phase of apoptosis which starts with the activation of caspase-3 from procaspase-3 (Fulda & Debatin, 2006). Activated caspase-3 plays a significant role as primary executioner of apoptosis through various events. These include apoptosis-associated chromatin margination, DNA fragmentation, and nuclear collapse (Slee et al., 2001).

The intrinsic pathway, the mitochondrial pathway, involves mitochondria as the key organelle that determines its execution phase. Intrinsic pathway is induced by non-receptor mediated stimuli like DNA damage, ischemia, and oxidative stress (Loreto et al., 2014). These stimuli produce intracellular signals that result in perturbation of mitochondrial membrane, resulting in the release of cytochrome C into the cytosol. In association to that, sequestered Bad and overexpressed Bax contribute further to the reduction of mitochondrial membrane potential (Pawlowski & Kraft, 2000). The released cytochrome C binds with Apaf-1 and caspase-9 and activates both of them consequently. This binding forms a complex called apoptosome, potentially triggering the execution phase of apoptosis, reflected in degradation of chromosomal DNA, nuclear and cytoskeletal proteins of cells. Apart from cytochrome C, in the late event of apoptosis, mitochondria releases proteins such as apoptosis inducing factor (AIF), endonuclease G, and caspase activated Dnase (CAD) for aiding in the DNA fragmentation process (Elmore, 2007). The apoptosis mechanism is also induced via the p53 cascade, mediated by puma and noxa proteins (Liu, Newland, & Jia, 2003; Oda et al., 2000). On the other hand, Smac/DIABLO, and htrA2/Omi promote apoptosis through the inhibition of IAPs (inhibitors of apoptosis protein) (Schimmer, 2004)

The event of apoptosis occurs as a result of upregulation of pro-apoptotic proteins and downregulation of anti-apoptotic proteins. This sort of balance promotes and enhances the apoptosis execution. However, cancer cells evade apoptosis by causing disruption in this balance (Wong, 2011). This include an upregulation of several anti-apoptotic proteins like Bcl-2, Bcl-xL, Bcl-w, Bid, Bim, Puma, Mcl-1, Noxa, and Bad or downregulation of several pro-apoptotic proteins like as Bax, and Bak or a combination of both in the same time. In particular to our target cancer,

cervical cancer has been found to upregulate the expression of anti-apoptotic proteins such as Bcl-xL and c-IAP2 for its survival (Tuohetimulati et al., 2018). The expression of Bcl-xL and c-IAP2 were significantly high in cervical cancer tissue compared to normal cervical tissue (Tuohetimulati et al., 2018). Bcl-2, was found highly expressed in malignant cervical lesion compared to premalignant lesion (Kamaraddi et al., 2016). According to Alibek et al. (2014), the disruption of Bcl-2 regulation is driven by the HPV viral protein, known as E6 protein. Another anti-apoptotic protein known as Mcl-1, has also been found to overexpress in cervical cancer tissue compared to normal tissue (Zhang et al., 2011). Mcl-1 overexpression is positively correlated with proliferative protein, Ki-67 (Zhang et al., 2011). The inability of cells to auto-reverse this dysregulation of expression causes it to progress further to become immortal cancerous cells. The evasion of apoptosis by cancer cells can also occur through the impairment of death receptor signaling. Abnormalities in the death signalling pathways can include downregulation of the receptor, impairment of receptor function, and reduction of death signals level (Wong, 2011). For instance, the loss of Fas and the dysregulation of FasL, DR4, DR5, and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in the cervical intraepithelial neoplasia (CIN) was hypothesized to be the culprit for cervical carcinogenesis (Reesink-Peters et al., 2005). Based on the given facts and past evidence, it can be hypothesized that cervical cancer may have been able to evade apoptosis through the dysregulation of pro- and anti-apoptotic proteins as well as death receptor signaling. Therefore, when it comes to targeting cervical cancer, it is important that any particular anticancer agent possess the ability to induce apoptosis, so does the *Streblus asper* root extract.

2.6 Selection of an ideal animal cancer model

For many decades, human cancer cell lines have been utilised to study various cancer mechanisms. The *in vitro* model for cancer research has been an interest for researchers across the globe because it provides controlled conditions, homogeneity, and good reproducibility. However, this system carries several disadvantages such as selection of phenotypic and genotypic cells during adaptation, accumulation of mutations over time in culture, a homogeneous population of cells, and isolation of cells from the tumour microenvironment (Cekanova & Rathore, 2014). The *in vivo* model for cancer research has been used to compensate for the limitations exhibited by the *in vitro* model, and it is more likely to resemble an actual complete biological system. Cancers are developed in rodents through several techniques such as chemically induced, genetically engineered, and xenograft. The chemically induced model encompasses the introduction of carcinogen into the rodent's body. Carcinogen such as diethylstilboestrol (DES) has been proved to cause cervical carcinogenesis in mice and this makes the model suitable for the cancer study (Zulfahmi et al., 2013). With various types of carcinogenic substances available in the market, developing a cancer tumour is considered as achievable. However, the time required for a tumour to grow to desirable sizes may differ from rodent to rodent. On top of that, this model requires skills and precaution as the use of carcinogens may be risky to laboratory personnel. The genetically engineered model utilises oncogenes or tumour suppressor genes inhibitors. These DNA materials are then microinjected into the pronuclei of fertilized zygotes, allowing them to be transgened into the host system. However, this model which is normally used to evaluate carcinogenesis and drug resistance does not eliminate the possibility of having transgenic rodents with unexpected phenotypes (Cheon & Orsulic, 2011).

The xenograft model involves transplantation of human or animal cancer into subcutaneous area (ectopic) or into the organ (orthotopic) of immunocompromised rodents (Bibby, 2004; Huynh et al., 2011; Ruggeri, et al., 2014). Unlike orthotopic, in an ectopic model, the site of transplantation is different from the origin of the cultured cells. Besides, this model is a standard model used in oncology research (Jung, 2014). The immunocompromised rodent is used in the xenograft model to eradicate the possibility of newly transplanted cancer to be rejected by the host's immune system. Transplanting HeLa cells into mice has been proved to be successful with 94.4% tumour take rate (Arjomandnejad et al., 2014). Besides, the HeLa xenograft model has been observed to produce tumours with similar characteristics to the one derived from humans. Histologically, the HeLa tumours present with highly malignant hyperchromatic epithelial cells. The cells appear with numerous nucleoli and atypical mitosis (Arjomandnejad et al., 2014). Similar to cervical malignancy found in humans, the cells undergoing mitosis form pathological morphology and this has been associated with oncogenic HPV infection (Jenkins, 2007). In addition, tumour developed from HeLa cells was found to express positive expression for cytokeratin (CK) and vimentin (Arjomandnejad et al., 2014). Positive expression of CK shows that the tumours are epithelial origin (Barak et al., 2004) whereas positive expression of vimentin confirms the involvement of epithelial-mesenchymal transition (EMT), both of which are associated with tumour invasion and metastasis found in human cervical carcinoma (Lee & Shen, 2012). Arjomandnejad et al. (2014) pointed out that 80 percent of tumour tissue developed from HeLa xenograft model was Ki-67-positive, coincides with the reaction discovered in high grade squamous intraepithelial lesion (HSIL) and squamous cell carcinoma (SCC) from patients samples (Shi et al., 2019).