ELUCIDATING THE MECHANISM OF BAICALEIN-RICH FRACTION FROM *Oroxylum indicum* LEAVES IN CERVICAL CANCER CELL LINES

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by

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS

%	percent
°C	degree Celsius
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar
AIF	apoptosis-inducing factor
ANOVA	Analysis of variance
Apaf-1	apoptosis protease-activating factor 1
APS	ammonium persulphate
ASEAN	Association of Southeast Asian Nations
ATCC	American Type Cell Culture
Bad	Bcl-XL/Bcl-2-associated death promoter
Bak	Bcl-2-associated killer
Bax	Bcl-2-associated X protein
BCA	bicinchoninic acid
BCG	Bacillus Calmette-Guerin
Bcl-2	B cell lymphoma 2
BH3	Bcl-2 homology domain 3
Bid	Bcl-2 interacting death domain agonist
Bim	Bcl-2 interacting mediator of cell death
BRF	baicalein-rich fraction
Ca^{2+}	calcium

CAD	caspase-activated DNase
cAMP	cyclic adenosine monophosphate
Cdk	cyclin-dependent kinase
CIN	cervical intraepithelial neoplasia
cm	centimeter
cm^2	centimeter square
c-Myc	cellular-Myelocytomatosis
CO_2	carbon dioxide
CREB	cAMP-responsive element-binding protein
DISC	death-inducing signalling complex
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E	early region
E6AP	E6-associated protein
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
FACS	fluorescence-activated cell sorting
FADD	Fas-associated death domain
Fas	fibroblast associated antigen
FasL	fibroblast associated antigen ligand
FBS	fetal bovine serum
FTIR	fourier Transform Infrared

g	gram
GCDA	glycochenodeoxycholate
GLOBOCAN	Global Cancer Incidence, Mortality and Prevalence
GSK3	glycogen synthase kinase 3
HCl	hydrochloric acid
HETE	hydroxyeicosatetraenoic acid
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
HPV	human papillomavirus
HRP	horseradish peroxide
HSCCC	high-speed counter-current chromatography system
HSIL	high-grade squamous intraepithelial lesion
HUVEC	human umbilical vein endothelial cell
IC_{50}	half maximal inhibitory concentration
ICAD	inhibitor of caspase-activated DNase
IDV	integrated density values
IFN	interferon
IFNG	interferon Gamma
IL	interleukin
IL-12R	IL-12 receptor
IL-6Rα	IL-6 receptor α
IPHARM	Malaysian Institute of Pharmaceuticals and Nutraceuticals
JAK	janus kinases
JNK	c-Jun N-terminal kinase

L	late region
LCR	long control region
LMIC	low- and middle-income countries
LOX	lipoxygenase
LPPKN	National Population and Family Board
LSIL	low-grade squamous intraepithelial lesion
M	Molar
MAP2K	MAP kinase kinase
MAP3K	MAP kinase kinase kinase
MAPK	mitogen-activated protein kinase
MBA	methylene blue assay
MCE	methanol crude extract
MCL1	myeloid leukemia 1
MEK	mitogen-activated protein/extracellular signal regulated kinase
mg	miligram
min	minutes
ml	milliliter
MLK	mixed lineage protein kinase
mm	millimeter
MMP	matrix metalloproteinase
MRI	mean relative intensity
mRNA	microRNA
NaCl	sodium chloride
NCI	National Cancer Institute

NGF	nerve growth factor
NK	natural killer
nm	nanometer
NO	nitric oxide
O. indicum	*Oroxylum indicum*
ORF	open reading frame
OX-LDL	oxidised low-density lipoprotein
Pap	Papanicolaou
PBS	phosphate buffer saline
p-ERK	phosphorylated ERK
p-JNK	phosphorylated JNK
p-p38	phosphorylated p38
pRb	retinoblastoma protein
PTLC	preparative thin layer chromatography
PUMA	p-53 upregulated modulator of apoptosis
PVDF	polyvinylidene difluoride
R_f	retention factor
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
ROS	reactive oxygen species
RPA	replication protein A
RSK	ribosomal protein S6 kinase
RT	retention time
SD	standard deviation
SDS	sodium dodecyl sulfate

SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sIL-6Rα	soluble IL-6Rα
siRNA	small interfering RNA
Smac	second mitochondria-derived activator of caspases
SNpc	substantia nigra pars compacta
STAT	signal transducer and activator of transcription
tBid	truncated Bid
TBS	tris-buffered saline
TEMED	tetramethylethylenediamine
TF	total flavonoid
TFC	total flavonoid content
Th	T helper cells
Thr	threonine
TLC	thin layer chromatography
TMB	3,3’,5,5’-Tetramethylbenzidine
TNF	tumour necrosis factor
TYK	tyrosine kinase
Tyr	tyrosine
URR	upstream regulatory region
US	United State
UV	ultraviolet
v	volume
V	volt
VEGF	vascular endothelial growth factor

VLP	virus-like-particle
w	weight
WHO	World Health Organization
x *g*	g force

PENGENALPASTIAN MEKANISMA FRAKSI-KAYA BAICALEIN DARI EKSTRAK DAUN *Oroxylum indicum* KE ATAS SEL SELANJAR KANSER SERVIK

ABSTRAK

Oroxylum indicum (*O. indicum*) telah diimplikasikan sebagai ajen anti-kanser untuk rawatan kanser termasuk kanser servik. Kajian terdahulu telah menunjukkan kebolehan tumbuhan ini dalam merencat proliferasi sel kanser dengan mengaruh apoptosis. Ciri-ciri terapeutik anti-kanser oleh *O. indicum* sangat berkait dengan kandungan kimia utamanya seperti chrysin, oroxylin A dan baicalein. Dalam kajian ini, fraksi-kaya baicalein (BRF) daripada daun *O. indicum* telah diekstrak untuk mengenalpasti aktiviti anti-kansernya terhadap sel kanser servik, SiHa (positif HPV 16) dan HeLa (positif HPV 18). Dengan menggunakan kaedah kromatografi lapisan nipis persediaan (PTLC) (n-hexane: ethyl acetate; 50:50), fraksi ini telah disediakan daripada ekstrak metanol dan diteruskan kepada kromatografi cecair berprestasi tinggi (HPLC) untuk kuantifikasi baicalein. Aktiviti anti-proliferasi oleh BRF telah diuji menggunakan asai metilina biru (MBA). Seterusnya, aktiviti pro-apoptosis oleh BRF dalam memodulasi ekspresi protein telah ditentukan melalui analisis pemblotan Western ke atas ekspresi onkoprotein HPV (E6 dan E7), protein penindas tumor (p53 dan pRb) dan protein lain yang penting untuk laluan apoptosis melalui pengantaraan mitokondria (Bax, Bcl-2, caspase-9 and caspase-3). Selanjutnya, kebergantungan apoptosis yang disebabkan oleh BRF dalam sel kanser serviks kepada subfamili protein kinase teraktif-mitogen (MAPK) yang terdiri daripada kinase yang terkawal isyarat ekstraselular (ERK), kinase c-Jun N-terminal kinase (JNK) dan p38 juga telah ditentukan melalui analisis pemblotan Western

melalui penambahan perencat MAPK yang tertentu. Selain itu, aktiviti pro-apoptosis BRF terus dikaji melalui tindakan modulasinya pada ekspresi interleukin (IL)-6 dan IL-12 dengan menjalankan asai enzim imunosorben berkaitan enzim (ELISA). Hasilnya, BRF dengan 75% baicalein merupakan kompaun yang paling poten berbanding cisplatin dan MCE berdasarkan nilai IC_{50} yang diperolehi daripada kedua-dua sel. Selepas 24 jam tempoh rawatan, analisis Western blot menunjukkan bahawa sel SiHa dan HeLa yang dirawat dengan BRF telah menurunkan pengekspresan E6 dan E7 dan memulihkan aruhan proses apoptosis melalui peningkatan pengekspresan p53 dan pRb dalam sel terawat. Dari segi laluan apoptosis melalui pengantaraan mitokondria, BRF meningkatkan laluan pengaktifan tersebut dengan menurunkan pengekspresan protein anti-apoptosis Bcl-2 dan meningkatkan pengekspresan komponen utama seperti Bax, caspase-9 dan caspase-3. Bagi isyarat MAPK, aktiviti pro-apoptosis BRF dalam sel SiHa and HeLa didapati bergantung kepada MAPK kerana semua subfamilinya terlibat dalam apoptosis yang disebabkan oleh BRF secara berbeza. BRF mengaruh apoptosis dalam sel kanser serviks melalui perencatan ERK dan pengaktifan JNK. Selain itu, BRF meningkatkan pengaktifan Bax melalui laluan ERK/p38 dan menindas ekspresi Bcl-2 sebahagiannya melalui perencatan ERK dan pengaktifan p38. Sementara itu untuk sel HeLa, pengaktifan laluan ERK/JNK/p38 diperlukan untuk mengaktifkan Bax dan perencatan kedua-dua isyarat ERK/p38 menggalakkan penurunan kawal atur Bcl-2 dalam sel HeLa yang dirawat BRF. Aruhan apoptosis oleh BRF juga ditingkatkan oleh penurunan pengekspresan IL-6 dan peningkatan kawal atur IL-12. Oleh itu, penemuan yang dibentangkan ini telah membuktikan keupayaan BRF yang difraksikan daripada daun *O. indicum* untuk dieksploitasikan sebagai calon anti-kanser berasaskan tumbuhan yang berpotensi untuk rawatan kanser servik.

ELUCIDATING THE MECHANISM OF BAICALEIN-RICH FRACTION FROM *Oroxylum indicum* LEAVES ON CERVICAL CANCER CELL LINES

ABSTRACT

Oroxylum indicum (*O. indicum*) has been implicated as a promising anti-cancer agent for cancer treatment including cervical cancer. Previous studies have shown that this plant has been able to inhibit the proliferation of cancer cells by acting as an apoptosis inducer. The therapeutic anti-cancer properties of *O. indicum* is strongly associated due to its major chemical constituents such as chrysin, oroxylin A and baicalein. In this present study, the baicalein-rich fraction (BRF) from *O. indicum* leaves has been extracted to elucidate its anti-cancer activity against cervical cancer cells, SiHa (HPV 16 positive) and HeLa (HPV 18 positive) cells. Using the preparative thin layer chromatography (PTLC) (n-hexane: ethyl acetate; 50:50), this fraction was prepared from the methanol crude extract (MCE) of *O. indicum* and proceeded to high performance liquid chromatography (HPLC) for baicalein quantification. At first, anti-proliferative activities of BRF were tested using methylene blue assay (MBA). Then, the pro-apoptotic activity of BRF in modulating protein expression was determined by Western blot analysis on the expression of HPV oncoproteins (E6 and E7), tumour suppressor proteins (p53 and pRb) and key proteins of mitochondrial signalling apoptosis pathway (Bax, Bcl-2, caspase-9 and caspase-3). Next, the dependency of BRF-induced apoptosis in cervical cancer cells to mitogen-activated protein kinase (MAPK) subfamilies consisting of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 were also determined by Western blot analysis with the addition of specific MAPK inhibitors. Moreover, the pro-apoptotic activity of BRF continued to be examined through its

modulation action on the expression of interleukin (IL)-6 and IL-12 by conducting an enzyme-linked immunosorbent assay (ELISA). As a result, BRF with 75% of baicalein was found to be the most potent compound compared to cisplatin (anti-cancer drug) and MCE based on the IC_{50} values obtained from both cells. After 24 hours treatment period, Western blot analysis showed that BRF-treated SiHa and HeLa cells downregulated the expression of E6 and E7 and restored the induction of apoptosis process through the up-regulation of p53 and pRb in treated cells. In terms of mitochondrial-mediated apoptosis pathway, BRF has positively enhanced the activation pathway by downregulating Bcl-2 anti-apoptotic protein and upregulating the required key components such as Bax, caspase-9 as well as caspase-3. In regard to MAPK signalling cascade, the pro-apoptotic activities of BRF in SiHa and HeLa cells have been found to be MAPK-dependent as all its subfamilies involved in BRF-induced apoptosis but in different approach. BRF induced apoptosis in cervical cancer cells predominantly through ERK inhibition and JNK activation. Besides, BRF induced Bax activation by ERK/p38 dependent pathway and suppressed Bcl-2 expression through inhibition of ERK and activation of p38. Meanwhile for HeLa cells, activation of ERK/JNK/p38 pathways are required for Bax activation and inhibition of both ERK/p38 signalling promoted Bcl-2 downregulation in BRF-treated HeLa cells. Apoptosis induction by BRF also enhanced through IL-6 downregulation and IL-12 upregulation. Thus, these presented findings have proved the ability of BRF fractionated from *O. indicum*'s leaves to be exploited as a potential plant-based anti-cancer candidate for cervical cancer treatment.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Cancer has become the major leading cause of death among women worldwide in both developing and non-developed countries. Over thousands of cancers have been found to date, cervical cancer is one of the most life-threatening diseases as it leads to the fourth-largest cause of death among women worldwide, especially in low-income countries (Torre *et al.*, 2017). Cervical cancer, also known as carcinoma of uterine cervix, remains a major public health concern for women throughout the world, despite the existence of efficient prevention and screening methods (Koh *et al.*, 2015). The burden of cancer continues to increase in developing countries due to the adoption of cancer-associated lifestyles such as smoking, high parity and long-term contraceptive use (Torre *et al.*, 2015).

However, the majority of reported cases of cervical cancer resulted from human papillomavirus (HPV) infection as HPV deoxyribonucleic acid (DNA) was detected in approximately 90% of malignant cervical lesions extracted from cervical cancer-positive patients (Petry, 2014). Even though most of HPV infections will be cleared by the host defense immune system, however persistent infection with high-risk group of HPV can contribute to viral immune evasion and allow the development of malignant conditions at the infected area of the squamocolumnar junction at the cervix tissue lining (Small *et al.*, 2017). High-risk group of HPV; HPV 16 and HPV 18 account for 72% and 90% of all HPV-attributable cases of

cervical cancer with different proportions among global populations (de Martel *et al.*, 2017).

HPVs are the sexually transmitted viruses and infected under the epithelium surface in the basal layer through microwounds as the entrance route. The basal layer consist of actively-dividing cells and acts as the reservoir of cells for the suprabasal regions (Longworth and Laimins, 2004). Following infection, the life cycle of HPV is closely dependent on the differentiation programme of the infected host cells as a part of the viral replication strategy. The viral genetic materials will be integrated with the host genome, resulting in the lifetime persistence of certain viral genes within the cells and causing genomic instability by interfering with the regulation of host homeostasis and providing a favourable condition for cancer development (Moody and Laimins, 2010).

HPV's genomic organisation encode for eight proteins with six early proteins (E1, E2, E4, E5, E6, E7) and two late proteins (L1 and L2). E2 is a replication factor that acts as a viral transcriptional repressor in controlling the expression of two main contributor oncoproteins; E6 and E7. The integration of HPV genomes into host DNA resulted in the disruption of E2's open reading frame (ORF) and led to the loss of E2 expression, consequently induced uncontrollable cell growth due to the overexpression of E6 and E7 (Sasagawa *et al.*, 2012). Although the expression of oncoproteins in the basal layer is considered low, the persistent infection that continues for years and even decades can certainly accumulate a high level of expression of E6 and E7 that is sufficient enough to induce mutation and immortalise the cells (Narisawa-Saito and Kiyono, 2007). These consequences

occurred due to the major tumourigenic mechanism of both oncoproteins by inactivating the function of tumour suppressor proteins; p53 and retinoblastoma protein (pRb) respectively. Abrogation of these key proteins abolish cell proliferation control and promote apoptosis evasion in HPV-infected cells (Martinez-zapien *et al.,* 2016).

Apoptosis is a crucial programme cell death that can be executed via several pathways through extracellular and intracellular activation signal (Matsuura *et al.,* 2016). Intracellularly, mitochondrial-mediated apoptosis pathway is triggers by the interactions between B cell lymphoma (Bcl)-2, Bcl-2-associated X protein (Bax) and caspases (Kiraz *et al.,* 2016). In normal condition, cytosolic Bax translocate to the mitochondrial membrane and induce pore formation to enable the release of cytochrome c. Bcl-2 functions as an antagonist for Bax activities. Next, cytochrome c forms an apoptosome complex by associating with apoptosis protease-activating factor 1 (Apaf-1). This complex then activates caspase-9 and subsequently leads to the activation of caspase-3. The activated caspase-3 then cleaves myriad of cellular proteins to manifest DNA fragmentation as apoptosis representative (Goldar *et al.,* 2015; Kiraz *et al.,* 2016; Pfeffer and Singh, 2018).

Apoptosis execution can be driven by multiple upstream activators including mitogen-activated protein kinase (MAPK) signalling. MAPK is a signal transduction cascade that responsible for sustaining human cancer cell survival, metastasize and resistance against the chemotherapy drugs (Burotto *et al.,* 2014). This complex pathway signals for wide range of cellular activities regulations including cell proliferation, differentiation and death through three main subfamilies

with their respective functions; extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 (Kim and Choi, 2010). ERK was shown to be critically important for cell survival meanwhile JNK and p38 were believed as major contributors in the regulation of stress-induced apoptosis pathway (Roskoski, 2012; Sui *et al.*, 2014).

Apart from that, apoptosis also can be influenced by the imbalance secretion of immunoinhibitory and immunostimulatory cytokines. Overexpression of immunoinhibitory cytokines such as interleukin (IL)-6 and deprivation of immunostimulatory cytokines like IL-12 promotes apoptosis evasion and regularly associated with chemoresistance in cancer cells (Carpenter and Lo, 2014; Kursunel and Esendagli, 2016; Lin *et al.,* 2017). Due to the link between these mediators with apoptosis induction, modulation of the related proteins has become one of the main targets for cervical cancer therapy to overcome the resistance developed by HPV oncoproteins (Baig *et al.*, 2016).

As we know, cervical cancer is a preventable disease. HPV vaccination has been introduced to control the disease. Yet, the vaccination has limited action as it has no therapeutic effect on pre-existing HPV even before the onset of cancer (World Health Organization, 2019). Furthermore, a regular Papanicolaou smear (Pap smear) test can also provide an early detection for cervical cancer. However, women still lack the awareness about this medical screening (Lin, 2015). In terms of treatment aspects, radiotherapy and chemotherapy continue as the first line treatment against cervical cancer. Although these treatments have increased the long-term survival of cancer patients, it can cause major and long-lasting side effects such as

infertility and development of secondary cancer (Derks *et al.,* 2017; Manem *et al.,* 2017). Therefore, the searching for plant-based anti-cancer agent with comparable efficacy and less side effect may provide an alternative treatment against cervical cancer.

Plants are a significant reservoir for therapeutic phytochemicals that serve promising intervention for cancer treatments. Plant-derived curative treatment provides the basis for most early medicines such as aspirin from willow trees and morphine from opium poppy plants (Kabera *et al.,* 2014). Meant for clinically used anti-cancer drugs, vinca alkaloids were isolated from *Catharanthus roseus,* taxol from *Taxus brevifolia* and topotecan from *Campthotheca acuminate* (Butler, 2004; Manju *et al.,* 2012; Khazir *et al.,* 2014). In terms of cervical cancer treatment intervention, various plants were screened and numbers were scientifically proven to be able to combat this life-threatening cancer such as *Quercus infectoria* (Hazwani *et al.,* 2018), *Psidium guajava* (Correa *et al.,* 2016) *Clinacanthus nutans* (Mohd *et al.,* 2018) and *Oroxylum indicum* (Moirangthem *et al.,* 2013; Siriwatanametanon *et al.,* 2010; Zazali *et al.,* 2013).

In this regards, *O. indicum* becomes one of the potential candidates to be developed as a new anti-cancer drug. *O. indicum* is a medium-sized tree, known as 'beko' in Malaysia and can be eaten as vegetable (Zazali *et al.,* 2013). Multiple studies have previously demonstrated the anti-cancer potentials of *O. indicum* in in vitro studies. *O. indicum* was found to exert cytotoxicity effects on a wide range of cancer cells such as human leukemia (Roy *et al.,* 2007), human breast cancer (Bhavita *et al.,* 2002; Kumar *et al.,* 2013; Nagasaka *et al.,* 2018), mouse

hepatocarcinoma (Li *et al.,* 2018) and human laryngeal carcinoma (Kamkaen *et al.,* 2006) cell lines. The pro-apoptosis potentials of *O. indicum* in cancer cells were observed through its anti-proliferative activities and morphological changes such as cell shrinkage, and reduction in size and shape of the *O. indicum*-treated cells (Zazali *et al.,* 2013). These therapeutic capabilities are strongly associated with the abundance of its anti-cancer flavonoid content such as chrysin, oroxylin A and baicalein (Gao *et al.,* 2016; Qiao *et al.,* 2016; Xuan *et al.,* 2016).

1.2 Rationale of the study

Despite government-led prevention approaches such as HPV vaccination and scheduled Pap smear screening, cervical cancer remained the third most diagnosed cancer among Malaysian women with approximately 2,000 new cases reported annually (Kessler, 2017; Manan *et al.,* 2016; Mezei *et al.,* 2017). Current chemotherapy methods offer the most effective treatment to treat cancer but the main disadvantage is the severe side effects (Mikkelsen *et al.,* 2017; Muliira *et al.,* 2017). Therefore, these limitations triggered the urge for the plant-derived anti-cancer agent development from ethnobotanical plants as an alternative.

Based on the statements above, *O. indicum* becomes one of the most promising candidates to be exploited as a new anti-cancer drug due to its diverse distribution and promising anti-cancer properties (Kalra and Kaushik, 2017). Certainly, *O. indicum* was discovered to have the highest cytotoxicity to cancer cells among eleven medicinal plants from Bangladeshi folk medicine and nine species of

Thai medicinal plants from prior reports (Costa-lotufo *et al.,* 2005; Siriwatanametanon *et al.,* 2010).

Every part of *O. indicum* has been discovered to possess anti-proliferative effects on cancer cells in vitro (Costa-lotufo *et al.,* 2005; Kumar *et al.,* 2010; Moirangthem *et al.,* 2013; Nagasaka *et al.,* 2018; Rajkumar *et al.,* 2011; Siriwatanametanon *et al.,* 2010). The pro-apoptosis activities of this beneficial plant mainly contributed by its major compounds such as chrysin, oroxylin A and baicalein (Gokhale *et al.,* 2017). Yet, only baicalein was selected to be investigated in this study. Baicalein is one of the major bioactive compounds that can be found abundantly in the leaves of *O. indicum* (Dinda *et al.,* 2015). It is also a common flavonoid that can be isolated from *Scutellaria baicalensis* (Jianjun and Huiru, 2008) and *Thymus vulgaris* (Fujita *et al.,* 2005). Anti-neoplastic effects of baicalein from other sources such as from *S. baicalensis* or synthetically produced were extensively studied in vitro in colon (Palko-Labuz *et al.,* 2017; Taniguchi *et al.,* 2008), promyelocytic leukemia (Li *et al.,* 2004), ovarian (Chen *et al.,* 2013a), hepatoma (Kuo *et al.,* 2010; Zheng *et al.,* 2014), oral (Cheng *et al.,* 2012) and cervical (Yong *et al.,* 2011) cancer cell lines. *O. indicum*'s baicalein exerted cytotoxic effects on bladder cancer (Yang *et al.,* 2017), promyelocytic leukemia (Roy *et al.,* 2007) and colorectal carcinoma cells (Lalou *et al.,* 2013).

Apart from that, baicalein was shown to be more a potent apoptosis inducer in cancer cells as compared to chrysin and oroxylin A (Parajuli *et al.,* 2009; Sanoda *et al.,* 2004). In a study done by Sanoda *et al.* (2004), the anti-proliferative activity of baicalein on human leukemia cells (HL-60) increased in a dose-dependent manner. Nevertheless, oroxylin A had stimulated the proliferation of cancer cells

(Sanoda *et al.,* 2004). On the other hand, higher percentage of apoptotic cells were detected in baicalein-treated malignant glioma cells as compared to chrysin-treated cells after 96 hours treatment period (Parajuli *et al.,* 2009). From this finding, baicalein showed higher apoptotic activity rather than chrysin which make baicalein a better target to be studied.

However, the knowledge regarding the anti-cancer activity of baicalein in cervical cancer is still limited. In SiHa and HeLa cervical cancer cells, baicalein was found to inhibit proliferation and induce cell cycle arrest at G0-G1 phase by inhibiting the phosphorylation of protein kinase B and glycogen synthase kinase 3ß that eventually caused the suppression of cyclin D1 (Wu *et al.,* 2017). Indeed, baicalein acted as an apoptosis inducer in treated HeLa cells by alleviating the expression of important elements in extrinsic apoptosis pathway which were Fas, FasL as well as caspase-8 and subsequently increased the cell's sensitivity towards apoptosis (Peng *et al.,* 2015). The ability of baicalein to initiate apoptosis was also observed through the increment of apoptotic bodies formation in HeLa cells and tumour growth inhibition in mice bearing U14 cervical cancer following baicalein treatment (Peng *et al.,* 2015; Yong *et al.,* 2011). Due to this limited understanding on how baicalein induced apoptosis in cervical cancer model, the underlying mechanism related to pro-apoptosis activities of baicalein in HPV-associated cervical cancer cells were further elucidated in this present study.

1.3 Objectives of the study

1.3.1 General objective

1. To elucidate the mechanism of baicalein-rich fraction (BRF) from *Oroxylum indicum* on the apoptosis pathway in human cervical cancer cell lines; SiHa and HeLa**.**

1.3.2 Specific objectives

1. To isolate BRF from *O. indicum* leaves through bio-assay guided fractionation.
2. To determine the anti-proliferative activity of BRF in human cervical cancer cell lines; SiHa and HeLa.
3. To measure the expression of HPV oncoproteins (E6, E7), tumour suppressor proteins (p53, pRb) and proteins related in mitochondrial-mediated apoptosis pathway (Bax, Bcl-2, caspase-9 and caspase-3) in BRF-treated SiHa and HeLa cells.
4. To determine the involvement of mitogen-activated protein kinase (MAPK) signalling in BRF-induced apoptosis in SiHa and HeLa cells.
5. To measure the expression of cytokines; IL-6 and IL-12 in BRF-treated SiHa and HeLa cells.

1.4 Hypotheses

1.4.1 Null Hypothesis (H_0)

1. BRF from *Oroxylum indicum* does not affect the mechanism of apoptosis pathway in human cervical cell lines; SiHa and HeLa.

1.4.2 Alternative hypotheses (H_1)

1. BRF will be fractionated from *Oroxylum indicum* leaves.
2. BRF shows a potent anti-proliferativ activity in treated human cervical cancer cell lines; SiHa and HeLa.
3. BRF-treated SiHa and HeLa cells will express low level of HPV oncoproteins and high level of apoptotic mediators as compared to the untreated cells.
4. BRF's activity in inducing cell death in SiHa and HeLa cells will be dependent on MAPK signaling cascade.
5. SiHa and HeLa cells treated with BRF will express low level of IL-6 and high level of IL-12 as compared to the untreated cells.

CHAPTER 2

LITERATURE REVIEW

2.1 Cervical cancer

The word cervix originated from a Latin word meaning “neck,” representing its anatomical function as a narrow connection between the body of the uterus and the vagina as shown in Figure 2.1. This key component of the women’s reproductive system plays a crucial role in protecting the foetus during pregnancy and facilitating childbirth delivery (Myers *et al.,* 2015). Stratified squamous epithelium that forms the first lining of cervix covers the mucus-secreting columnar epithelium of endocervix canal and the exocervix. The transition area between these types of cells that known as squamocolumnar junction was found to be the most susceptible point for viral evasion (Mirkovic *et al.,* 2015).

According to World Health Organization (WHO) (2019), cervical cancer is a slow-growing cancer that has developed in cervix tissues. It begins with the growth of abnormal cells on the cervix’s surface lining which also known as cervical intraepithelial neoplasia (CIN). As the time pass by, accumulation of the abnormal cells leads to the development of cancerous cells when the cells start to grow and spread deeply into the cervix. Cervical cancer signs and symptoms may include abnormal vaginal bleeding after sexual intercourse, irregular menstrual cycle, vaginal discomfort and a single swollen leg (WHO 2019).

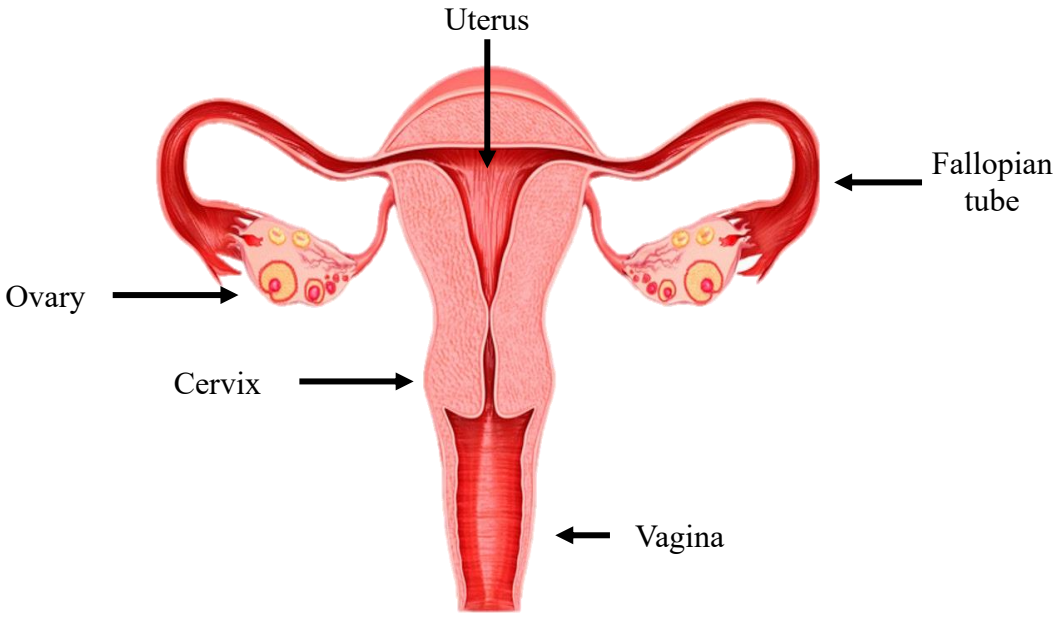


Figure 2.1 The female reproductive system's anatomic structure. The uterus, ovaries, fallopian tubes, cervix and vagina are among the organs in the female reproductive system. Modified from Briceag *et al.*, (2015).

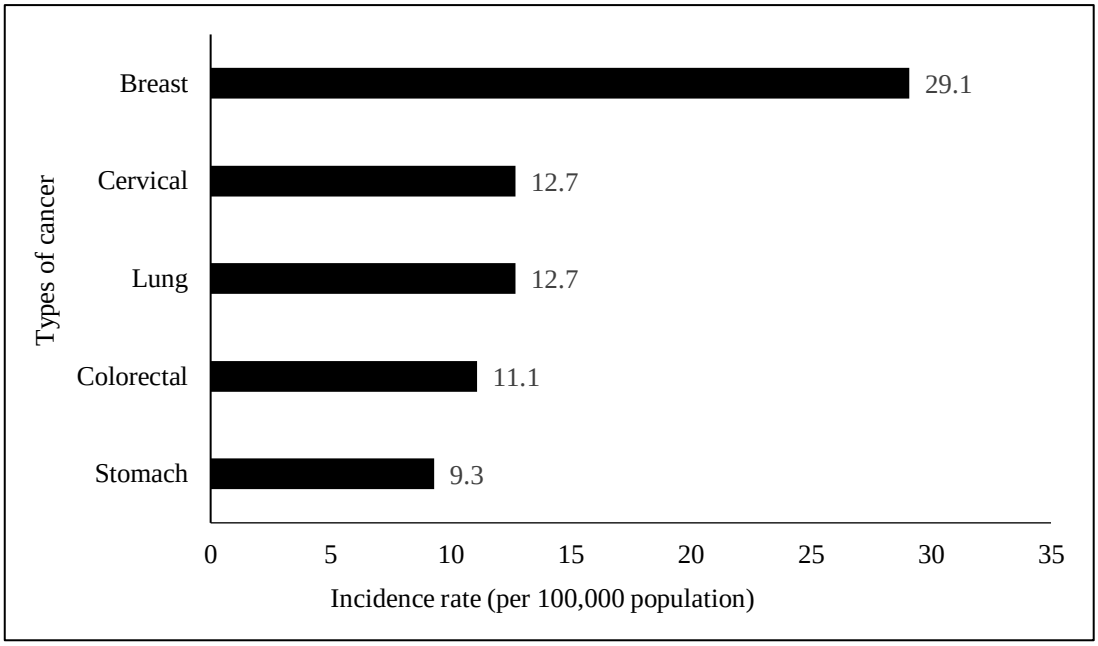
2.1.2 Cervical cancer statistics

Global Cancer Incidence, Mortality and Prevalence (GLOBOCAN) 2018 showed cervical cancer as the fourth most frequently diagnosed cancer among women worldwide with 570,000 cases, and 311,000 deaths after breast cancer, colorectal cancer and lung cancer. Nevertheless, cervical cancer was second in incidence and mortality rates in less developed countries and third in transition countries (Bray *et al.,* 2018). Despite that, cervical cancer leads the rank for new cases reported in 28 over 31 countries and based on the mortality profile, it is the leading cause of death in 42 countries including Sub-Saharan Africa and South Eastern Asia countries.

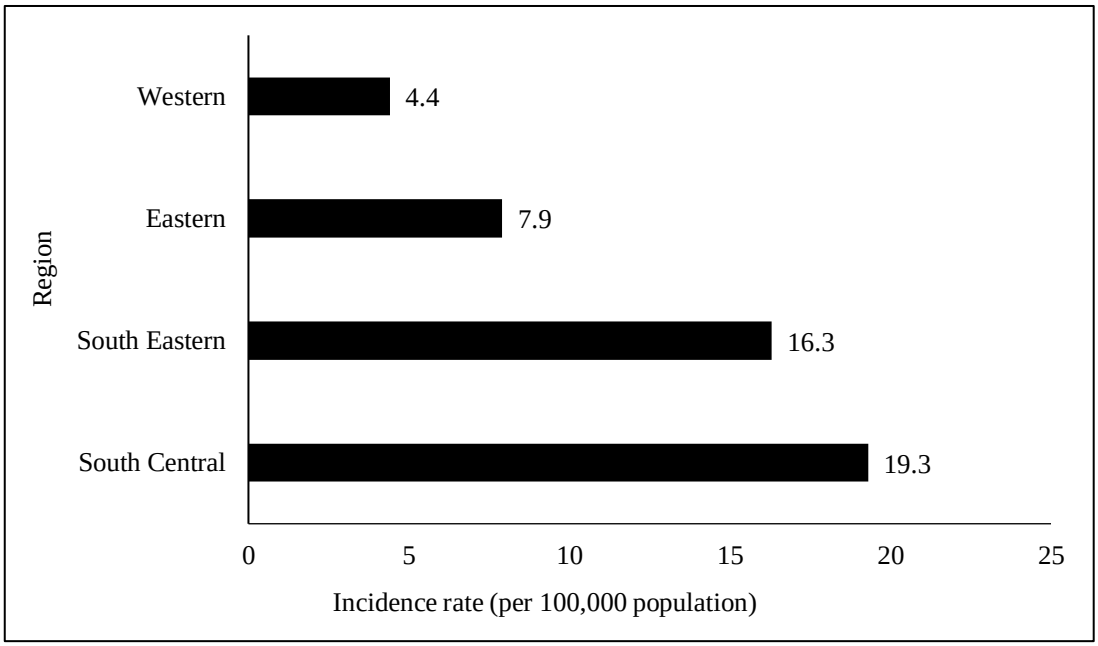
Africa reported the highest regional incidence and mortality cases and this observation particularly contributed by cases from Southern Africa country (Swaziland), Western Africa countries (Guinea, Burkina Faso and Mali) and Eastern Africa countries (Malawi and Zimbabwe). Yet, the statistics are 10 times lower in the developed countries such as in Australia, North America, New Zealand and Western Asia countries such as Saudi Arabia (Bray *et al.,* 2018). These facts demonstrate the negative relationship between cervical cancer incidence and mortality rates and country revenues, since cervical cancer cases have increased in low-income countries in comparison to developing and developed countries (Ng *et al.,* 2015).

As shown in Figure 2.2(a), cervical cancer ranked second among Asian women over top five common cancer in Asian countries. South Central Asian countries such as India, Bangladesh, Pakistan and Afghanistan recorded the highest

incidence of cervical cancer in Asia as India alone accounted for nearly 27% of the global incidence of cervical cancer as shown in Figure 2.2(b) (Ng *et al.*, 2015). Within the Association of Southeast Asian Nations (ASEAN) countries, cervical cancer incidence was the second most reported for female-specific cancers with 44, 351 cases after breast cancer (86, 842 cases) and followed by colorectal cancer (34, 493 cases). It was ranked third in terms of mortality rates with 22, 473 deaths following breast cancer (36, 723 deaths) and lung cancer (27, 837 deaths) (Kimman *et al.*, 2012). In this regard, most cervical cancer mortality was reported in Cambodia, followed by Myanmar, Thailand and Laos, as shown in Figure 2.3(a). The increase in cervical cancer incidence rates among ASEAN women has been observed with the increasing age when the chart is dominated by women older than 45 years (Figure 2.3(b)).



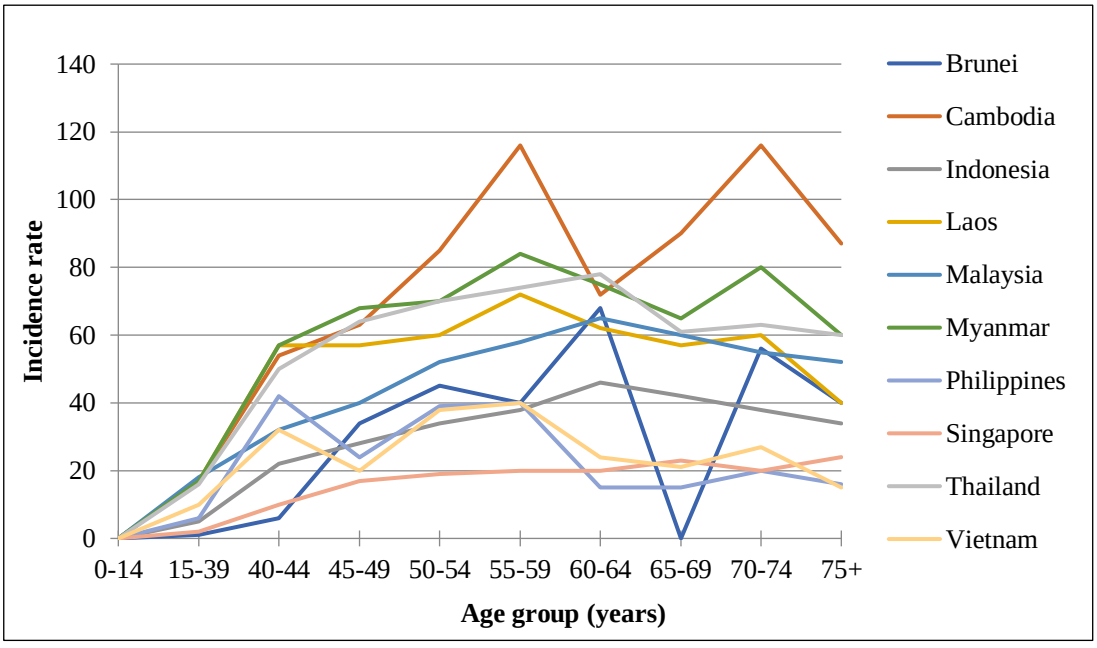
(a)



(b)

Figure 2.2 Cervical cancer in Asia. **(a)** Estimated age-standardised incidence of cervical cancer in the top five cancers **(b)** Regional estimated age-standardised cervical cancer incidence. Modified from Ng *et al.,* (2015).

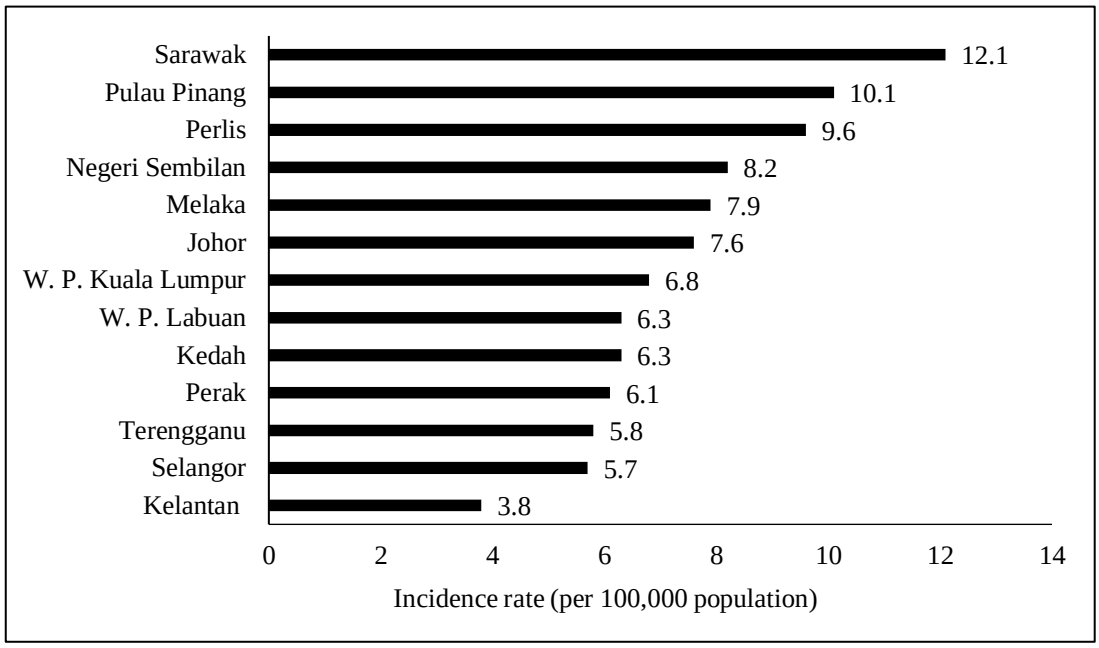
(a)



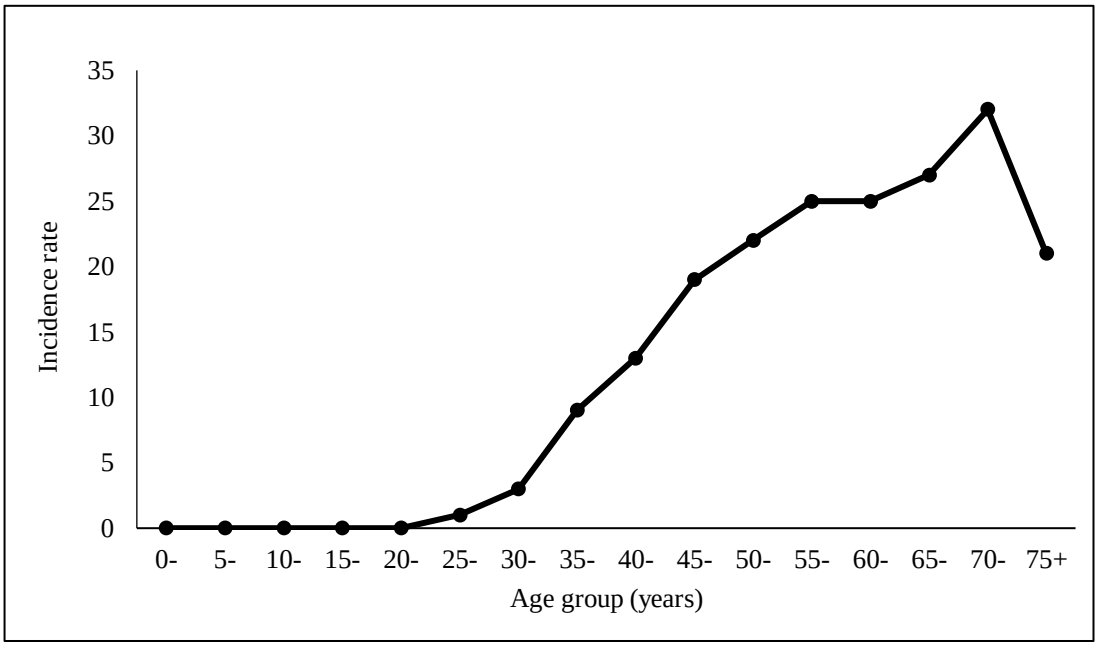
(b)

Figure 2.3 Cervical cancer in ASEAN countries. **(a)** Regional-based estimated age-standardised mortality rate **(b)** Age-based cancer incidence rate per 100,000 population. Modified from Kimman *et al.*, (2012).

Meanwhile in Malaysia, cervical cancer is the third most common cancer among Malaysian women with an incidence rate of 19.7 per 100,000 women and the seventh among total cancer cases reported across the general population (Manan *et al.,* 2016). About 4, 325 cases were reported in between 2007-2011 which contributed 9.7% from total cancer cases for top ten diagnosed cancer in Malaysia with most of the cases were reported in Sarawak and least in Kelantan (Figure 2.4(a)). The incidence rate of cervical cancer was started to be detected in 25 years old women and reached the peak at the age of 70-74 years old as shown in Figure 2.4(b). Literally, no cases were reported for women under the age of 24, but it is the second common cancer for 25 to 59 years old women, and the fourth for women aged 60 to 74. By ethnicity, it ranked fourth among Malay, third among Chinese, third among Indian and second among other Bumiputera ethnics (Manan *et al.,* 2016). The highest incidence rate of cervical cancer was detected in Chinese population (9.8), followed by Indian community (7.6) and Malay (5.1) per 100, 000 women (Raub *et al.,* 2014).



(a)



(b)

Figure 2.4 Cervical cancer in Malaysia. **(a)** Regional-based estimated age-standardised incidence rate **(b)** Age-based cancer incidence rate per 100,000 population. Modified from Manan *et al.,* (2016).

2.2 Causative agent of cervical cancer

Cervical cancer is a multifactorial cancer (Figure 2.5). Unhealthy sexual behaviors such as high parity and first intercourse at a very young age (under 15 years old) elevate the risk of cancer development as 54% of reported cases in India involved with two or more parity. India is one of the countries with the highest number of cervical cancer cases worldwide (Nath *et al.,* 2015). Besides, immunosuppressed patients with low counts of CD4+ T cells especially for women infected with human immunodeficiency virus (HIV) were found to be an important enhancer for cervical carcinogenesis as HIV also induced systemic natural killer (NK) and T cells defects in patients (Bere *et al.,* 2014; Clifford *et al.,* 2016). On the other hands, epidemiological studies persistently associating the period of smoking with the progression of invasive cervical cancer as tobacco content in the smoke provided a favourable condition for malignancies through induction of oxidative stress and deoxyribonucleic acid (DNA) damage (Appleby *et al.,* 2006; Carnevale *et al.,* 2016; de Marco, 2013; Roura *et al.,* 2014). Tobacco by-products and carcinogenic chemiscals in cigarettes found in the cervical mucosa of smoking women supported the potential for cervical cancer (Kessler, 2017).

Despite all these risk factors, a strong and specific association relating human papillomavirus (HPV) with cervical cancer was shown beyond any reasonable doubt by consistent evidences from epidemiological studies (Clifford *et al.,* 2003; de Martel *et al.,* 2017; Hu *et al.,* 2015). HPV-DNA was detected in 90-100% of cervical cancer cases in investigated population by using state-of-the-art amplification techniques in clinical and epidemiological studies (de Martel *et al.,* 2017; Hooi *et al.,* 2017).

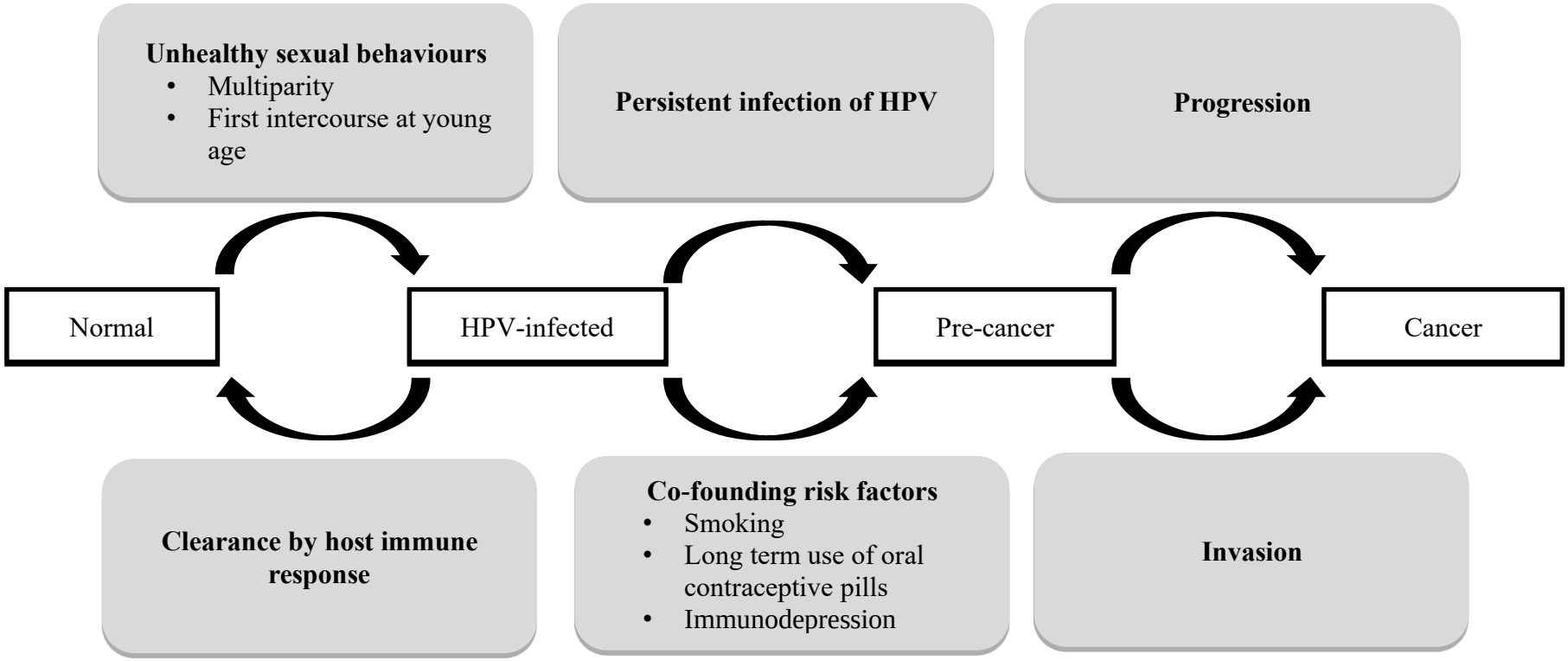


Figure 2.5 Natural history of cervical cancer development. Unhealthy sexual activities and other co-founding risk factors elevate the tendency for cervical cancer development and progression. Modified from Small *et al.*, (2017).

2.2.1 Human papillomavirus (HPV)

HPV belongs to *Papillomaviridae* family, small size and double stranded DNA virus. Up to date, more than 200 types of HPV have been identified based on their common DNA sequence and about 40 of it were implicated with genital infections (de Martel *et al.,* 2017). HPV can be classified into several groups based on its associated diseases ranging from innocuous lesions to its ability in inducing malignant transformation in the cells of infected host (Table 2.1). Infection with low-risk HPV types results in the proliferation of epithelial cells and typically manifested as papillomas or benign warts on the skin (Sanjose *et al.,* 2018). Yet, these infections are self-limiting and rarely associated with malignancies.

In contrast, persistent infection of high-risk HPV types can lead to cancerous development as proven by the detection of all 15 HPV types in HPV-related cervical carcinoma cases (Clifford *et al.,* 2003; Mirkovic *et al.,* 2015). HPV 16 and HPV 18 were the most prevalent high-risk HPV types identified in multistage carcinogenesis of cervical cancer reported worldwide as demonstrated in Figure 2.6 (de Martel *et al.,* 2017) and the same trend of HPV distribution also detected in Asian countries (Bao *et al.,* 2008) including Malaysia (Raub *et al.,* 2014; Tan *et al.,* 2018).

Persistent infection of HPV 16 integration was significantly associated with malignant progression, with increased frequency from pre-neoplastic lesions to invasive tumors. From a study done by Badaracco *et al.,* (2002), HPV 16 DNA was found either as pure integrant, pure episome or both meanwhile all lesions containing

HPV 18 showed pure integrated forms (Badaracco *et al.*, 2002). Nevertheless, the complete HPV 18 integration indicates a different behaviour in genital transformation compared to HPV 16 and may reflect a major aggressiveness of this viral type. It is suggested that HPV 18 causes a greater chromosome instability compared to HPV 16 that may in turn explain the major pathogenetic role of HPV 18 in term of aggressiveness (Shima *et al.*, 2000).

Table 2.1 Classification of HPV types. Summarised from Bathula *et al.*, (2015).

Group	**HPV type**
High-risk	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82
Probable high- risk	26, 53, 66
Low-risk	6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81
Undetermined risk	34, 57, 83

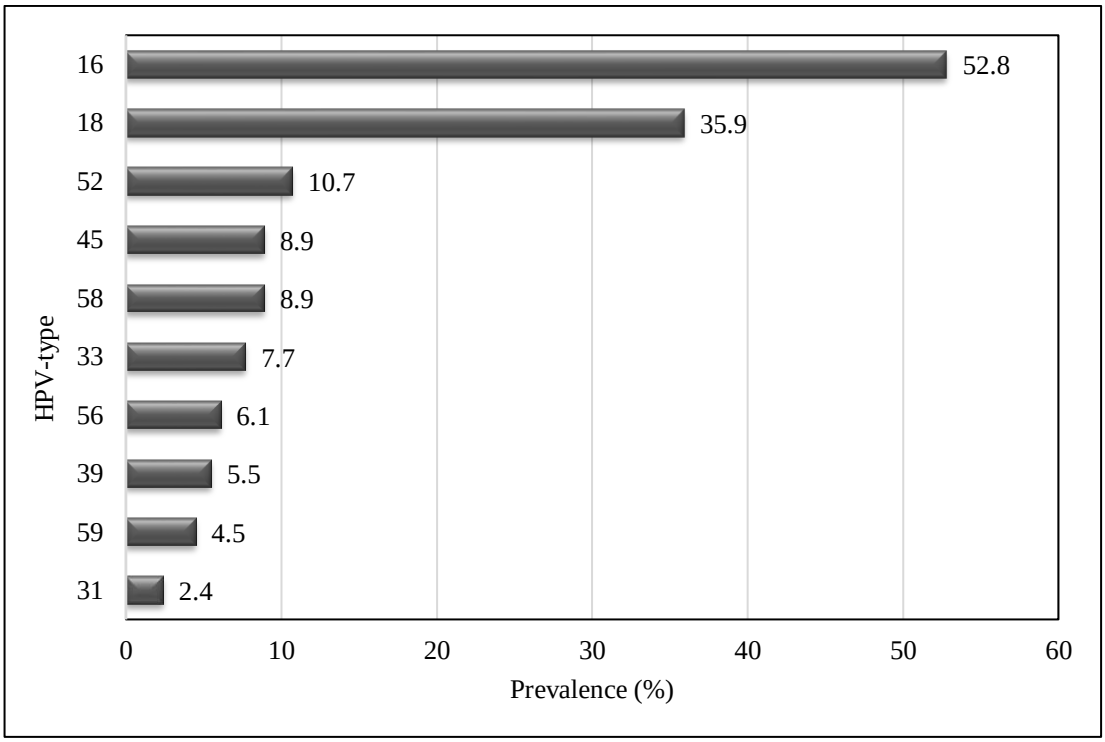


Figure 2.6 Distribution of HPV genotypes reported throughout the world in cervical cancer cases. Modified from de Martel *et al.*, (2017).

2.2.1(a) Genetic structure of HPV

The HPV genome exists as a covalently closed circle that consists of approximately 8000 base pairs of double-stranded circular DNA associated with histones *(*Doorbar *et al.,* 2012). The genomic structure of HPV is divided into three regions depending on its expression time and their particular functions in controlling the replication of viral genetic materials (Figure 2.7). The three regions are consist of non-coding long control region (LCR), early region (E) and late region (L) (Yuan *et al.,* 2012). LCR contains p97 and p670 core promoters that include enhancer and silencer sequences that are responsible for regulating the transcription of the early and late region respectively.

Meanwhile, the early region of the HPV genome encoded for non-structural proteins (E1, E2, E4, E5, E6 and E7) is transcribed early in the infection phase and responsible for modulating keratinocyte differentiation, promoting viral evasion from the host immune system and accelerating the replication of viral DNA. (Sanjose *et al.,* 2018). The expression of early gene products is therefore a crucial factor in the malignant transformation of host cells (Kranjec and Doorbar, 2016). In the meantime, the structural proteins encoded in the late-expressed region, L1 and L2, play a central role in forming the viral capsid and assembling the virus particles. L1 is the primary structural element of the virus coat proteins containing infectious virions made up of 72 capsomeres carrying 360 protein copies (Mirkovic *et al.,* 2015). Table 2.2 summarises the general functions for each encoded HPV protein.