

**CYTOTOXICITY MECHANISM OF GALLIC
ACID AND METHYL GALLATE COMBINED
WITH CISPLATIN ON CERVICAL CANCER
(HeLa) CELLS**

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UNIVERSITI SAINS MALAYSIA

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ACID AND METHYL GALLATE COMBINED
WITH CISPLATIN ON CERVICAL CANCER
(HeLa) CELLS**

by

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LIST OF SYMBOLS

A	Absorbance
LO•	Alkoxy
cells/mL	Cells per millilitre
C	Carbon atom
Ca ²⁺	Calcium ion
CO ₂	Carbon dioxide
Cu ⁺	Cupric ions
°C	Degree celcius
Δ	Delta
dH ₂ O	Distilled water
E	Efficiency of primer
=	Equal to
Fe ²⁺	Ferrous
g	Gram
HO•	Hydroxyl radical
H ₂ O ₂	Hydrogen peroxide
HOCl	Hypochlorous acid
<	Less than
L	Litre
μg/mL	Microgram per millilitre
μM	Micromolar
mM	Milimolar
mg/mL	Milligram per millilitre

mg/kg	Milligram per kilogram
mL	Millilitre
M	Molar
>	More than
≥	More than or equal to
nm	Nanometer
O ₂	Oxygen
O ₃	Ozone
%	Percentage
LOO•	Peroxy
±	Plus minus
NaCl	Sodium chloride
O ₂ •-	Superoxide anion
H ₂ O	Water
R ²	Correlation coefficients

LIST OF ABBREVIATIONS

Akt	Ak strain transforming
AML	Acute myeloid leukemia
AQPs	Aquaporins
ASR	Age-standardized rate
APS	Ammonium persulfate
APAF	Apoptosis protease activating factor
ATCC	American Type Culture Collection
ATPase	adenosine triphosphatase
BAX	Bcl-2-associated X
BCL-2	B cell lymphoma 2
BEL-7402	Human hepatocellular carcinoma
CAS	Chemical abstracts service
CBDCA	Bidentate dicarboxylate
BMI	Body mass index
cAMP	Cyclic adenosine 3',5'-monophosphate
CGIN	Cervical glandular intraepithelial neoplasia
CI	Combination index
CIN	Cervical intraepithelial neoplasia
CIS	Cisplatin
CIS-MG	Cisplatin and methyl gallate
CIS-GA	Cisplatin and gallic acid
Ct	Threshold cycle
DCFH	Dichlorofluorescein

DCFH-DA	2',7' –dichlorofluorescein diacetate
DR	Death receptor
DISC	Death-inducing signaling complex
DMEM	Dulbecco modified eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
E	Early region
ECM	Extracellular matrix
EGCG	Epigallocatechin gallate
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
E6AP	E6-associated protein
EPR	Electron paramagnetic resonance
FADD	Fas-associated death domain
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
GA	Gallic acid
GAPDH	Glutaraldehyde phosphate dehydrogenase
GLOBOCAN	Global Cancer Observatory
GSH	Glutathione
GSSG	Glutathione disulfide
GPX	Glutathione peroxidase

H446	Human small-cell lung cancer
HeLa	Cervical cancer cells
HIV	Human immunodeficiency virus
HSIL	High-grade squamous epithelial lesion
HSV-1	Herpes Simplex Virus type 1
HPV	Human papillomavirus
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cells
IC ₅₀	Half maximal inhibitory concentration
IDV	Integrated density value
IL	Interleukin
kDa	Kilodalton
L	Late region
LCR	Long control region
LMIC	Low and middle-income countries
LO2	Normal human hepatocyte
LSIL	Low-grade squamous intraepithelial lesion
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MDCK	Madin-Darby canine kidney
MG	Methyl gallate
MGC-803	Human gastric cancer
MIC	Minimum inhibitory concentration
MMP	Matrix metalloproteinase

MOMP	Mitochondrial membrane permeabilization
MRI	Mean relative intensity
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium Bromide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NCI	National Cancer Institute
Nrf2	Nuclear factor erythroid 2-related factor 2
NF-kB	Nuclear factor-kappa B
NIH/3T3	Mouse embryonic cells
NO	Nitric oxide
NOX	Nicotinamide Adenine Dinucleotide Phosphate Oxidases
OD	Optical density
ORF	Opening reading frames
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PEITC	Phenethyl isothiocyanate
PI	Propidium iodide
PPAR- γ	Peroxisome proliferator-activated receptor gamma
pRb	Retinoblastoma tumor suppressor gene
PS	Phosphatidylserine
PVDF	Polyvinylidene difluoride
p53	Tumor suppressor protein
RIPA	Radio-Immunoprecipitation Assay
RM	Ringgit Malaysia
RNA	Ribonucleic acid

ROS	Reactive oxygen species
RT-PCR	Real-time polymerase chain reaction
SCC	squamous cell carcinoma
SCJ	Squamocolumnar junction
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
TA	Tannic acid
TAE	Tris-acetic EDTA
TEMED	Tetramethylethylenediamine
TNF- α	Tumor necrosis factor-alpha
TNFRSF	Tumor necrosis factor receptor superfamily
(TRAIL-R1/2)	Tumor necrosis factor-related apoptosis-inducing ligand
USD	United States dollar
UV	Ultraviolet
WHO	World Health Organisation
XIAP	X-linked inhibitor of apoptosis protein

**MEKANISME SITOTOKSISITI KOMBINASI ASID GALIK DAN METIL
GALAT DENGAN CISPLATIN KE ATAS TITISAN SEL KANSER SERVIKS
(HeLa)**

ABSTRAK

Kanser serviks merupakan kanser yang keempat paling kerap dihadapi oleh wanita di seluruh dunia. Cisplatin merupakan antara ubatan kemoterapi utama yang digunakan dalam rawatan kanser serviks. Walaubagaimanapun, cisplatin dilaporkan toksik kepada sel normal dan membentuk kerintangan sel di samping kesan sampingan yang lain. Kombinasi agen kemoterapi merupakan salah satu strategi bagi meningkatkan keberkesanan dadah antikanser melalui kesan sinergistik. Asid galik dan metil galat merupakan sebatian fenolik yang dilaporkan mempunyai aktiviti antioksidan dan antikanser yang bagus. Jadi dalam kajian ini, asid galik dan metil galat telah dipilih sebagai bahan kombinasi dengan cisplatin. Kajian ini dijalankan untuk menjelaskan mekanisme sitotoksisiti kombinasi asid galik dan metil galat dengan cisplatin terhadap kanser serviks (HeLa) menerusi mod kematian secara apoptosis. Selain itu, kapasiti antioksidan sel yang dirawat dengan rawatan tunggal dan kombinasi turut dinilai. Aktiviti sitotoksisiti asid galik, metil galat dan cisplatin ke atas sel HeLa dan NIH/ 3T3 ditentukan melalui asai MTT. Kesan kombinasi asid galik dan metil galat dengan cisplatin kemudian ditentukan dengan perisian CompuSyn. Morfologi dan peratusan sel apoptotik dinilai menggunakan pewarnaan Hoechst dan asai annexin V/PI. Pengekspresan protein apoptotik dan anti-apoptotik (Bax, Bcl-2, caspase-3, caspase-9 dan p53) ditentukan dengan analisis western blot. Kesan perencatan migrasi oleh kombinasi juga ditentukan melalui asai penyembuhan luka. Aktiviti antioksidan asid galik dan metil galat diukur menggunakan asai DPPH.

Spesies oksigen reaktif (ROS) dan aktiviti enzim antioksidan diukur secara spektrofotometri manakala pengepresan gen antioksidan (*SOD1* and *hCAT*) dalam sel HeLa yang dirawat kemudian dinilai menggunakan RT-PCR. Asid galik dan metil galat menunjukkan kesan sitotoksiti yang kuat terhadap sel HeLa. Nilai IC_{50} bagi asid galik, metil galat dan cisplatin ke atas sel HeLa adalah masing-masing 13.44 $\mu\text{g/mL}$, 16.55 $\mu\text{g/mL}$ and 8.04 $\mu\text{g/mL}$ manakala bagi sel NIH/3T3 adalah 32.90 $\mu\text{g/mL}$, 35.70 $\mu\text{g/mL}$ and 6.57 $\mu\text{g/mL}$. Kombinasi asid galik dan metil galat dengan cisplatin telah merencat proliferasi sel HeLa dengan lebih baik berbanding rawatan cisplatin sahaja dengan kesan sinergistik diperhatikan dalam kombinasi dengan cisplatin pada kepekatan 0.51-4.02 $\mu\text{g/mL}$. Pemerhatian morfologi bagi pewarnaan Hoechst menunjukkan wujud beberapa ciri apoptosis pada semua sel yang dirawat. Secara konsisten, analisis flowsitometri menunjukkan peratusan sel apoptotik awal bagi kombinasi cisplatin-asid galik (28.72 ± 1.14) and cisplatin-metil galat (23.37 ± 9.72) adalah lebih tinggi secara signifikan dari kumpulan kawalan (6.00 ± 0.95). Rawatan kombinasi juga secara signifikan meningkatkan pengepresan Bax, caspase-3, caspase-9 dan p53 serta menurunkan Bcl-2 berbanding dengan kumpulan kawalan. Selain itu, rawatan ini telah menunjukkan kesan perencatan migrasi selepas 24 jam. Asid galik dan metil galat menunjukkan aktiviti antioksidan yang kuat dengan nilai EC_{50} masing-masing adalah 18.23 μM dan 19.39 μM . Rawatan kombinasi secara signifikan meningkatkan aras ROS dan menurunkan aras SOD dan katalase dalam asai enzimatik. Keputusan ini adalah konsisten dengan hasil penemuan RT-PCR yang menunjukkan penurunan pengepresan gen *SOD1* and *hCAT* dalam semua sel yang dirawat. Kesimpulannya, hasil kajian ini mencadangkan bahawa kombinasi asid galik dan metil galat dengan cisplatin secara sinergistik merencat proliferasi melalui aruhan apoptosis dan ROS dalam sel kanser serviks.

**CYTOTOXICITY MECHANISM OF GALLIC ACID AND METHYL
GALLATE COMBINED WITH CISPLATIN ON CERVICAL CANCER
(HeLa) CELLS**

ABSTRACT

Cervical cancer is the fourth most common cause of cancer-related death affecting women worldwide. Cisplatin is one of the chemotherapy drugs used in the treatment of cervical cancer. However, cisplatin was reported to cause toxicity to normal cells and develop cell resistance with other side effects. Combining chemotherapy agents is one of the strategies to increase the effectiveness of anticancer drugs through synergistic effects. Gallic acid and methyl gallate are the most abundant phenolic compounds that have been reported to have good antioxidant and anticancer activities. Hence, in this study, gallic acid and methyl gallate were selected as combination substances with cisplatin. This study was conducted to elucidate the cytotoxicity mechanism of gallic acid and methyl gallate combined with cisplatin on cervical cancer (HeLa) cells through apoptosis mode of cell death. Furthermore, the antioxidant capacity of the cells treated with single and combination treatment was also evaluated. Cytotoxicity activity of gallic acid, methyl gallate and cisplatin on HeLa and NIH/ 3T3 cells was determined using MTT assay. The effects of gallic acid and methyl gallate combined with cisplatin were then determined using CompuSyn software. The morphology and percentage of apoptotic cells were evaluated using Hoechst staining and annexin V/PI assay. The expression of apoptotic and anti-apoptotic proteins (Bax, Bcl-2, caspase-3, caspase-9 and p53) was further determined by western blot analysis. The migration inhibitory effect of the combinations was also evaluated using scratch wound healing assay. The antioxidant activity of gallic acid

and methyl gallate was measured using DPPH assay. Reactive oxygen species (ROS) and antioxidant enzyme activity were measured spectrophotometrically while the expression of antioxidant genes (*SOD1* and *hCAT*) in HeLa-treated cells was then evaluated using RT-PCR. Gallic acid and methyl gallate showed strong cytotoxicity effects on HeLa cells. The IC₅₀ values of gallic acid, methyl gallate and cisplatin on HeLa cells were 13.44 µg/mL, 16.55 µg/mL and 8.04 µg/mL whereas in NIH/3T3 cells were 32.90 µg/mL, 35.70 µg/mL and 6.57 µg/mL respectively. Gallic acid and methyl gallate in combination with cisplatin inhibited greater HeLa cell proliferation than cisplatin alone with synergistic effects seen in combination with cisplatin at concentrations of 0.51-4.02 µg/mL. Morphological observation of Hoechst staining then revealed the appearance of several apoptotic features in all treated cells. Consistently, flowcytometry analysis showed that the percentages of early apoptotic cells in the combination of cisplatin-gallic acid (28.72 ± 1.14) and cisplatin-methyl gallate (23.37 ± 9.72) groups were significantly higher than the control group (6.00 ± 0.95). The combination treatments significantly upregulated Bax, caspase-3, caspase-9 and p53 expressions and downregulated bcl-2 expressions as compared to the untreated group. Moreover, the treatments were shown to have migration-inhibitory effects after 24 hours. Gallic acid and methyl gallate exhibited strong antioxidant activity with EC₅₀ values of 18.23 µM and 19.39 µM respectively. The combination treatments significantly increased intracellular ROS levels and reduced the level of SOD and catalase in an enzymatic assay. This result was consistent with RT-PCR result that showed the downregulation of *SOD1* and *hCAT* genes in all treated samples. In conclusion, these findings suggest that the combination of gallic acid and methyl gallate with cisplatin synergistically inhibited proliferation by inducing apoptosis and ROS in cervical cancer cells.

CHAPTER 1

INTRODUCTION

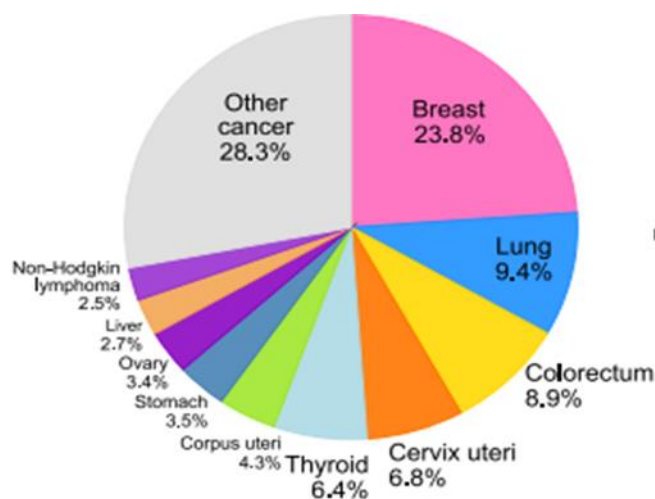
1.1 Background of the study

Cancer has now become one of the death-leading causes worldwide. According to the Global Cancer Observatory (GLOBOCAN) 2022, cancer was responsible for 9.7 million deaths globally. It is estimated that 1 in 6 deaths is due to cancer. It has caused major health problems and economic burdens, especially for less developed countries. The cancer incidence is rapidly growing globally with approximately 20 million new cases diagnosed in 2022. Generally, in both sexes, lung cancer is the most frequently diagnosed cancer (12.4% of total cases) followed by breast cancer (11.6%) and colorectum cancer (9.6%). Apart from that, cervical cancer is the fourth most frequent cancer and also ranks as the fourth highest cause of death in women globally with an estimated 660,000 (6.8%) new cases and 350,000 (8.1%) deaths in 2022 (Figure 1.1) (Bray et al., 2024).

The majority of the incidence and mortality cases due to cervical cancer are in Sub-Saharan Africa and South-Eastern Asia. In this regard, the African region represents the highest number of cases, with the increasing rates seen in Eastern Africa (Malawi and Zimbabwe), Southern Africa (Swaziland) and Western Africa (Guinea, Burkina Faso and Mali). The highest rates are recorded in Swaziland and Malawi. In the Southeast Asia region, cervical cancer ranks as the second most common type of cancer among females. In fact, in low and middle-income countries (LMIC), cervical cancer has become one of the causes of mortality related to cancer among women

(Shrestha et al. 2018). In 2015, the mortality rate from cervical cancer was 18 times higher in middle and low-income countries than in developed countries, accounting for an estimated 90% of the 270,000 fatalities from the disease (Cohen et al. 2019).

A)



B)

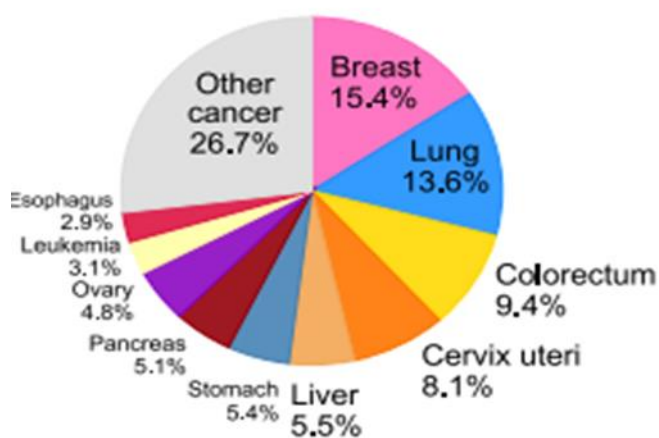


Figure 1.1 Distribution of incidence cases (A) and mortality (B) for the 10 most common cancers in women worldwide. Adapted from Bray et al. (2024).

In Malaysia, the prevalence of cervical cancer is also notably high, as it is the third most common cancer among women. Approximately 3,981 cervical cancer cases had been diagnosed in women from 2012 to 2016. According to the National Cancer Registry (2019), the age-standardized rate (ASR) for cervical cancer in females is 6.2 per 100,000. The incidence and mortality rates of this cancer are low among young women under the age of 30, increase after 30 and reach their peak between the ages of 70 to 75 (Figure 1.2). By ethnicity, Chinese women had the highest ASR (6.8/100000) followed by Indian women (5.5/100000) and Malay women (4.6/100000) (Figure 1.3) (Azizah et al. 2019).

Cervical cancer cases recorded an average of 2000 to 3000 hospital admissions per year, with an annual death rate of 5.6 per 100,000, as reported by The Ministry of Health Malaysia. This statistic shows that the mortality rate in Malaysia is more than twice as high as in Finland, United Kingdom and the Netherlands. Despite being preventable, especially with many screening and vaccination programs available, the mortality rate due to cervical cancer remains high in Malaysia. The economic burden of this disease is also high as it costs about RM312 million (USD76 million) annually for the cervical cancer management (Mustafa et al. 2022).

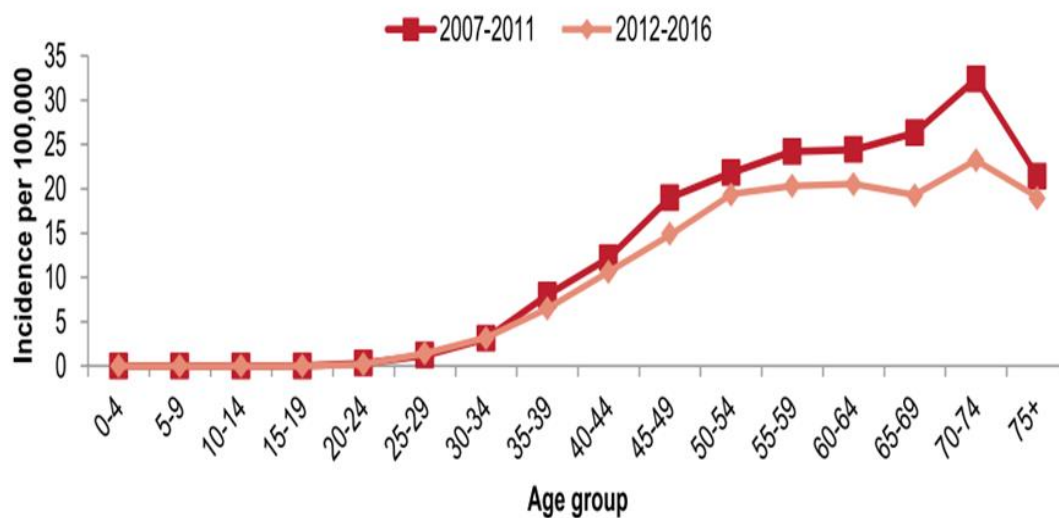


Figure 1.2 The age-specific incidence rate for cervical cancer in Malaysia from 2012-2016. Adapted from Azizah et al. (2019).

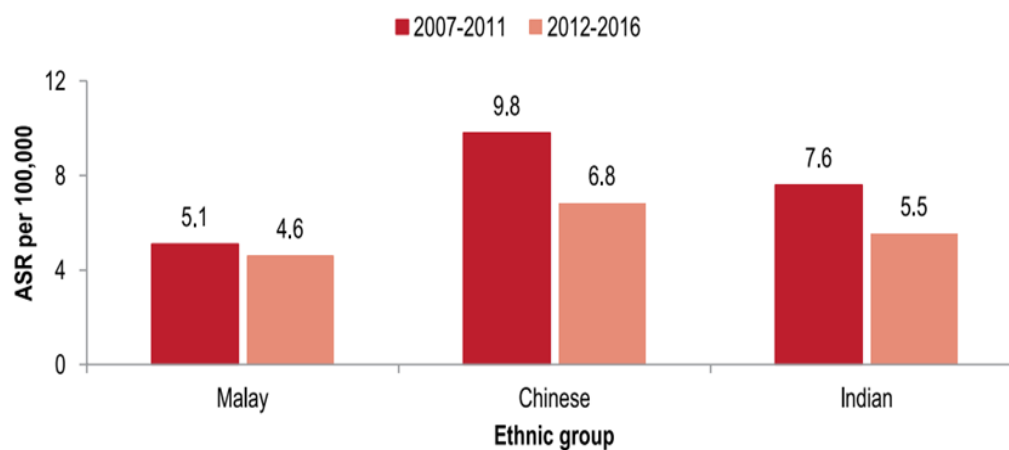


Figure 1.3 The age-standardised rate (ASR) of cervical cancer by ethnicity in Malaysia from 2007-2011 to 2012-2016. Adapted from Azizah et al. (2019).

There are several treatments available for cervical cancer including surgery, radiotherapy, chemotherapy and targeted therapy. Chemotherapy is one of the cancer treatments that uses drugs to kill the cancer cells. It works by stopping and slowing the cancer growth. Despite its effectiveness on cancer cells, chemotherapy unfortunately causes many undesired side effects to the patients. It does not only kill cancer cells but also kills the surrounding healthy cells. Currently, chemotherapy is frequently given as a combination of drugs rather than a single drug because different drugs effectively kill cancer cells using distinct mechanisms (National Cancer Institute United States, 2021). Cisplatin (CIS), a platinum-based drug, is the most active anticancer agent and is widely used to treat different types of cancers. Nevertheless, the development of chemoresistance and numerous side effects such as severe renal damage, hearing loss, allergic reactions, decreased immune system, gastrointestinal disorders, and haemorrhage have become its major limitations. Thus, CIS and its combination with oxaliplatin, carboplatin and other chemotherapeutic drugs, have commonly been used in cancer treatment (Dasari & Bernard, 2014). The combination of anticancer drugs and other agents also might have the potential to alleviate metastasis.

Metastasis is the major cause of cancer-related deaths rather than primary tumours. The lungs, followed by bones are the most frequent site for cervical cancer metastases. Each single-site metastasis has a similar prognosis. Nevertheless, patients with single-site metastatic tumours have a better prognosis than those with multi-site metastatic tumours (Zhou & Peng, 2020). Metastasis occurs when tumour cells move from the initial site to form new tumours in other body areas, leading to organ failure. The metastatic cascade begins with invasion, which occurs when tumour cells pierce the basement membrane and travel through the extracellular matrix (ECM) into

surrounding tissue (Novikov et al., 2021). Tumour cells must develop malignant phenotypes to separate from the primary tumour before passing through the basal membrane, penetrating the ECM, and entering the circulation (Wu et al., 2021). In this regard, inhibiting cell migration can be a therapeutic approach to prevent cancer metastases. Drugs that prevent cancer cells from invading through the ECM and forming secondary tumours should be used in conjunction with treatment for solid cancer. Drug development efforts should also be divided into antiproliferative approaches and those that focus on processes related to motility, migration and/or invasion, and metastasis because of the different mechanisms that underlie cell proliferation, movement, and invasion (Gandalovičová et al., 2017). In this context, drug development was reported to rely mainly on natural products as one of the sources of antioxidants.

Antioxidants are substances that interact with free radicals and neutralize them to prevent them from causing harm. It is believed that antioxidant compounds can be one of the sources of anticancer drugs to counteract free radicals. Several previous studies also reported that natural and dietary antioxidants possess anticancer properties (Widowati et al., 2013; Wang et al., 2016). However, the efficacy of antioxidant supplements for cancer prevention has become a controversial issue since many clinical evaluations of antioxidant supplements among cancer patients showed unsatisfactory results. Some studies suggested that antioxidant supplementation during chemotherapy gives benefits while some studies reported that it is harmful to the patients. It has been shown that regular consumption of vitamin C and vitamin E throughout the time after breast cancer diagnosis has been linked to a lower chance of recurrence. On the other hand, regular use of a combination of carotenoids among

breast cancer patients was linked to a higher risk of death (Greenlee et al., 2012). Moreover, a study by Jung et al., (2019) found that postmenopausal breast cancer survivors who took antioxidants simultaneously during radiation therapy and/or chemotherapy have poor prognosis, hence proposing the use of antioxidants with caution during the treatment.

Antioxidants are important to counter-balance the free radical levels in the human body. Free radicals at moderate concentration play a major role in signalling processes as regulatory mediators. However, at high concentrations, the free radicals and their derivatives, especially reactive oxygen species (ROS) are dangerous for living species and can cause harm to all major cellular components. A high and/or persistent increase in ROS generation has been linked to the pathogenesis of cancers and various human diseases (Phaniendra et al., 2015). Excessive ROS, in an oxidative stress state, causes damage to lipids and proteins, contributing to chromosomal instability that promotes tumour formation. ROS also increases the cancer risk by interfering with various physiological processes such as cell division, protein synthesis and signal transduction (Didier et al., 2023).

For decades, natural products containing high antioxidants have become a great source of new medication development for several illnesses. Furthermore, many researchers have paid great attention to natural products due to their effectiveness in combating various diseases and lesser side effects. In cancer research, it has been proven that natural products have shown anti-cancer properties with minimal side effects. They have been clinically used for cancer treatment since there is a significant number of anti-cancer drugs from natural origin have been recognized. The discovery

of new anti-cancer drugs keeps increasing as more compounds derived from natural products have been discovered and tested for their anticancer properties. Despite the effectiveness of conventional chemotherapy drugs, the existence of dose-limiting side effects and drug resistance have become their limitation. Hence, combining chemotherapeutic drugs with natural compounds is considered a new promising cancer therapeutic strategy (Kojima-Yuasa et al., 2015). Due to these reasons, many researchers have recently been interested in exploring more phytochemical substances that have the potential to be combined with the current chemotherapeutics drugs to develop as new anticancer agents. It has been suggested that combining chemotherapeutic drugs with natural compounds may increase antitumor activity through synergistic reactions while simultaneously compensating for the adverse effects. The lower drug dosage used in the combination treatment may also reduce the systemic toxicity caused by chemotherapies (Sarkar & Li, 2006).

Two natural compounds, gallic acid (GA) and its methyl ester, methyl gallate (MG) are the common phenolic acids in various herbs and plants. GA and MG are recognized as strong antioxidant compounds as numerous studies have previously reported their antioxidant activity (Badhani et al., 2015). To date, there are various pharmacological activities of GA and MG have been discovered, including their antimicrobial and antifungal (Karamać et al., 2005), cardioprotective, anti-inflammatory, neuroprotective and gastroprotective effects (Kahkeshani et al., 2019). However, the most attention to GA and MG is gained due to their antitumour property. They have shown good antiproliferative activity on several cell lines including cervical cancer (HeLa) cells.

Generally, in this study, GA and MG in combination with CIS were investigated for their cytotoxicity and migration effects on HeLa cells. The apoptosis mechanism involved including apoptotic and anti-apoptotic proteins, and related antioxidant enzyme levels in HeLa-treated cells by single and combination compounds were evaluated through several *in-vitro* experimental procedures to postulate their potential role as anticancer agents for cervical cancer.

1.2 Rationale of the study

Cervical cancer is a malignancy of the cervical cells that develops slowly over time. Despite the available screening programs and advanced treatment, cervical cancer remains one of the leading causes of cancer-related death in women mostly in developing countries. Apart from surgery, immunotherapy and radiotherapy, one available cancer treatment is chemotherapeutic drugs such as CIS, carboplatin, paclitaxel and topotecan. Although these agents are generally effective, they also present serious side effects and develop chemoresistance by the cells. These undesired side effects may cause poor prognosis and affect patients' quality of life. Alternatively, the active substances from natural sources with high antioxidant properties are good candidates for exploring their anticancer effects. Numerous studies have recently reported the anti-cancer properties of antioxidant compounds. In this context, antioxidants can have beneficial effects on cancer patients by reducing or preventing harmful side effects caused by ROS (Yasueda et al., 2016).

To date, the efficacy of antioxidant supplementation in cancer treatment remains unsatisfactory and has become controversial among health practitioners. The

action of some chemotherapeutic drugs involves the generation of ROS, which contradicts the mechanism of antioxidants. Hence, it is worried that treatment with antioxidant compounds combined with chemotherapeutic drugs will antagonize or inhibit its antiproliferative effect. Besides, many oncologists today recommend to their patients to avoid herbs and supplements with antioxidant properties during chemotherapy treatment as they will interfere with the efficacy of the anticancer agents. Conversely, many integrative practitioners suggest consuming antioxidant supplements for the patients, to tolerate the high doses of chemotherapeutic drugs in order to get better effects and thus increase the survival rate (Singh et al., 2018). Therefore, it is necessary to clarify whether the use of antioxidants as cancer therapy agents may interfere with or suppress the chemotherapy effect.

Earlier research from Abdullah et al., (2023) revealed that GA and MG isolated from *Quercus infectoria* ethyl acetate extract exhibited good anticancer activity towards cervical cancer (HeLa) cells. This finding provides a new insight into the potential of GA and MG to be explored to postulate their anticancer effect on cervical cancer cells. Combination chemotherapy is one of the strategies to increase the efficacy of anticancer drugs from the different reactions of the combination compounds produced. The synergistic effect of the combination may enhance the tumour's responses to the treatment, thereby overcoming the side effects by lowering the drug dosage. Hence, combining GA and MG, which have high antioxidant capacity with CIS is a strategy proposed in this study to enhance the cytotoxicity effect on HeLa cells.

To the best of our knowledge, there has been no study regarding the combination of GA and MG with CIS, particularly towards HeLa cells. Thus, this study was conducted to evaluate the cytotoxicity effect of GA and MG combined with CIS on HeLa cells. This present study was also carried out to elucidate the mechanism of cell death in the HeLa-treated cells associated with reactive oxygen species and antioxidant enzyme levels.

1.2.1 Research questions

1. Do GA and MG have cytotoxicity effects on HeLa cells?
2. Are there any differences between single and combination treatments on HeLa cell growth inhibition?
3. Do the single and combination treatments inhibit migration and induce apoptosis in HeLa-treated cells?
4. How do single and combination treatments affect the apoptotic and tumour suppressor protein expression levels in HeLa-treated cells?
5. How do single and combination treatments affect intracellular ROS and antioxidant enzyme levels, and the expression of antioxidants genes (SOD and catalase) in HeLa cells treated cells?

1.2.2 Hypotheses of study

The hypotheses of the study are:

1. GA and MG exhibit cytotoxicity effects on HeLa cells.
2. Combination treatments of GA and MG with CIS are expected to inhibit more HeLa cell proliferation than single treatments.
3. Combination treatments are expected to inhibit migration and induce apoptosis in HeLa cells through apoptotic features and a high percentage of apoptotic cells.
4. Single and combination treatments are expected to upregulate the expression of apoptotic proteins (Bax, caspase-3, caspase-9) and tumour suppressor protein (p53), and downregulate Bcl-2 expression in HeLa cells.
5. Single and combination treatments are expected to increase ROS levels, decrease antioxidant enzyme levels and inhibit the expression of antioxidant genes in HeLa cells.

1.3 General and specific objectives

1.3.1 General objective

To elucidate the cytotoxicity mechanism of gallic acid (GA) and methyl gallate (MG) combined with cisplatin (CIS) on cervical cancer (HeLa) cells.

1.3.2 Specific objectives

- i. To determine the cytotoxicity effects of GA and MG on cervical cancer (HeLa) cells.
- ii. To assess the combination effect of GA and MG with CIS (CIS-GA and CIS-MG) towards HeLa cells.

- iii. To evaluate anti-migratory and apoptotic effects in HeLa cells treated with single and combination treatments.
- iv. To investigate the expression of apoptotic proteins (Bax, Bcl-2, caspase-3, caspase-9) and tumour suppressor protein (p53) in HeLa cells treated with single and combination treatments.
- v. To measure the intracellular ROS and antioxidant enzyme levels, and the expression of antioxidants genes (SOD and catalase) in HeLa cells treated with single and combination treatments.

1.4 Research framework

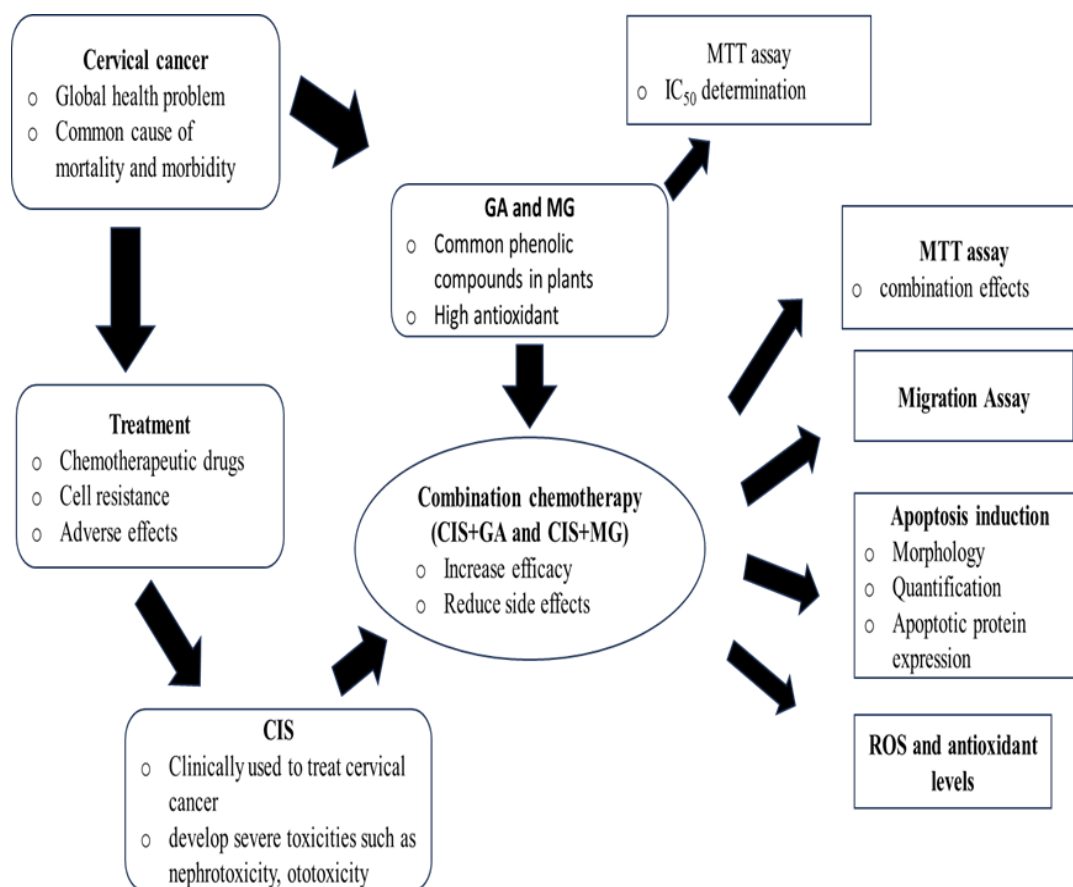


Figure 1.4 Research framework of the study.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

Cancer is an uncontrolled growth of abnormal cells that can spread or invade other cells. In regulating the human body's homeostasis, healthy cells normally divide and proliferate to replace the dead cells. Cancer develops when the body's normal mechanism stops working thus the cell grows abnormally out of control to form tumours (Raj et al., 2021). A tumour can be classified as benign or malignant. A benign tumour does not spread or invade other body sites. In contrast, malignant tumours invade the nearby healthy tissue and metastasis throughout the body through circulatory or lymphatic systems. Hence, only malignant tumours are appropriately classified as cancer. Besides, benign tumours are easily removed by surgery compared to malignant tumours that have been metastasized making them resistant to localized treatment (Cooper, 2000). There are three major stages in cancer; the initiation phase, followed by the promotion and cancer progression. Several factors may contribute to uncontrolled cell proliferation including gene mutations, gene hypermethylation, silencing of certain genes, overexpression and post-translational alteration in the proteins (Kumar et al., 2016).

2.2 Cervical cancer

Globally, cervical cancer is one of the leading causes of morbidity and mortality among women. The majority of cervical cancer cases occur due to human papillomavirus (HPV) infection (Okunade, 2020). Cervical cancer originates initially in the cervix. The cervix is the organ that joins the uterine cavity and vagina. It is a 2-3 cm long cylindrical shape composed of stroma and epithelium. The cervix is classed into two regions; the ectocervix and the endocervical canal as shown in Figure 2.1. The ectocervix is the lower part of the cervix and is covered by stratified squamous epithelium. While the other part, the endocervical canal is lined by columnar epithelium that connects the uterine cavity and the vagina lumen through the internal and external cervical orifices. The squamocolumnar junction (SCJ) is the area in which squamous and glandular epithelium join (Devine et al., 2019). The cervical transformation zone is the region of metaplastic tissue that lies between the glandular tissue of the endocervical canal and the squamous epithelium of the vagina. The cervical transition zone is more susceptible to carcinogenesis even though HPV can infect the entire anogenital epithelium (Schiffman & Wentzensen, 2013).

Adenocarcinoma and squamous cell carcinoma (SCC) are the two most prevalent forms of cervical cancer. SCC arises from the precursor cervical intraepithelial neoplasia (CIN) in squamous cells and accounts for 85% of all cervical cancers while adenocarcinomas that arise from the precursor cervical glandular intraepithelial neoplasia (CGIN) in glandular mucus-producing cells, are less common with nearly 15% (Kotsopoulos et al., 2020).

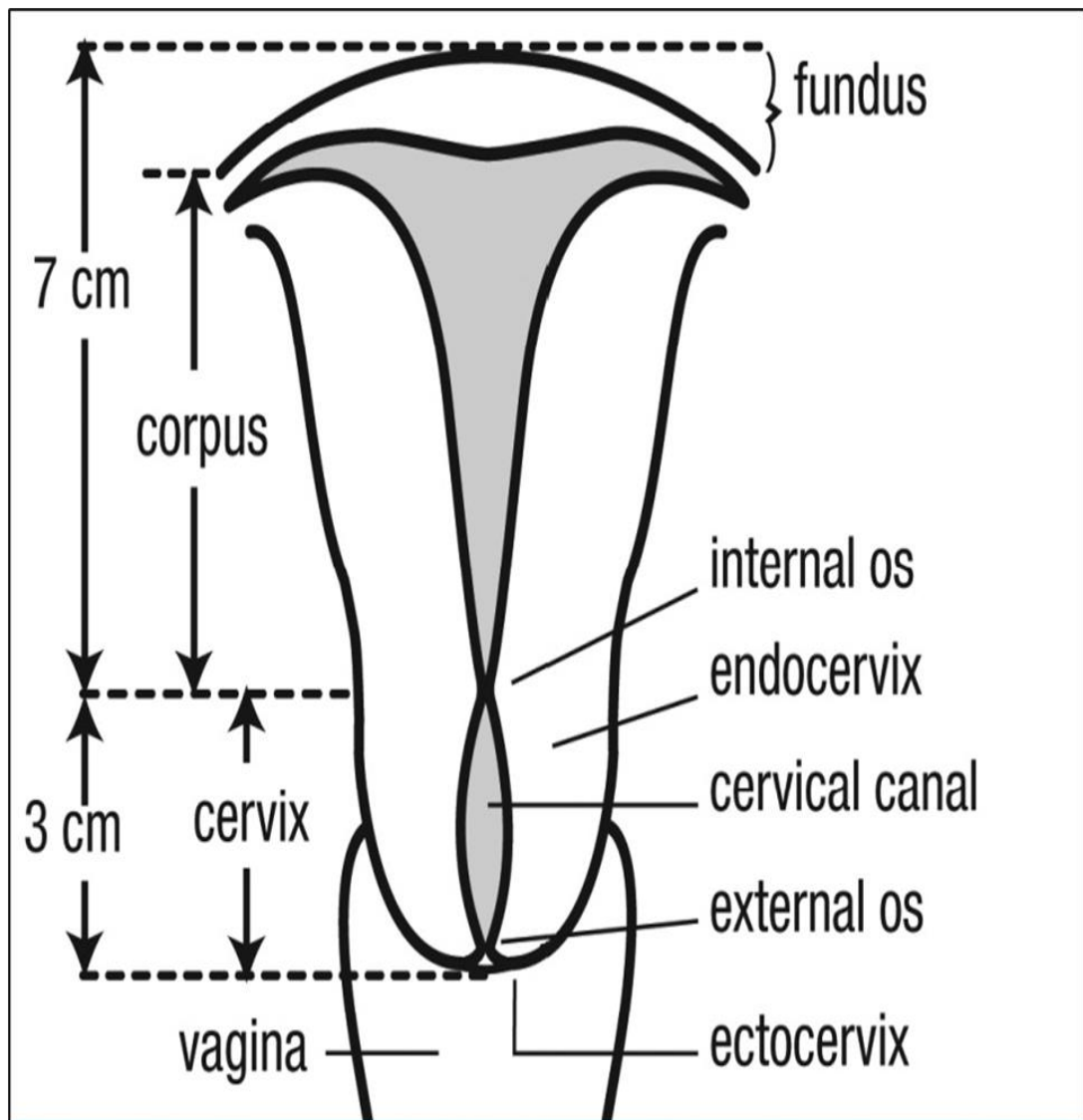


Figure 2.1 Cervix of a woman (World Health Organization, 2014).

The development of cervical cancer generally occurs in 4 steps; metaplastic epithelium infection at the cervical transformation zone, virus persistence, cervical precancer formation from the infected epithelium, and cancer invasion through the epithelium basement membrane. There are also backward steps, namely clearance of HPV infection and the regression of precancer to normal (Schiffman & Wentzensen, 2013). Cervical cancer develops continuously from cervical intraepithelial neoplasia (CIN), a non-invasive precursor lesion form to early invasive cancer and then progresses to the more established cancer. These development steps involve a long and reversible precancerous lesion phase. The progression of early invasive cancer from CIN or the development of cervical cancer can be prevented if there is early detection of CIN and the precancerous lesion stage is properly treated (Husaiyin et al., 2018).

Basically, CIN is classified into three groups (CIN 1-CIN3) according to the level of differentiation of the cervical squamous epithelium. CIN 1, represents mild dysplasia; CIN 2 is characterized by moderate dysplasia; and CIN 3 called as carcinoma in situ, is described as severe dysplasia that may develop into an invasive squamous cell carcinoma (Silva et al., 2018). Currently, the classification of intraepithelial lesions has been modified by the WHO histological classification of 2014 into two grades; Low-Grade CIN that corresponds to low-grade squamous intraepithelial lesion (LSIL) or CIN1 and High-Grade CIN which corresponds to high-grade squamous epithelial lesion (HSIL) or CIN2 and CIN3 (Shiraz & Majmudar, 2017).

2.2.1 Risk factors

Cervical cancer occurs due to several risk factors. The most common aetiological factor for cervical cancer is persistent human papillomavirus (HPV) infection that is acquired through sexual activity. The association between cervical cancer development and HPV infection has been very well established. The detection of high-risk HPV deoxyribonucleic acid (DNA) proves this association in 99.7% of cervical cancer specimens (Chan et al., 2019). In addition to HPV infection, cervical cancer incidence is strongly related to sexual behaviour. The age of the first sexual intercourse and the number of sexual partners are the common factors for cervical cancer development. Engaging in their first sexual activity before the age of 18 was associated with a twofold higher chance of acquiring cervical cancer than those after the age of 21. Additionally, compared to having sex with one partner, the risk of developing cancer doubles when having sex with two partners and triples with six or more partners (Johnson et al., 2019).

Cigarette smoking is another factor that poses a significant risk of cervical cancer. Cigarette smoking, both active and passive, has been linked to an increased risk of cervical cancer. Current and past smokers are thought to have a two to three times higher risk of developing invasive cancer among women with HPV (Mustafa et al., 2016). The cervical mucus or epithelium of smokers has shown the presence of polycyclic aromatic hydrocarbons and tobacco-specific carcinogens that can react to and cause cellular DNA damage. These compounds could act with HPV to cause cancerous transformation (Strander, 2008).

Additionally, women who have prolonged use of oral contraceptives also have a high tendency to develop cervical cancer. Accordingly, the risk of invasive carcinoma is three times higher among women who have used oral contraceptives for five to nine years, while those who have used them for more than 10 years have four times the risk. Another risk factor that contributes to this cancer is multiple pregnancies. It has been reported that the risk of cervical cancer is roughly four times higher for women with HPV infection who had seven or more full-term pregnancies, compared to two times higher for those who had one or two full-term births (Mustafa et al., 2016). Besides, obesity also might increase the risk of cervical carcinoma and is an important factor in cervical adenocarcinoma incidence. It is believed that this factor is strongly associated with hormonal risk factors. It was discovered that women who are overweight (BMI ≥ 25) and obese (BMI ≥ 30) have a two-fold increased chance of getting cervix adenocarcinoma compared to other women (Momenimovahed & Salehiniya, 2017). An immunosuppressive condition such as patients treated with immunosuppressive drugs and those who are human immunodeficiency virus (HIV)-positive also increases the risk of developing cervical cancer (Kessler, 2017).

2.2.2 Basic virology of HPV as a causative agent

HPV belongs to the *Papillomaviridae* family. It is a small and non-capsulated virus. The genome of HPV is comprised of double-stranded DNA with an approximate size of 8000 base pairs (Wang et al., 2020). It consists of three regions; early region (E), late region (L) and upstream regulatory region or also known as long control region (LCR) (Figure 2.2). The early region consists of several opening reading frames (ORFs) that encode the replication proteins (E1, E2 and E4) and the oncoproteins (E5,

E6 and E7). The major (L1) and minor (L2) viral capsid proteins are encoded by the late genes (L1 and L2) found in the late region meanwhile the long control region (LCR) is involved in regulating viral DNA replication and transcriptional regulatory elements (Narisawa-Saito & Kiyono, 2007).

To date, more than 200 HPVs have been discovered with an estimated more than 40 types colonizing the genital tract. Generally, HPVs are classified into low-risk and high-risk types according to their carcinogenic characteristics. High-risk types include 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 68, and 59 while 53, 66, 70, 73, and 82 are classified as potential high-risk types (Chan et al., 2019). Among these, HPV types 16 and 18 are considered the most virulent high-risk genotypes that account for 70% of the overall prevalence (Muñoz et al., 2003). Low-risk types include 6, 11, 42, 43, and 44 which are also occasionally found in cervical carcinomas (Okunade, 2020).

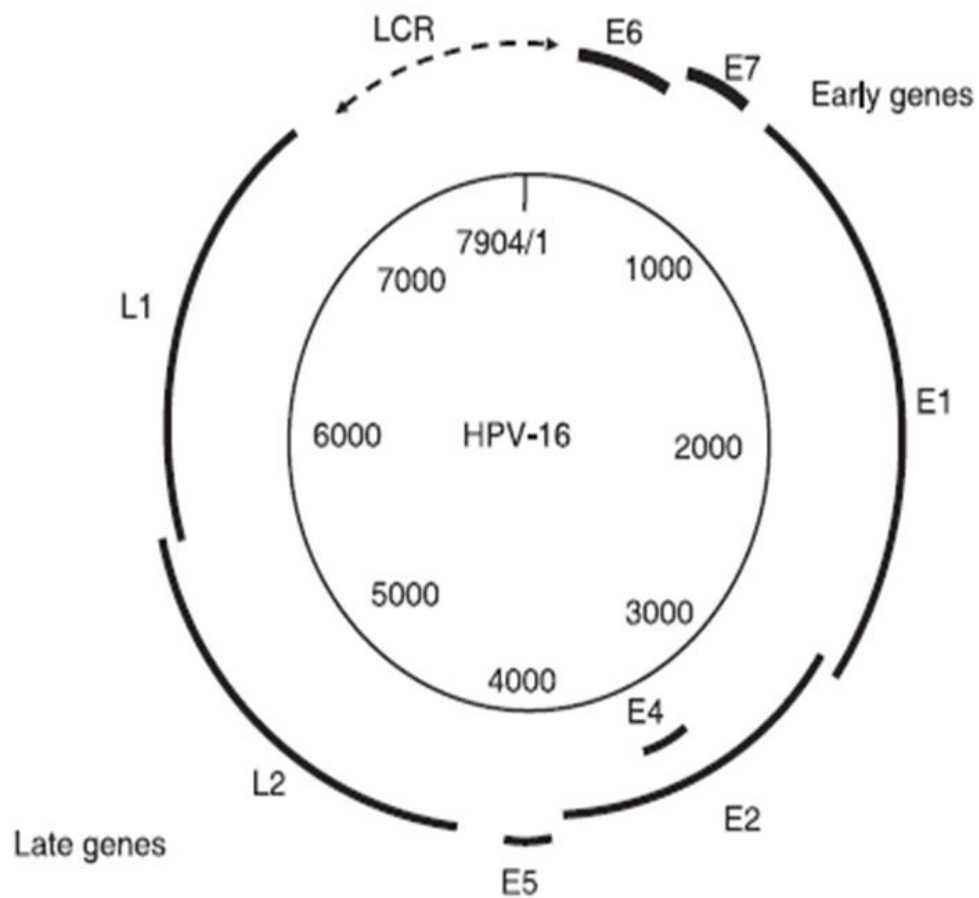


Figure 2.2 The genome structure of human papillomavirus (HPV) consists of early (E1, E2, E4, E5, E6, E7) region and late (L1, L2) region. Adapted from Narisawa-Saito & Kiyono, (2007).

2.2.3 Pathogenesis of cervical cancer

Sexual contact with an infected partner might expose women to HPV. HPV infection begins when the viruses penetrate the basal epithelial cells through the epithelial lesion. Following infection, the productive lifecycle exhibits expression of the early HPV genes (E1, E2, E4, E5, E6, and E7). Progeny viral particles are created after the replication of the viral genome in the higher layers of epithelium and the late

HPV genes (L1 and L2) and E4 are expressed (Wang et al., 2020). Viral replication within infected cells is regulated by E1 and E2 expression, which then triggers the expression of additional early-stage proteins. At the same time, E5, E6 and E7 oncoproteins contribute to cell survival and uncontrolled proliferation (Almeida et al., 2019). The viral late genes L1 and L2 are transcribed as the infected basal cells migrate up and differentiate, initiating the vegetative stage of the life cycle, marked by a significant amplification of the genome. It shows that the host cell's level of differentiation may influence the expression of late genes. The newly generated viral DNA is encapsulated to create new virions, which are released once the cell reaches the outermost layer of the epithelium. At this point, the life cycle is repeated (Wang et al., 2018).

The E6 and E7 oncoproteins have a major role in contributing to the development and progression of cervical cancer. By interacting with a cellular protein, E3 ubiquitin ligase called E6-associated protein (E6AP), the E6 protein mostly facilitates the degradation of p53. It binds to E6AP and forms ternary complexes with p53 and becomes ubiquitinated. The inactivation and degradation of p53 interfere with its function as an apoptosis signalling cascade regulator, resulting in the mutation and inhibition of apoptosis (Narisawa-Saito & Kiyono, 2007). In addition, E6 triggers telomerase activity, therefore forming the immortalized epithelial cells by maintaining telomere length. E6 also inhibits apoptosis by interfering with other pro-apoptotic protein functions, such as Bak, Fas-associated death domain (FADD) and pro-caspase 8 (Ramakrishnan et al., 2015).

By contrast, E7 proteins are involved in inhibiting retinoblastoma tumour suppressor gene product (pRb) function. The primary mechanism by which pRb acts is the deactivation of transcription factors such as E2F that regulate cell division. E7 is expressed when a virus infects a cell and attaches itself to pRb, which causes increased phosphorylation and degradation. Destruction of pRb causes the E2F family of transcription agents to be released, which in turn activates genes that facilitate cell division (Yim & Park, 2005). Furthermore, through interaction with E2F transcription factor, E7 interferes with the functions of the proteins p107 and p130, which control cell cycle proliferation. In addition to pRb degradation, E7 oncoprotein-induced p107 and p130 inhibition promotes uncontrolled cell proliferation and the development of malignant transformation in HPV-infected cervical epithelium (Almeida et al., 2019).

2.2.4 Clinical presentation and symptoms of cervical cancer

Cervical cancer is often asymptomatic in its early stage. The most typical symptom, though, is excessive or irregular vaginal bleeding, especially after sexual activity. Some patients may experience mucoid, watery, malodorous and purulent vaginal discharge. In the advanced stage, patients may have pelvic or lower back pain. Additionally, patients may also exhibit bladder and bowel abnormalities such as vaginal passage of urine or stool, haematuria, haematochezia, or pressure-related complaints (Johnson et al., 2019). The lower limb oedema, sciatica and flank pain may suggest the pelvic sidewall invasion has occurred. The urine passage through the vagina may suggest bladder invasion while the passage of faeces through the vagina may suggest rectum invasion has occurred (Cohen et al., 2019).