COMBINATION EFFECT OF GALLIC ACID AND CISPLATIN ON ROS AND GSH PRODUCTION OF CERVICAL CANCER CELLS, HeLa

BATRISYIA BINTI MOHD NAZRI

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by

BATRISYIA BINTI MOHD NAZRI

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KESAN GABUNGAN ASID GALIK DAN CISPLATIN TERHADAP PENGELUARAN ROS DAN GSH SEL KANSER SERVIK, HeLa

ABSTRAK

Kanser serviks adalah salah satu punca utama kematian dalam kalangan wanita di seluruh dunia. Malangnya, kebanyakan rawatan kanser yang ada menyebabkan kesan sampingan kepada pesakit dan kurang berkesan. Oleh itu, pesakit mencari rawatan alternatif dengan menggunakan produk semula jadi yang mengandungi bahan fitokimia seperti asid galik (GA). Asid galik (3,4,5- trihydroxy benzoic acid; GA), ialah sebatian fenolik semula jadi yang berasal dari tumbuhan yang telah dilaporkan menghalang pertumbuhan pelbagai jenis kanser. Aktiviti antikanser dilaporkan berkaitan dengan regulasi spesies oksigen reaktif (ROS) dan glutation (GSH) di peringkat selular. Walaubagaimanapun, mekansime tindak balas masih kurang jelas. Oleh itu, kajian ini bertujuan untuk menilai pengawal atur ROS dan GSH pada gabungan cisplatin dan GA dalam sel HeLa yang dirawat dalam tempoh 24 dan 48 jam menggunakan kepekatan perencatan 50% pertumbuhan (IC_{50}). Tahap ROS, termasuk kepekatan hidrogen peroksida (H_2O_2) diukur dalam sel HeLa kerana variasi ini kelihatan mempengaruhi kerentanan kanser serviks terhadap rawatan cisplatin dalam selang masa 24 jam dan 48 jam. Selain itu, tahap GSH diukur dalam sel HeLa yang dirawat kerana ia memberi maklumat tentang keseimbangan antara percubaan badan untuk menentang tekanan oksidatif dan tindak balas sel kanser serviks terhadap kerosakan yang disebabkan oleh rawatan. Keputusan menunjukkan bahawa gabungan cisplatin dan GA membawa kepada corak pengeluaran ROS yang berbeza bagi kedua-dua selang 24 jam dan 48 jam. Ini menunjukkan kesan ketara tahap ROS terhadap keberkesanan rawatan. Tambahan pula, tahap H_2O_2 menunjukkan peningkatan dalam tempoh 48 jam apabila cisplatin dan GA digabungkan. Lonjakan ini bertepatan dengan induksi

apoptosis yang menunjukkan kebergantungan masa bagi tahap H_2O_2 disebabkan peningkatan kepekatan selepas 48 jam. Selain itu, dalam tempoh rawatan 48 jam rawatan gabungan yang melibatkan cisplatin dan GA, tahap GSH menunjukkan pengurangan. Keseimbangan antara tahap ROS dan GSH membantu mewujudkan keseimbangan redoks untuk rawatan gabungan cisplatin dan GA pada sel-sel kanser serviks dalam masa 48 jam. Keputusan ini boleh dijadikan asas untuk kajian lanjutan dalam mekanisme tindak balas rawatan gabungan GA dan cisplatin kerana ia menonjolkan interaksi antara ROS dan GSH, menekankan kesan ke atas proses selular.

COMBINATION EFFECT OF GALLIC ACID AND CISPLATIN ON ROS AND GSH PRODUCTION OF CERVICAL CANCER CELLS, HeLa

ABSTRACT

Cervical cancer is one of the leading causes of death among women worldwide. Unfortunately, most of the available cancer treatments reported to cause side effects. Therefore, patients turn to alternative treatments by utilizing natural products. Gallic acid (3,4,5-trihydroxy benzoic acid; GA), a plant-derived natural phenolic compound, has been reported to prevent the development and progression of various types of cancers. Anticancer activities of GA were reported due to regulation of reactive oxygen species (ROS) and glutathione (GSH) at the cellular level. However, its mechanism of action is still unclear. Thus, this study aimed to evaluate the regulations of ROS and GSH on the combination of cisplatin and GA in HeLa cells at 24 and 48 hours treatment period at IC_{50} concentration. ROS levels, including H_2O_2 concentrations were measured in HeLa cells as these variations appear to influence cervical cancer's vulnerability to cisplatin treatment over both the 24-hour and 48-hour intervals. Moreover, GSH levels were measured in treated HeLa cells as it helps to provide information about the intricate balance between the body's attempts to counter oxidative stress and the response of cervical cancer cells to the damage caused by the treatment. The results revealed that the combination of cisplatin and GA led to distinct patterns of ROS production for both the 24-hour and 48-hour intervals. This highlights the substantial influence of ROS levels on the treatment efficacy. Additionally, H_2O_2 levels was elevated during the 48-hour period when cisplatin and GA were combined. This increase aligned with the initiation of apoptosis, highlighting the time-dependent nature of H_2O_2 levels production, as the combination treatment demonstrated increased concentration after 48 hours. Moreover, during the 48-hour treatment

period, GSH levels has decreased. The intricate balance between ROS and GSH levels helps creating a redox balance for the combination treatment of cisplatin and GA on cervical cancer cells within 48 hours. These results may serve as a fundamental study for advanced study in combination treatment of Gallic acid and cisplatin as it highlights the interplay between ROS and GSH, emphasizing the impact on cellular processes.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
Akt	Serine/threonine kinase
AMPK	AMP-activated protein kinase
ANGPTL4	Angiopoietin-like 4
ANOVA	One-way analysis of variance
AsA	Ascorbic acid
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
CR	Cisplatin-resistant
CT	Computed Tomography
DCFDA	2',7'-dichlorofluorescin diacetate
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECC	Endocervical curettage

EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial-to-mesenchymal transition
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FASN	Fatty acid synthase
FBS	Fetal bovine serum
FDA	Food and Drug administration
FIGO	The International Federation of Gynaecology and Obstetrics
GA	Gallic acid
GCL	Glutamate cysteine ligase
GPx	Glutathione peroxidase
GSH	Glutathione
GSSG	Glutathione/glutathione disulphide
GST	Glutathione S-Transferase
GSTP1	Glutathione S-Transferase Pi
Hela	Cell line grew from cervical cancer cells sample

HIF	Hypoxia-inducible factor
HIV	Human immunodeficiency virus
HRP	Horse radish peroxidase
HPV	Human papillomavirus
H_2O_2	Hydrogen peroxide
IC_{50}	Extract concentration required to inhibit 50% of cells populations
IgE	Immunoglobulin E
KEGG	Kyoto Encyclopaedia of Genes and Genomes
KOH	Potassium hydroxide
LC	Local control
LDHA	Lactate dehydrogenase
MAPK	Mitogen-activated protein kinases
MDSC	Myeloid-derived suppressor cells
miR-23b	MicroRNA 23b
MMP	Matrix metalloproteinases
MRI	Magnetic resonance imaging

NAC	N-acetylcysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NSCLC	Non-small cell lung cancer
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
Nox4	NADPH oxidase 4
OD	Optical density
OS	Oxidative stress
PARP	Poly-ADP ribose polymerase
Pap	The Papanicolaou test
PBS	Phosphate-buffered saline
PCA	Perchloric acid
PDXK	Reduced pyridoxal kinase
PEITC	Phenylethyl isothiocyanate
PET	Position emission tomography
PFH	Lignan-extracted purified flaxseed hydrolysate
PLP	Pyridoxal 5'-phosphate

PMP	Pyridoxamine 5'-phosphate
Pra-B	Praeruptorin-B
Pt-GSH	GSH S-conjugate
P13K	Phosphatidylinositol-3-kinase
RAC-1	Ras-related C3 botulinum toxin substrate 1
ROS	Reactive oxygen species
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
SCC	Squamous cell carcinoma
SE	Standard error
SPSS	Statistical Package for the Social Sciences
SOD	Superoxide dismutase
TAK1	Growth factor-β-activated kinase 1
TGF-1	Tumor growth-1
TME	Total mesorectal excision
TMZ	Temozolomide

TNF	Tumour necrosis factor
TRX1	Thioredoxin-1
UA	Unsymmetrical bisacridines
uPA	Urokinase-type plasminogen activator
UPLC-MS/MS	Ultra-performance liquid chromatography- mass spectrometry
UV	Ultraviolet
2-ME	2-Methyoxyestradiol

CHAPTER 1

INTRODUCTION

1.1 Background Study

Cervical cancer is still among the top gynecologic cancers globally (Brisson & Drolet, 2019). According to current statistics, it ranks fourteenth among all malignancies and fourth among women globally (Fowler et al., 2023). Intervention for cervical cancer relies on both primary and secondary prevention. Primary prevention and screening are the most effective ways to reduce the burden of cervical cancer and mortality. Most screening and diagnostic efforts in the United States and other developing nations are focused on early detection of high-risk human papillomavirus (HPV) lesions by HPV testing and Pap smears. Although HPV testing is not advised for women under 30, low-risk younger women should begin screening with Pap tests at the age of 21 and continue until the age of 65, according to the United States Preventive Services Task Force guidelines (Fowler et al., 2023). Newer guidelines advocate screening every 3 to 5 years, depending on earlier results and the utilization of pap and HPV co-testing. Cervical cancer is preventable because a sexually transmitted infection causes it. Disease burden can be reduced by targeted education, screening, and intervention (Wang et al., 2022). However, as with many diseases and malignancies, disparities exist in screening rates, early diagnosis, and timely treatment. Screening rates are lower in low socioeconomic and low-resource communities with ethnic and age differences (Fowler et al., 2023).

On the other hand, patients who have been diagnosed with invasive illness require a thorough staging evaluation. The International Federation of Gynaecology and Obstetrics (FIGO) staging system provides a variety of approaches to patient staging. Traditionally, this was determined by a combination of pelvic examination, cystoscopy, proctoscopy, chest x-ray, intravenous urography, and basic labs (CBC, CMP, etc.). Advanced imaging modalities, like

magnetic resonance imaging (MRI) and position emission tomography (PET) scans, have recently been approved for staging (Fowler et al., 2023). A pelvic MRI is very useful for detecting local tumor extension. It is also helpful in assessing tumor response. PET scans detect nodal and visceral metastases more accurately than computed tomography (CT) scans. This is crucial since the existence of nodal illness can have a significant impact on the prognosis. After thorough staging evaluation, the cancer's stage would determine the treatment options, the woman's overall health, and her reproductive objectives (Fowler et al., 2023).

Surgery and radiation therapy are therapeutic options for cervical cancer. Chemotherapy, targeted therapy, immunotherapy, and clinical trials are essential in extending treatment options. Early-stage cervical cancer is usually treated with surgical resection, ranging from a conization to a modified radical hysterectomy. At the same time, women with high-risk characteristics after resection may require adjuvant treatment with chemotherapy and radiation (Fowler et al., 2023). On the other hand, locally aggressive and node-positive cancer is frequently treated with concurrent platinum-based chemoradiotherapy followed by brachytherapy. Compared to radiotherapy alone, the addition of chemotherapy following definitive radiotherapy resulted in significant gains in overall survival (Marth et al., 2017). The advanced stage of cervical cancer treatment focuses more on stopping and killing malignant cells from dividing and growing. Hence, the only treatment options are radiation and chemotherapy (Amjad et al., 2023). Chemotherapy is an essential component of standard cervical cancer treatment. It is typically administered as adjuvant therapy following surgery when poor prognostic tumor features increase the risk of recurrent disease, in combination with radiotherapy, as previously mentioned, and as a standalone treatment for locally advanced disease (Figure 1.1).

Cisplatin, platinum-based chemotherapy, has been the most effective single medication for cervical cancer over the previous three decades (Burmeister et al., 2022). Despite early

patient response to cisplatin, several adverse side effects in the short and long term are frequently noted. Thus, several studies explored that combining chemotherapy and radiation, immunotherapy, or targeted therapy in cervical cancer may help reduce tumor volume, inhibit micro-metastasis, prevent damage repair and drug resistance, and increase the radio-sensitivity of hypoxic cells in the cervix (Burmeister et al., 2022). Cervical cancer is a complicated and resilient disease that is hard to treat. Moreover, it causes several adverse side effects on cancer patients partly because some tumors resist drugs used in monotherapies. A combination of medicines may be more effective than a single therapy because it is more likely to disrupt various redundant signalling pathways necessary for cervical cancer cell survival. Furthermore, combining therapeutic techniques lowers the intensity, cost, number of cycles, and side effects associated with high monotherapy doses.

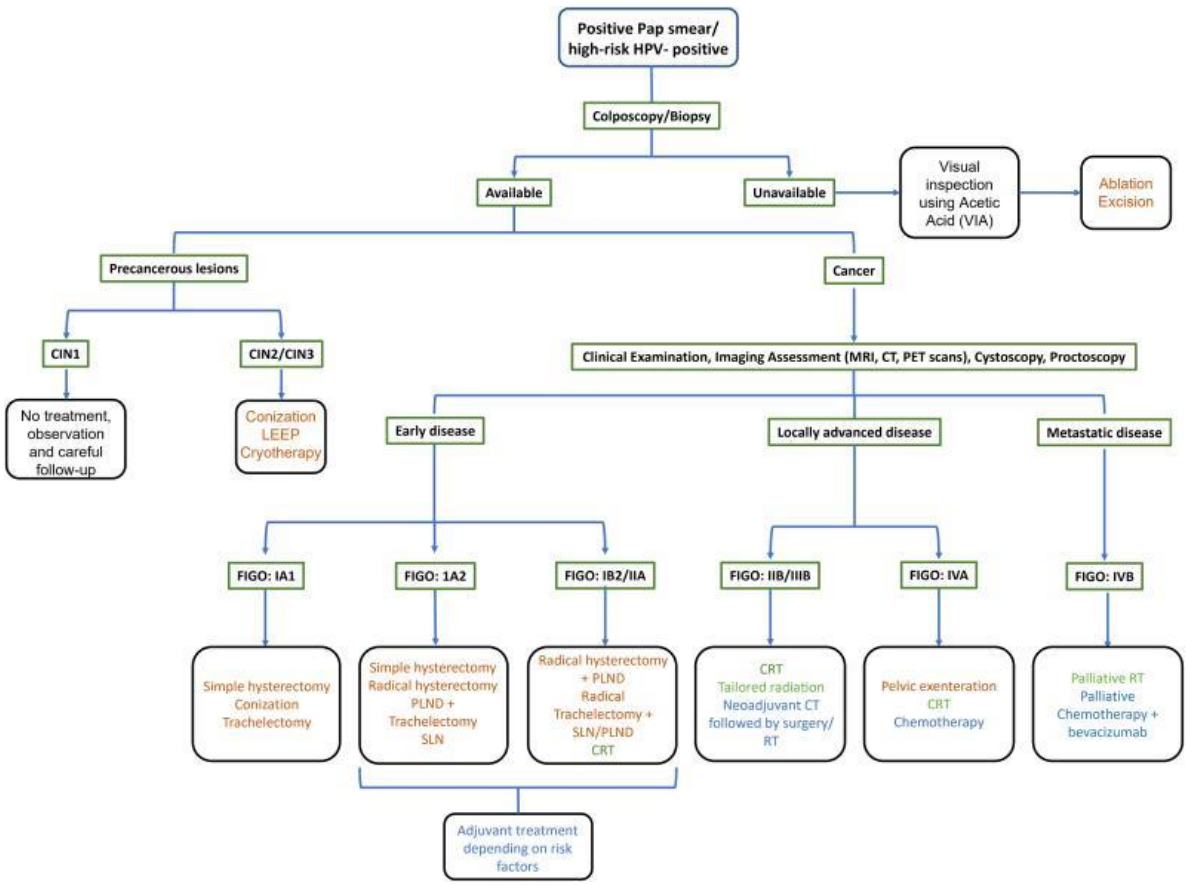

Figure 1.1: Overview of the management and treatment of cervical cancer based on the stage of the disease (Marth et al., 2017).

Innovative cancer treatment strategies, such as combined therapy of cisplatin and natural products shown some effectiveness (Dasari et al., 2022). Many medicinal plants contain bioactive chemicals that are prospective candidates for treating human ailments and constitute a good source for drug discovery. Natural products such as polyphenols have been shown in preclinical investigations to boost the therapeutic effectiveness of cisplatin and reduce its chemotherapy-induced toxicity (Dasari et al., 2022). Tocopherols, flavonoids, carotenoids, and phenolic substances are natural antioxidants which able to scavenge free radicals, without affecting the anticancer efficacy of cisplatin (Hajian et al., 2014). Moreover, natural antioxidants protect biomolecules from free radical damage caused by reactive oxygen species (ROS) and reactive nitrogen species (RNS). Thus, combination of natural products and cisplatin could have potential therapeutic applications (Hajian et al., 2014).

Cisplatin is the most effective medicine for treating both locally advanced and metastatic cervical cancer. Cisplatin primarily works by forming platinum-DNA adducts, which cause double-strand DNA breaks (Chen et al., 2021). However, a significant proportion of patients frequently experience relapse and mortality due to drug resistance or toxicity to numerous organs, including the liver, kidneys, gastrointestinal tract, cardiovascular, hematologic, and neurological systems (Dasari et al., 2022; Jing et al., 2019). Following these advantages of these conventional drugs, many researchers have been focusing their interest on natural compound as anti-cancer agent.

Gallic acid (GA) is a polyhydroxyphenolic molecule found in various plants, including green tea, grapes, strawberries, bananas, and other fruits. GA and its derivatives have been shown in several studies to have antioxidant properties, both *in vivo* and *in vitro* (Bhattacharya et al., 2016). GA has been shown to have anticancer activity in various cancer cells, including cervical cancer cells. GA's anticancer effect has been demonstrated to be owing to its capacity in suppressing cancer and promote apoptosis by activating ROS generation, activating redox

processes that consume glutathione (GSH) and produce ROS such as hydrogen peroxide (H_2O_2), which damage cellular components and deplete GSH which involved in the ROS detoxification (Ashrafizadeh et al., 2021). In short, the redox equilibrium would be attained when GSH levels are diminished and ROS levels are heightened, including H_2O_2.

1.2 Problem statement

Current cancer treatments have detrimental effects on the patients, either short- or long-term such as fatigue, vomiting, hair fall, and permanent neuronal and renal damage. This may be due to chemotherapy drugs such as cisplatin killing fast-growing cells, whereas healthy cells are also damaged as they cannot differentiate between healthy and tumor cells. Moreover, conventional monotherapy approaches target active proliferating cells in a non-selective manner, resulting in the death of both healthy and malignant cells. Thus, chemotherapy can be high-risk to the patient, causing various side effects. Thus, finding another concomitant drug used along with cisplatin is essential to enhance the effectiveness of the treatment, which ultimately prevents the toxic effects on normal cells while simultaneously producing cytotoxic effects on cancer cells (Mokhtari et al., 2017). Other than that, gallic acid can act as an antioxidant or a prooxidant, depending on the conditions under which it reacts with other molecules. However, the antioxidant mechanism of this combination drug is yet to be explored. Thus, this study selects a combination of cisplatin and polyphenols, GA, to explore how this combination drug can potentially improve cancer treatment outcomes by considering the interplay between ROS generation by cisplatin, the antioxidant properties of polyphenols, and their combined effects on cancer cells. Moreover, the antioxidant pathway involves the action of various antioxidant enzymes and molecules, including hydrogen peroxide and GSH were studied. The research aims to identify the most practical combination of strategies and

mechanisms involved to optimise therapeutic approaches and minimize the potential harm caused by ROS.

1.3 Rationale of study

Cisplatin-based therapy is one of the prevailing methods for addressing cervical cancer globally. It is a significant problem, adversely impacting individuals with cervical cancer patients. However, there have been reports of improved cisplatin efficacy and reduced adverse effects on non-cancerous cells when combined with other medications or natural compounds. Thus, the significance of the study is to create a combination of chemotherapeutic drugs utilising low concentrations of cisplatin and GA to alleviate the side effects of chemotherapy, enhance cisplatin efficacy, and ultimately reduce the toxic effects on normal cells while simultaneously producing cytotoxic effects on cancer cells. In addition, combination therapy would help enhance cisplatin's efficacy as GA is a natural antioxidant that exhibits anticancer effects, as widely reported. Therefore, searching for a safer modality for cervical cancer treatments is an effort. Combining cisplatin with natural products was proven to increase the efficacy of cisplatin, which potentiates anticancer activity and induces apoptotic effects on cancer cells (Norlida et al., 2021). However, understanding the antioxidant mechanism through ROS and GSH levels are necessary to understand this new combination of chemotherapeutic drugs that may be a potential approach for new cervical cancer treatments.

1.4 Objectives

1.4.1 General objective

The general objective of this study is listed below:

1. To evaluate the regulation of ROS and GSH on a combination of cisplatin and GA in HeLa cells

1.4.2 Specific objectives

The specific objectives of this study are listed below:

1. To determine cellular ROS levels in HeLa cells upon administration of different incubation time of cisplatin and GA at IC_{50} concentration.
2. To quantify hydrogen peroxide production levels in HeLa cells upon administration of different incubation time of cisplatin and GA at IC_{50} concentration.
3. To measure GSH levels in HeLa cells upon administration at different incubation times utilising cisplatin and GA at IC_{50} concentration.

1.5 Hypothesis

1. There is a significant effect on cellular ROS levels in HeLa cells upon administration of different incubation time of cisplatin and GA at IC_{50} concentration.
2. There are significant H_2O_2 production levels in HeLa cells upon administration at different incubation times of cisplatin and GA at IC_{50} concentration.
3. There is a significant effect on GSH levels in HeLa cells upon administration at different incubation times of cisplatin and GA at IC_{50} concentration.

CHAPTER 2

LITERATURE REVIEW

2.1 Cervical cancer

Cervical cancer is the fourth most common malignancy in women worldwide, with an anticipated 604,000 new cases in 2020. Around 90% of the anticipated 342,000 cervical cancer deaths in 2020 will occur in low- and middle-income countries. HIV-positive women face a sixfold higher risk of cervical cancer development than those without HIV, and roughly 5% of all cervical cancer cases are linked to HIV. Furthermore, the impact of HIV on cervical cancer is particularly pronounced among younger women across various regions worldwide (Sung et al., 2021). These figures are anticipated to surge, projecting a 67% rise and a twofold increase in anticipated maternal mortality from pregnancy-related issues, resulting in an estimated 443,000 annual fatalities by the year 2030 (Wang et al., 2022).

Radical hysterectomy, lymph node dissection, radiation therapy, and chemotherapy are the gold standard treatments for patients with early-stage cervical cancer. External beam radiotherapy, combined with cisplatin-based chemotherapy and brachytherapy, is the standard of care for patients with locally advanced cervical cancer. However, the results of cervical cancer treatment that omits brachytherapy in favour of external beam radiation therapy are below than average. Patients with early-stage cervical cancer have a 3-year local control (LC) rate of 87% to 95% with staging and treatment, while those with advanced-stage cervical cancer have a 74% to 85% rate. In many developing countries, the overall 3- to 5-year survival rate for cervical cancer is less than 50%. In cervical cancer, local disease development is expected and associated with substantial suffering, including ureteral blockage, pain, fistulas, and death (Small et al., 2017).

2.1.1 ROS and cervical cancer

Researchers are actively investigating the role of ROS in cervical cancer and exploring potential therapeutic strategies that target ROS to mitigate their harmful effects on cellular processes. Understanding the intricate relationship between ROS and cervical cancer could lead to developing more effective approaches for this disease treatment. Many cellular functions produce ROS as natural by-products. As a result of an unbalance between oxidants and antioxidants, cancer cells typically show greater basal levels of ROS compared to normal cells. ROS operate as signal transducers at low to moderate levels to drive cell proliferation, migration, invasion, and angiogenesis, demonstrating their dual role in cellular metabolism. In contrast, excessive ROS contributes to cell death by damaging proteins, nucleic acids, lipids, membranes, and organelles. Extensive research has shown that anticancer treatments, such as immunotherapies, that control ROS levels show potential *in vitro* and *in vivo* effects (Bhattacharyya et al., 2014).

2.1.1.1 Oxidative stress and cervical cancer development

An imbalance in the production and removal of oxidant species is the cause of oxidative stress (OS). Due to the accumulation of oxidative changes in various biomolecules, their accumulation may cause cell dysfunction (Bhattacharyya et al., 2014; Pizzino et al., 2017). The formation of free radicals is a constant physiological process resulting from metabolic processes and inflammation. ROS and RNS are primarily produced in the mitochondria by cytochrome P450 and peroxisomes, which are endogenous. Radiation, nicotine use, chemotherapy, and diet are significant external variables that induce free radical generation. Metabolic control, the cell cycle, and intracellular signaling pathways all rely on intermediate reactive species created normally under physiological settings (Silva et al., 2018; Aranda-Rivera et al., 2022).

Protective chemicals known as "antioxidant defenses" keep ROS and RNS generation and clearance in equilibrium. Antioxidant mechanisms within cells are classified as either enzymatic or non-enzymatic. Catalase, superoxide dismutase (SOD), glutathione peroxidase (GPx), and Glutathione-S-transferase (GST) are all members of this family of enzymes. Vitamins C and E, lipoid acid, carotenoids, flavonoids, and others make up the non-enzymatic category of chemicals (Sharifi-Rad et al., 2020).

The primary cause of OS is an accumulation of free radicals and cellular active intermediates that have not been neutralized. Alterations in aerobic metabolism, inflammatory response, exposure to UV radiation, hypoxia, atypical cell growth, and viral infections are all linked to elevated levels of oxidized biomolecules. Therefore, OS is directly linked to several clinical diseases, such as HPV-associated tumors (Silva et al., 2018).

Figure 2.1 describes several factors, such as environmental pollutants, chemicals, tobacco smoking, and chronic inflammation, among others, that generate reactive oxygen and nitrogen species (ROS/RNS). An imbalance between oxidant species and the antioxidant system results in DNA, RNA, and protein damage, which may accumulate genetic alterations and promote malignant transformation (Silva et al., 2018).

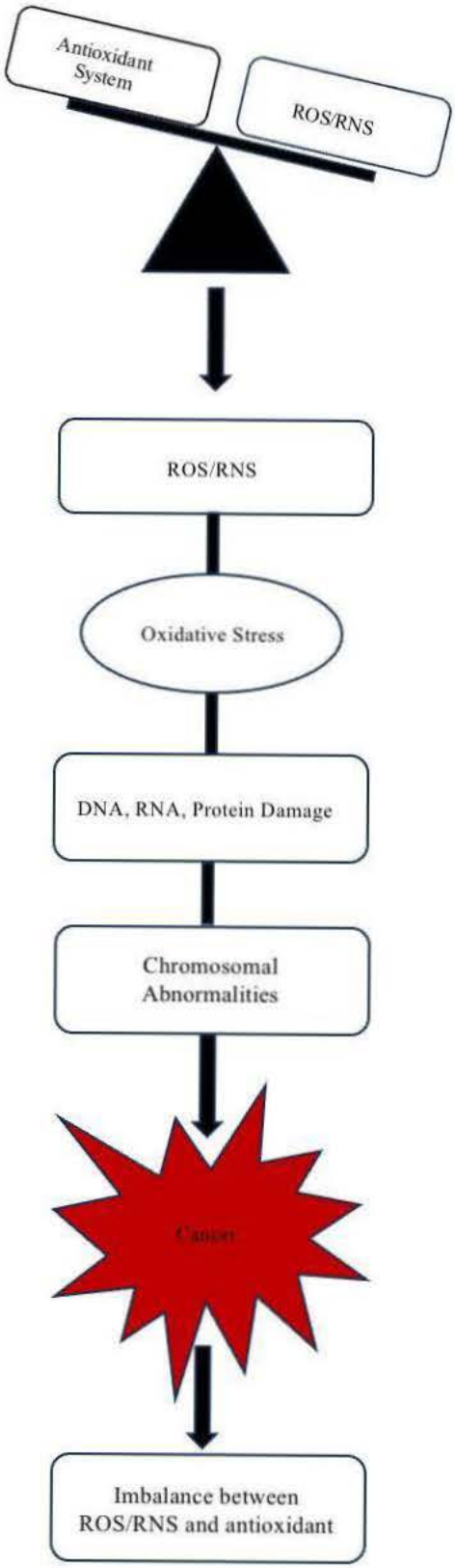

Figure 2.1 Relationship between oxidative stress and cancer modified from Silva et al., 2018

2.1.1.2 ROS and cervical cancer progression and metastasis

The primary cause of cancer death and morbidity is metastasis, which is the spread of cancer cells from the primary tumor to other organs (Clark & Vignjevic, 2015). The likelihood of cancer relapse in distant organs increases dramatically once metastasis has occurred, and surgical removal of the main tumor no longer ensures disease-free longevity. Cancer cells in the primary tumor undergo epithelial-to-mesenchymal transition (EMT) as the first step in the complex, multi-phase process of metastasis (Clark & Vignjevic, 2015). The basement membrane is detached and degraded, resulting in an invasive tumor epithelial phenotype. Invasive cancer cells eventually invade nearby blood vessels and lymphatics, undergo intravasation, and spread throughout the body. Cancer cells in circulation can avoid death by avoiding basement membrane attachment. They can defy immune surveillance until they reach a secondary site, at which point they can extravasate and colonize distant organs. However, metastases cause most cancer-related deaths and researchers have mainly concentrated on the primary tumor (Smith & Kang, 2013).

EMT, a trans-differentiation program in which epithelial cancer cells lose cell-cell adhesion and simultaneously gain mesenchymal properties of motility and invasion, is the first step in metastasis (Yazaki et al., 2021). Substantial evidence links ROS to EMT in cancer cells. It is common knowledge that TGF-1 is a crucial factor in EMT induction. TGF-1 promotes cell migration and invasion by activating NF-B via a Rac1-NOXs-ROS-dependent pathway, which regulates urokinase-type plasminogen activator (uPA) and matrix metalloproteinase 9 (MMP-9). The non-canonical TGF-1-TGF-activated kinase 1 (TAK1) pathway, in which ROS participates, is also critical in controlling EMT. TAK1 loss causes a cascade of signals that expedite EMT by increasing integrin-Rac-induced ROS. Invasive squamous cell carcinomas (SCCs) were found to have lower TAK1 expression than benign SCCs consistently. ROS control EMT by activating nuclear factor (erythroid-derived 2)-like 2 (Nrf2), which in turn

promotes Notch signaling and, by extension, EMT (Yazaki et al., 2021). TGF-1 can also be induced by exposure to exogenous ROS from sources like ionizing radiation. Evidence for ROS's varied role in EMT has been mounting in recent years. The involvement of ROS in several pathways that connect to numerous important EMT-inducing pathways demonstrates the centrality of ROS in this process (Robertson & Rifkin, 2013).

The integrin-mediated extracellular matrix interaction required for the survival and proliferation of circulating cancer cells is lost, and the cells develop anoikis resistance. Numerous investigations have established that ROS are a significant factor in sensitivity to anoikis. Angiopoietin-like 4 (ANGPTL4) is a gene that has been demonstrated to play a significant role in metastasis. The ANGPTL4 protein interacts with integrin to increase ROS generation, stimulating PI3K/Akt and ERK to protect tumor cells from apoptosis via an outside-in signaling mechanism. Recent research has linked increased NOX4-induced ROS generation to anoikis resistance in gastric cancer cells (Du et al., 2018). By elevating ROS levels, NOX4 promotes cell viability and suppresses apoptosis by upregulating EGFR. Another study found that EGFR is directly related to enhanced cell survival when no extracellular matrix is present. An essential step in cancer spread is anoikis resistance and intracellular ROS plays a part in this by, for example, regulating growth factors (Adeshakin et al., 2021).

When metastasis is complete, circulating cancer cells extravasate into the new secondary tumor site and colonize it because of favorable conditions (Du et al., 2018). It has been discovered that main tumor site interactions can establish a pre-metastatic niche in the secondary tumor site, impacting the survival of disseminated tumor cells at the new site. What happens to a cancer cell after it has spread depends on the primary total mesorectal excision (TME) but also on the secondary TME (Delibegovic, 2017). There is growing evidence that ROS play a role in establishing soil in distant organs, facilitating the growth of a tumor that has spread to other body parts. One of these is the release of miR-23b from cells into the

environment via exosomes. MiR-23 b, a microRNA, inhibits tumorigenesis because it controls ROS.

A metastatic niche favoring breast cancer cell dormancy could be enabled by exosome transfer of miR-23b from bone marrow mesenchymal stem cells, a common secondary tumor site in breast cancer (Li et al., 2019). Increased production of ROS by hemopoietin-derived MDSCs in the milieu of metastatic niches reduces the activity of cytotoxic $CD8^+$ T-cells, thereby promoting the survival of disseminated cancer cells at the secondary tumor site. Due to their participation in multiple stages of tumor metastasis, ROS are crucial participants in this process (Du et al., 2018) This discovery is significant because it may help direct the design of future clinical trials and the creation of redox-based therapies that precisely target metastasis (Delibegovic, 2017).

2.1.2 Glutathione in cervical cancer

The antioxidant glutathione or GSH is often hailed as a critical participant in the detox process. The level of GSH in a cell affects how sensitive the cell is to anticancer treatments and how much harm the treatment might cause. For instance, with low levels of GSH, the treatment may be toxic to the patient. To predict whether malignant cells will be sensitive to the action of the medicine or whether the drug will not affect normal cells, determining the level of GSH is vital.

Patients with cervical cancer who have a complete response to treatment have significantly lower GSH levels in their blood and tumors than those who achieve only a partial response. Enzymes involved in GSH metabolism that use tripeptide as a substrate can influence GSH levels. Since the solubility of drugs and other toxic materials in water increases during the enzyme-catalyzed conjugation reaction with GSH, the effect on the organism is reduced, which can lead to a poor response to the treatment and a shorter survival time when chemotherapy is used to treat cancer. (Daukantienė et al., 2014).

2.1.2.1 Role of glutathione in antioxidant defense

The optimal approach for countering the impact of free radical degradation involves utilizing a healthy lifestyle to ensure the body's antioxidant systems work in harmony. Cells can be safeguarded against apoptosis triggered by stress, courtesy of the collaborative efforts of GSH and a diverse array of alternative antioxidants and denitrosylases. This cooperative defense mechanism shields the functionality of numerous vital proteins that are susceptible to impairment caused by oxidative harm (Raj Rai et al., 2021). The study of GSH's role as an antioxidant in animals other than humans is a rapidly growing area of research. In order to slow the development of cancer, several treatment plans already rely on GSH administration, as seen in Figure 2.2, where its potential use in developing novel drugs to treat diseases and disorders caused by oxidative and nitrosative stress (Raj Rai et al., 2021).

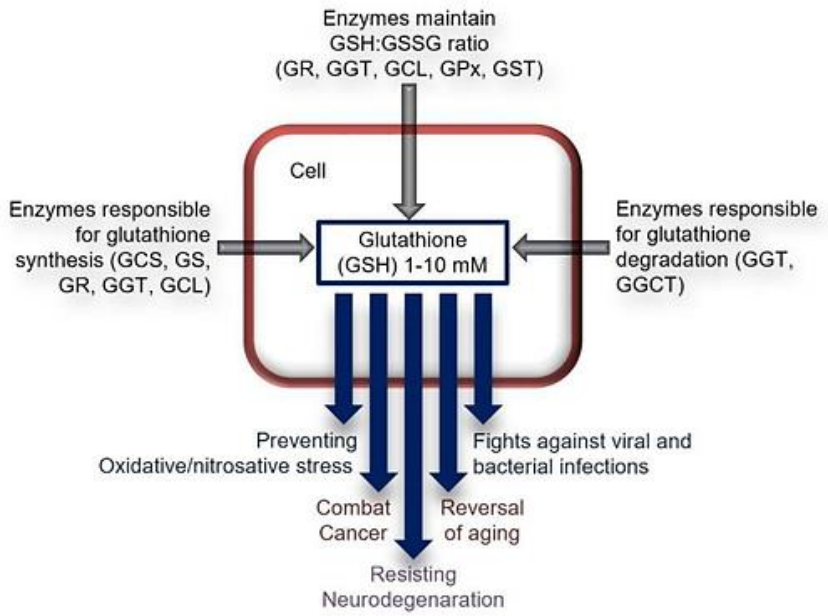

Figure 2.2 GSH: Role in Oxidative/Nitrosamine Stress, Antioxidant Defence, and Treatments (Raj Rai et al., 2021)

2.1.2.2 Altered glutathione metabolism in malignancies

Utilizing metabolomics and pharmacological investigations to understand better GSH metabolism is instrumental in elucidating how Oridonin effectively hinders cervical cancer

(Wang and Zhang, 2023). Oridonin is a natural tetracycline diterpenoid that is isolated from the Chinese herb Rabdosia rubescens (Wang et al., 2013). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis and network pharmacology to find overlapping targets and metabolic processes. Ultra-performance liquid chromatography – mass spectrometry (UPLC-MS/MS) metabolomics analysis is performed to identify modified metabolites following oridonin treatment. Changes in key molecules closely associated with changed metabolites can be uncovered using a variety of bioassays. In total, 75 overlapping targets between oridonin and cervical cancer were found. Treatment with oridonin dramatically alters the levels of twenty-one metabolites involved in the tricarboxylic acid cycle, GSH metabolism, branched-chain amino acid metabolism, and other metabolic pathways. The rate-limiting enzyme in GSH synthesis, the glutamine-cysteine ligase subunit, is inhibited by oridonin, leading to a dramatic decrease in cysteine levels. Eventually, it leads to a decrease in GSH levels. The antioxidant enzyme GSH constitutes a part of the antioxidant GSH system. When subjected to oridonin, Hela cells significantly reduce ATP production. The suppression of GSH metabolism could underlie the apoptosis observed in Hela cells (Wang and Zhang, 2023).

The continuation of the search for possible anticancer treatments, where they synthesized and created unsymmetrical bisacridines (UAs), a completely novel class of acridine derivatives with unusual structures (Potęga, 2022). They were classified based on their physicochemical features and the role of phase I and II metabolic transformations in their activity. In nude mice, UAs demonstrated significant cytotoxic activity against a variety of cancer cell lines as well as high anticancer efficacy against a variety of human cancer xenografts. These are primarily chemotherapy-resistant malignancies characterized by elevated activity of several GST isoenzymes compared to normal tissues. C-2028 (9-N-[(imidazo [4,5,1-de]-acridin-6-on-5-yl)aminopropyl]-N-methylaminopropylamino-1′-nitroacridine, a typical

UA) was shown to be capable of GST-mediated and direct GSH conjugation in preliminary investigations using rat liver microsomal and cytosolic subfractions (Potęga, 2022).

Moreover, GSH conjugation of xenobiotics and conversion of thioethers to mercapturic acids is generally recognized as a metabolic defense mechanism of organisms against potentially hazardous chemicals. In general, many chemicals lose their harmful qualities due to the detoxification process, either entirely or partially. However, in rare situations, a diversion of this pathway (e.g., by the activity of cysteine S-conjugate -lyase) may result in the bioactivation rather than detoxification of some anticancer medications, increasing their toxicity. These routes of GSH conjugation of anticancer medications, the potential outcomes of which include cancer treatment, resistance, or development, still need to be exploited more with a better understanding of the pharmacokinetics properties and lower overall toxicity targeting specific cancers. For instance, high-dose *cis*-DDP therapy causes side effects including nephrotoxicity, ototoxicity, and neurotoxicity which limit its efficacy. Renal cell damage has been confirmed to be caused by cysteine S-conjugate -lyase activity, which is overexpressed in this organ. The activation process of *cis*-DDP to nephrotoxic species is depicted in Figure 2.3, which follows the mercapturic acid production pathway, i.e., the conversion of cis-DDP to its GSH S-conjugate (Pt-GSH) and subsequently to cysteine S-conjugate (Pt-Cys), the highly reactive and cytotoxic thiol form of cisplatin (Ramsay & Dilda, 2014). Cysteine S-conjugate-lyase transforms a pharmacological intermediate into a thiol-reactive metabolite with a Pt-SH (or Pt-S) moiety that binds to thiophilic centers of mitochondrial proteins found in renal proximal tubule cells.

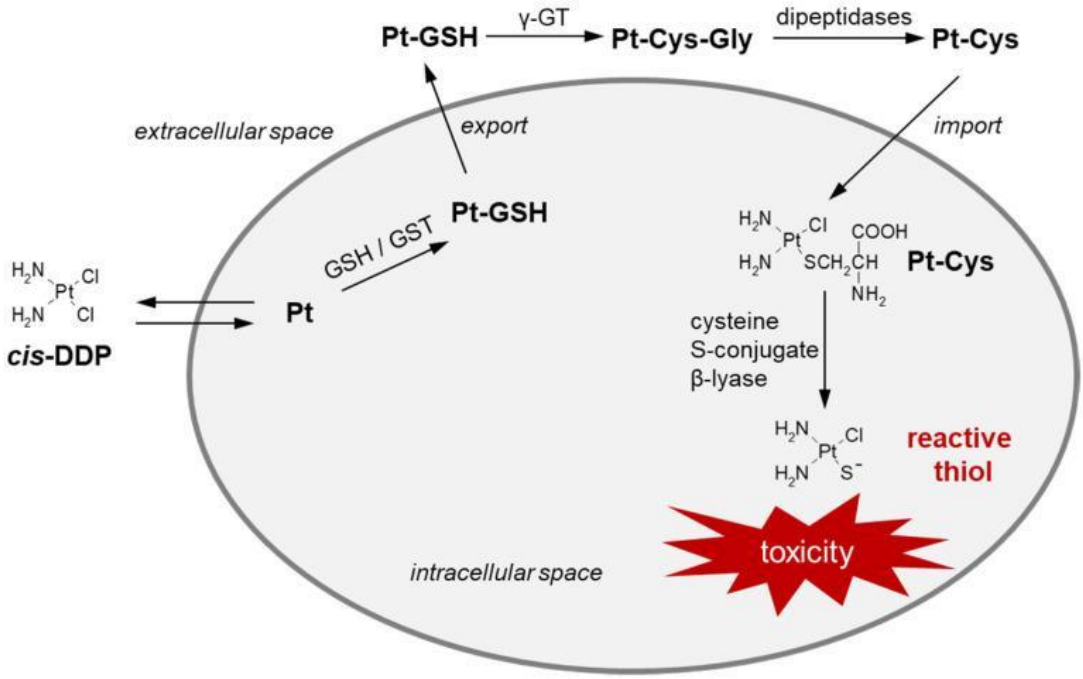

Figure 2.3: Cisplatin (*cis*-DDP) bioactivation through GSH S-conjugate (Pt-GSH) and cysteine S-conjugate (Pt-Cys) formation (Potęga, 2022). *cis*-DDP = cisplatin; GSH = glutathione; GST = glutathione S-transferase; γ-GT = γ-glutamyl transferase; Pt = platinum (II) aqua species.

2.2 Combination of anticancer drug and natural products for cervical cancer

Cisplatin, also known as (SP-4-2)-diamminedichlorido platinum(II), is one of the most promising and widely used drugs for treating a variety of solid tumors, including testicular, ovarian, head, and neck, bladder, lung, cervical, melanoma, and lymphomas (Ghosh, 2019). However, cisplatin's use and efficacy are limited by potentially serious side effects and the development of drug resistance.

Figure 2.4: Cisplatin: The first metal based anticancer drug (Ghosh, 2019)

Cancer cells resistant to cisplatin can reduce the drug's concentration inside the cell, inactivate the drug by reacting with GSH and metallothioneins, and repair DNA damage more quickly. Utilizing combination therapies aims to reduce the risk of adverse effects and counteract cisplatin resistance. These strategies prove more efficacious in cancer treatment by attaining a state of equilibrium in cellular redox levels, achieved through diminishing GSH and increasing ROS concentrations. Cisplatin's brief history, synthesis, action mechanism, resistance, applications, side effects, and modifiability are all covered in Ghosh's study (2019) where he provides a brief overview of the evolution of platinum medications, from the tiny cisplatin complex to the massive conjugated platinum nanocarriers of the following generation as seen in Figure 2.4.

However, the incidence of cervical cancer among females worldwide ranks fourth. Numerous natural substances have been documented for their efficacy in combating cervical cancer through their anti-cancer mechanisms (Park et al., 2021). Pubmed (including Medline) and google scholar articles published in the last three years were used to compile this list of

natural compounds with anti-cancer properties against cervical cancer. Their mechanisms were broken down into five groups: apoptosis induction, angiogenesis inhibition, metastatic inhibition, resistance reduction, and miRNA modulation as seen in Figure 2.5. Sixty-four natural compounds inhibited cervical cancer. *Cassia fistula L. Penicillium sclerotiorum* extracts, *Bauhinia variegate candida* ethanol extracts, *Nigella sativa thymoquinone*, *Pinellia pedatisecta Schott*. Lipid-soluble extracts and *Alpinia conchigera* 1′S-1′-acetoxy chavicol extracts have multiple effects against cervical cancer. Natural compounds might offer promising leads for developing new cancer treatments (Park et al., 2021).

Several active compounds in natural products derived from plants and animals have been hailed as viable substitutes for chemotherapeutic medications or compatible with their combined usage. In Hela cells, for instance, apoptosis is induced by Lignan-extracted purified flaxseed hydrolysate (PFH). *Nigella sativa*'s thymoquinone exhibited similar apoptotic and anti-proliferative properties in SiHa and CaSki cells. Natural remedies include the ethanol extracts of the *Bauhinia variegated* yeast, Praeruptorin-B (Pra-B), and ordinary tea (Park et al., 2021).

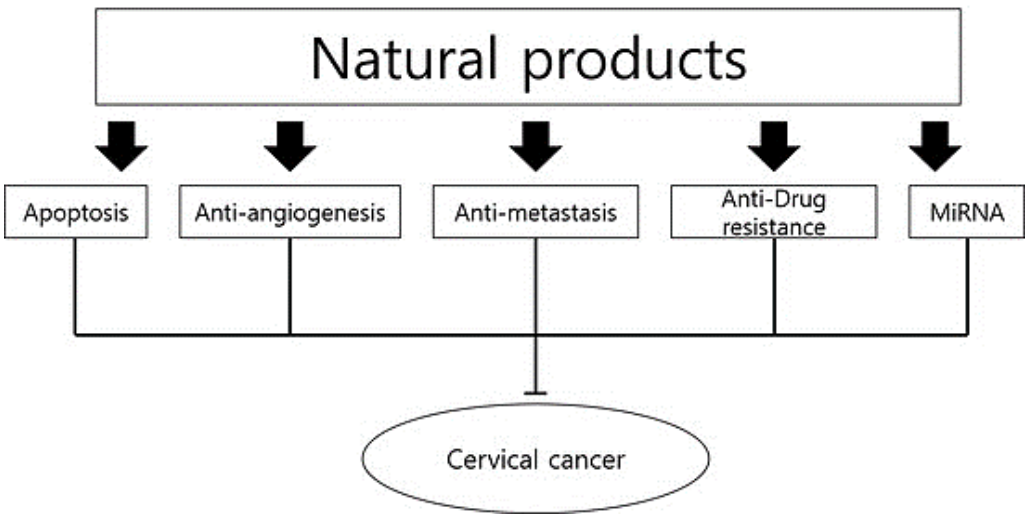

Figure 2.5: Potential of Natural Products in Treatment of Cervical Cancer (Park et al., 2021)

2.2.1 Cisplatin

Cisplatin, or cis-diamminedichloroplatinum(II), has a square planar structure and is a metallic (platinum) coordination compound (CAS No. 15663-27-1, MF-Cl2H6N2Pt; NCF-119875) as seen in Figure 2.6. At room temperature, the crystalline powder is either white or a deep yellow to yellow-orange color. It dissolves in dimethylprimanide and N, N-dimethylformamide, and only weakly in water. Cisplatin is stable at room temperature and pressure but may gradually change into a trans-isomer over time. However, the compound received severe scientific attention in the 1960s when Rosenberg at Michigan State University discovered that electrolysis products of platinum mesh electrodes could inhibit cell division in Escherichia coli. This finding sparked widespread interest in the potential use of these products in cancer chemotherapy. Much interest has been developed in using platinum, palladium, and other coordination complexes in cancer therapy since cis-dichlorodiammineplatinum (II) (cisplatin, r) was identified as the agent responsible for this activity.

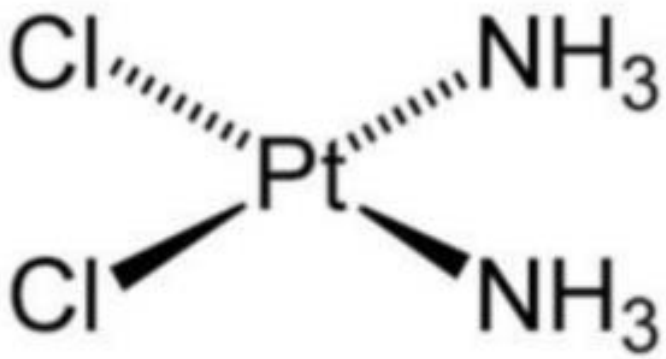

Figure 2.6: Chemical structures of platinum drugs: cisplatin (Dasari & Bernard Tchounwou, 2014)

Cisplatin has been particularly intriguing because it has demonstrated anticancer efficacy in various tumors, including ovarian and testicular malignancies and head and neck solid tumors. The cytotoxic effects of this compound were first identified in the 1960s, and by the late 1970s, it had become an integral part of the standard treatment protocol for germ cell

tumors. Cisplatin is one of the most compelling chemotherapeutic agents for cancer. In 1978, it was the first platinum compound to receive FDA approval for use in cancer therapy. Therefore, platinum (II) and other metal-containing compounds have recently gained attention as possible anticancer treatments.

Cisplatin, an alkylating agent and antineoplastic, is used to treat a variety of cancers. The ability of alkylating agents to add alkyl groups to multiple electronegative groups under cellular conditions is what lends them their name. Tumour growth is halted because the guanine nucleotides in the DNA double helix are being cross-linked. This prevents the strands from unwinding and breaking apart. This prevents cell division since it is essential for DNA replication to occur. These medications also introduce unnatural methyl or alkyl groups into molecules, which prevents them from being utilized appropriately during base pairing and results in incorrect DNA coding. The cell cycle has little effect on alkylating agents. The effects of alkylating chemicals, including disruption of DNA function and cell death, can be achieved in three distinct ways (Dasari & Bernard Tchounwou, 2014).

2.2.2 Mechanism action of cisplatin

The three mechanisms by which alkylating agents inhibit DNA synthesis and RNA transcription are as follows:

1. The attachment of alkyl groups to DNA bases, causes DNA fragmentation when repair enzymes try to replace the alkylated bases
2. DNA damage via the formation of cross-links (bonds between atoms in the DNA)
3. The induction of mispairing of the nucleotides (Dasari & Bernard Tchounwou, 2014)

Tchounwou et al. (2021) found that DNA binding followed by single-stranded breaks is thought to be the cause of cisplatin's cytotoxicity. When cisplatin enters the cytoplasm, it replaces chloride atoms with water molecules, creating an electrophile attraction to sulfhydryl

groups on proteins and nitrogen donor atoms on nucleic acids. Due to cisplatin's specific binding to 1, 2-intrastrand crosslinks of purine bases, cell division is inhibited, and apoptotic cell death is triggered. Covalent interaction between cisplatin and DNA has been observed most frequently at the N7 locations of the imidazole ring of two neighboring guanines. The toxicity of cisplatin has been linked to the formation of inter- and intrastrand crosslinks as well as nonfunctional adducts. The concentration-dependent effects of cisplatin on cell growth inhibition, DNA adduct induction, and cytotoxicity in leukemia cells were observed in their lab (Tchounwou et al., 2021).

2.3 Polyphenols in cervical cancer treatment

Polyphenols are natural compounds found in plants and is one of the most essential dietary components that have been extensively studied for their potential health benefits (Rana et al., 2022). They possess antioxidant properties and have been associated with the prevention of degenerative diseases, including cardiovascular diseases and cancers. Polyphenols have also shown anti-inflammatory effects and have been investigated for their epigenetic effects. Their different chemical compositions classified polyphenols into four primary groups of polyphenols are flavonoids, lignans, stilbenes, and phenolic acids. Several studies have been done thus far to examine their health effects, both *in vivo* and *in vitro*. Polyphenols protect the body from harmful environmental influences and neutralize disease-causing ROS. Tea, chocolate, fruits, and vegetables all contain polyphenols, which may benefit human health (Williamson, 2017). Over the previous 10-15 years, the association between polyphenol consumption and cancer risk incidence has been examined and meta-analyzed regularly. In a meta-analysis of prospective trials, isoflavone consumption was linked to a 19% lower risk of stomach cancer. Soy product consumption has been linked to a lower incidence of breast cancer in recent epidemiologic research. In cohort and case-control studies, Isoflavone and flavonol

consumption was linked to a 30% reduction in the risk of ovarian and endometrial malignancies (Rothwell et al., 2017; Messina, 2016). Consumption of soy isoflavones and soy-based foods was linked to a lower risk of colorectal cancer in two Asian population meta-analyses, one of which revealed a 23% reduction in risk from 13 case-control studies and four prospective studies. In addition, in a case-control Korean study, a high intake of total soy products was connected to a lower risk of colon cancer, particularly at the distal and rectal sites. A meta-analysis of green tea polyphenols found that drinking seven or more cups of green tea daily reduced the incidence of prostate cancer by 19%. A case-control study in Canada found that high dietary consumption of total flavonoids reduced the incidence of lung cancer. Overall, polyphenols such as GA, those found in olive oil, and oleuropein and hydroxytyrosol appeal for cancer treatment (Gorzynik-Debicka et al., 2018). However, polyphenols' health effects are determined by both their intake and bioavailability (Hung & Lai, 2020).

2.3.1 Gallic acid

Gallic acid (GA) is an antioxidant and natural phenolic compound found in various plants and common foods such as strawberry, grape, banana, hazelnut and many more. GA is a 3,4,5-trihydroxybenzoic acid, C_6H_2 $(OH)^3$ COOH chemical structure as seen in Figure 2.6. It has molecular weight of 170.12 g/mol and can be found relatively high concentrations in plant material. Besides that, GA also can be extracted from hydrolysis of tannic acid ($C_{76}H_{52}O_{46}$) by bioconversion with enzyme tannase (Govea-Salas et al., 2018). It can mediate different therapeutic properties involved in anti-inflammation and anti-cancer activities. GA was shown to have cytotoxic effects in cervical cancer cells without damaging the normal cells and can prevent the progression of cancer cells by downregulating the expression of molecular pathways involved in cancer progression, such as P13K/Akt (Zhao & Hu, 2013). Moreover, GA has been shown to exert anti-cancer activities via several biological pathways that include