Anticancer properties of newly synthesized Triazoles (14a) against cervical and breast cancer cell lines

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Thesis submitted in Partial Fulfillment of the requirements for the Master Degree of Science in Biological Sciences

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A Thesis submitted in partial fulfillment of requirements for the degree of Master of Biological Sciences

By

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Declaration

I hereby declare that this submission is my own work and to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree of the University or other institutes, except where due acknowledged has been made in the text.

Signature   Name        Date
Ghadeer     Ghadeer Idhair  2019
Dedication

To who stood beside me and supported me, and the reason for achieving my dreams, to my dear father,

To the symbol of love and healing balm, to the heart pure white, to my beloved mother,

To my beloved and my partner who shared my happiness, sadness, and moments of success and failure, to my fiancé,

To those who showed me the most beautiful thing in life, to my brothers and sisters,

To the supervisors who supported me Dr. Saleh N. Mwafy and Dr. Saeb Aliwaini,

To my friends who encouraged and supported me,

To the Palestinian people who still suffering until now for freedom.

Ghadeer S. Idhair
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Finally, I would like to express my sincere thanks and gratitude and my love to my dad and my dear mother, my fiancé, my brothers, and sisters and my friends for their unlimited support and encouragement.
Abstract

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Ghadeer Salem Idhair

Background: Cervical and breast cancers are leading causes of death among women. Although many current treatments used to treat these diseases, it is still rapidly spreading worldwide. Recently, triazole compounds have shown their ability to eliminate cancer.

Aim: This study aims to investigate the anticancer properties of newly synthesized Triazoles (14a) against cervical and breast cancer cell lines.

Methods: The cytotoxic effects of the triazole compound (14a) on cervical and breast cancer cell lines (Hela and MCF-7, respectively) were determined using MTT assay. Anti-viability properties examined using trypan blue, clonogenic assay. The ability of the compound to inhibit cancer cell migration was tested by scratch assays. To understand the mechanism of action of compound 14a, key apoptotic and cell cycle proteins were determined using the western blotting.

Results: Our results show that triazole 14a has a cytotoxic effect on both HeLa and MCF-7 cells with IC50s of 54.6μM and 110μM, respectively. The results of the trypan blue test show that 60μM of triazole 14a kills about 85% of HeLa cells after 48 hours of the treatment. However, treatment with 14a at 60μM and 100μM for 48 hours kills around 10% and 30% of MCF-7 cells respectively. In addition, triazole 14a inhibits the ability of cervical cancer cells to migrate in vitro. Clonogenic survival assay shows that 60μM of 14a decreases Hela survival rate by more than 90%. The extrinsic apoptosis and cell cycle arrest are induced by 14a treatment. This is evident by the increased level of cell cycle arrest proteins cyclin D1, P53, and P21 and apoptotic marker cleaved PARP.

Conclusion: A newly synthesized triazole (14a) is a promising compound against cervical and breast cancer cells.

Keywords: Cervical cancer, Breast cancer, Triazole (14a), cyclin D1 and apoptosis
ملخص الدراسة
دراسة الخصائص المضادة للسرطان لدى مركب التريازول (14أ) المصنّع حديثًا على خلايا سرطان عنق الرحم وسرطان الثدي.

ضارم سالم اضهير

خلفية البحث:
يعتبر سرطان عنق الرحم والثدي أحد الأسباب الرئيسية المؤدية للوفاة بين النساء. على الرغم من استخدام العديد من العلاجات المختلفة لعلاج هذا المرض، إلا أنه لا يزال يتطلب سرعة حول العالم.

أهداف البحث:
تهدف هذه الدراسة إلى تقييم الخصائص المضادة للسرطان لمركب التريازول (14أ) ضد خلايا سرطان عنق الرحم والثدي.

منهجية البحث:
أجريت تجربة MTT على خلايا سرطان عنق الرحم والثدي من أجل تحديد تأثيرات السامة لمركب تريازول (14أ) على الخلايا. باستخدام التريازول الأزرق، مولدة النشاط تم فحص الخصائص المضادة للحياة لهذا المركب. وتم اختيار قدرة هذا المركب على منع هجرة الخلايا السرطانية بواسطة فحوصات الخدش لفهم آلية عمل مركب (14أ)، تم تحديد بروتينات خلايا الموت المبرمج ودورة الخلية باستخدام تجربة أطخة وبيستر.

نتائج البحث:
أظهرت نتائج الدراسة أن مركب التريازول (14أ) له تأثير سام على كل من خلايا MCF-7 و Hela. تم تحديد IC50s مع MCF-7 و Hela (60 ميكرومول و 110 ميكرومول). كما أظهرت نتائج اختبار التريازول الأزرق أن مركب تريازول (14أ) يقلل حوالي 85% من خلايا Hela بعد 48 ساعة، ومع ذلك، فإن العلاج بالتريزول (14أ) عند 60μM و 100μM لمدة 48 ساعة يقلل حوالي 10% و 30% من خلايا Hela. علاوة على ذلك، مع تم تحديد نتائج التريازول (14أ) قدرة خلايا سرطان عنق الرحم على الهجرة. أظهر فحص مولدة النشاط أن (14أ) خفض معدل بقاء خلايا Hela أكثر من 90% عند تركيز 60 ميكرومول. تشير النتائج أيضًا إلى موت الخلايا المبرمج الخارجي ووقف دورة الخلية من خلال زيادة مستوى بروتينات أيقاف دورة الخلية cleaved P21 و P53 و P53cyclin D1 و P21ة و MCF-7.

خلاصة البحث:
خلصت نتائج الدراسة إلى أن مركب التريازول (14أ) ذو فعالية عالية ضد خلايا سرطان عنق الرحم والثدي.

الكلمات المفتاحية: سرطان عنق الرحم، سرطان الثدي، التريازول (14أ)، الموت المبرمج cyclin D1
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<tr>
<td>Apaf1</td>
<td>Apoptosis protease-activating factor 1</td>
</tr>
<tr>
<td>Bak</td>
<td>BCL-2 homologous antagonist/ Killer</td>
</tr>
<tr>
<td>Bax</td>
<td>BCL-2 Associated x</td>
</tr>
<tr>
<td>Bel-2</td>
<td>B- cell lymphoma-2</td>
</tr>
<tr>
<td>BRCA</td>
<td>Breast cancer susceptibility</td>
</tr>
<tr>
<td>CCA</td>
<td>Clear-cell adenocarcinomas of vagina or cervix</td>
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<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
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<tr>
<td>COXs</td>
<td>Cyclooxygenases</td>
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<tr>
<td>CRT</td>
<td>Cisplatin-based chemotherapy</td>
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<td>DCIS</td>
<td>Ductal carcinoma in situ</td>
</tr>
<tr>
<td>DD</td>
<td>Death domain</td>
</tr>
<tr>
<td>DDH1</td>
<td>Dihydrodiol dehydrogenase</td>
</tr>
<tr>
<td>DED</td>
<td>Death Effector Domain</td>
</tr>
<tr>
<td>DES</td>
<td>Diethylstilbestrol</td>
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<tr>
<td>DISC</td>
<td>Death-inducing signaling complex</td>
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<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
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<td>DMF</td>
<td>Dimethylformamide</td>
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<td>Dimethyl sulfoxide</td>
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<td>DOX</td>
<td>Doxorubicin</td>
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<tr>
<td>D-SET</td>
<td>D-secoestrone-triazole</td>
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<tr>
<td>EBRT</td>
<td>External beam radiation therapy</td>
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<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
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<td>ECG</td>
<td>Electrocardiographic</td>
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<td>ELIZA</td>
<td>Enzyme linked immunosorbant assay</td>
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<td>ER</td>
<td>Estrogen receptor</td>
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<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>GABA</td>
<td>Antagonist for gamma-amino-butyric acid</td>
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<tr>
<td>HBC-SS</td>
<td>Hereditary Breast Cancer Site Specific</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full name</td>
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<td>--------------</td>
<td>-----------</td>
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<tr>
<td>HBOC</td>
<td>Hereditary Breast Ovarian Cancer</td>
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<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
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<tr>
<td>HPV</td>
<td>Human papillomavirus</td>
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<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
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<tr>
<td>ICC</td>
<td>Invasive cervical cancer</td>
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<td>IC50</td>
<td>The half maximal inhibitory concentration</td>
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<tr>
<td>IDC-NST</td>
<td>Invasive ductal carcinoma of no special type</td>
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<td>IMRT</td>
<td>Intensity-modulated radiotherapy</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<tr>
<td>Ki-67</td>
<td>Kiel-the colone number in the 96 well plate</td>
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<td>LCIS</td>
<td>Lobular carcinoma in situ</td>
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<td>5-LOX</td>
<td>5-lipoxygenase</td>
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<td>MEKK1\JNK</td>
<td>MAPKERK Kinase Kinase\c-Jun N-terminal kinase</td>
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<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazoliumbromide</td>
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<td>NF-Y</td>
<td>Nuclear transcription factor Y subunit alpha</td>
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<tr>
<td>OAR</td>
<td>Organs at risk</td>
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<tr>
<td>OC</td>
<td>Oral contraceptives</td>
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<tr>
<td>HtrA2</td>
<td>High-temperature requirement protein A2</td>
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<tr>
<td>PS3</td>
<td>Tumor protein-53</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PBS/T</td>
<td>Phosphate buffer salin/ Tween</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
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<td>PTV</td>
<td>Planning target volume</td>
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<td>RH</td>
<td>Radical hysterectomy</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium media</td>
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<tr>
<td>SRB</td>
<td>Sulforhodamine B assay</td>
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<tr>
<td>SEM</td>
<td>Standard error of the means</td>
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<tr>
<td>SIL</td>
<td>Squamous epithelial lesions</td>
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<tr>
<td>Smac/DIABLO</td>
<td>Second mitochondria-derived activator of caspase/direct IAP-binding protein with low PI</td>
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<td>Abbreviation</td>
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<td>--------------</td>
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<tr>
<td>TNBCs</td>
<td>Triple negative breast cancers</td>
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<td>TNF</td>
<td>Tumor necrosis factor</td>
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<td>WHO</td>
<td>World Health Organization</td>
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Chapter One
Introduction
1. Introduction

1.1 Overview

Cancer is one of the most common causes of death in both developed and nondeveloped countries (Torre et al., 2015). According to the international agency for research on cancer (IARC), expected that 18.1 million of new cancers cases were diagnosed and 9.6 million cancers deaths in 2018 (Bray et al., 2018). About 550,000 deaths occur annually due to cancer, which indicates that cancer is the second leading cause of death in the world after cardiovascular disease (Akhtar et al., 2017). The number of new cancer cases worldwide is expected to reach 21.6 million by 2030 (Dizon et al., 2016; World Health Organization, 2017). The burden of cancer will increase all over the world due to population growth and aging, especially in the nondeveloped countries. The adaptation on unhealthy lifestyle behaviors, such as smoking, malnutrition, physical inactivity, and reproductive changes led to an increase in the number of cancer patients (Torre et al., 2015).

According to the report of the Palestinian Ministry of Health in 2017 in Gaza strip, the number of new diagnosed cases of cancer during the period of 2015 to 2016 was 3328 cases (males 1478 by 44.3%, and females 1851 by 55.6%). The most common cancers among males are colorectal, lung, leukemia, lymphoma, prostate, and urinary bladder cancers, while the most common types among women are breast, colorectal, thyroid, lymphoma, leukemia and cervical cancers (Palestinian Ministry of Health, 2017a).

Breast cancer ranks the first among women with cancer by 20.5% in Gaza strip (Palestinian Ministry of Health, 2017a). Globally, it ranked first in terms of incidence and mortality between women by 24.2% and 15%, respectively (Bray et al., 2018). Globally, cervical cancer ranks fourth among cancer-related to women, with 6.6% and 7.5%, respectively (Bray et al., 2018). In the Gaza strip, 21.9 cases of the disease were recorded per 100,000 among females with cervical cancer (Palestinian Ministry of Health, 2016).

Each type of cancer needs a special treatment that includes one or more of the following methods; surgery, radiotherapy, chemotherapy, hormone therapy, immune therapy, and targeted therapy (drugs that specifically interfere with the cancer cell growth). Choice of treatment methods depends on the type and phase of cancer that
the patient reached (National Cancer Institute, 2015; World Health Organization, 2014).

Further researches were needed for the development of new therapeutic compounds more effective, safe and selective to gain the battle against this fatal disease. Therefore, there is a need for new classes of drugs to treat cancer (Narsimha et al., 2016). One of the most important compounds that may promising cancer treatment strategies is the triazole compound, due to their very active biological effects and the simple synthesis of these compounds (Kamel and Abdo, 2014; Kharb et al., 2011).

Triazoles played a vital role in the cancer treatment and developing medicine. It have different biological activities such as antiviral (Wu et al., 2018), antimicrobial (Lal et al., 2018), antifungal (Szalaj et al., 2018), anticonvulsant (Plech et al., 2013), antidepressant (Deng et al., 2014), anti-inflammatory (Moussa et al., 2018) and anticancer activities (Parida et al., 2014).

1.2 Objectives of the study
1.2.1 General objective

This work aimed to investigate the anticancer properties of newly synthesized Triazoles (14a) against cervical and breast cancer cell lines.

1.2.2 Specific objectives
1. To investigate the effect of triazole compound (14a) on cell morphology.
2. To examine cytotoxicity activities of triazole compound (14a) on cervical cancer cell lines (Hela) and breast cancer cell lines (MCF-7).
3. To examine the anti-viability activities of triazole compound (14a) on cervical cancer cell lines (Hela) and breast cancer cell lines (MCF-7).
4. To examine the anti-migration activities of triazole compound (14a) on cervical cancer cell lines (Hela).
5. To examine the anti-survival activities of triazole compound (14a) on cervical cancer cell lines (Hela).
6. To investigate whether compound triazole (14a) induces apoptosis and cell cycle arrest in cervical cancer cell lines (Hela).
1.3 **Significance of the study**

Cancer is one of the most challenging and complex diseases, and it is still one of the leading cause of death worldwide. Breast and cervical cancer are diseases that have shown strong resistance against the current treatments that have found. Currently, there is a shortage in the anticancer studies in the Gaza strip and few laboratories provide data related to these diseases. Consequently, more efforts are needed to synthesize and examine new anticancer compounds including traizoles.

1.4 **Limitations**

1. The limited cell lines in the Gaza strip.
2. The search cost is high.
3. Delayed arrival of materials.
4. Inability to experience it *in vivo*. 
Chapter Two
Literature Review
2. Literature Review

Cancer is a considerable public health problem and one of the most prevalent diseases worldwide (Siegel et al., 2017). Cancer is a group of diseases characterized by the uncontrolled growth and the proliferation of abnormal cells in the body (American Cancer Society, 2019). The universal cancer burden is estimated to risen to 18.1 million new cases and 9.6 million deaths in 2018 (Bray et al., 2018; World Health Organization, 2018).

Normal cells grow and divide in a regular manner (Mohammed et al., 2016). There are two types of the tumor; benign tumor may be an unorganized encapsulated mass, however, it does not invade neighboring tissues. The second type is the malignancy tumor, which involves the growth of cells outside the infection site with partial or full loss of regulation. Mostly in advanced disease stages, malignant tumors invade the surrounding tissue (Mohammed et al., 2016). Only a few numbers of about 35,000 genes in the human genome are linked to cancer (Shen et al., 2018). Classified into three groups and they are proto-oncogenes, oncogenes and tumor suppressors.

2.1 The most common types of cancer among people in the world

Lung and breast cancer estimated at 2.09 million cases in 2018. Colon cancer with about 1.80 million cases, while prostate, melanoma, and stomach cancers, have an estimation of 1.28 million cases, 1.04 million cases, and 1.03 million cases, respectively (World Health Organization, 2018).

The total number of cancer patients in Gaza strip from 2015 to 2016 were 3328 patients (males 1478 by 44.3% of patients, while females 1851 by 55.6%) registered within the monitoring center of tumors in Gaza strip. West bank was reported 4000 cancer cases in 2017 (Palestinian Ministry of Health, 2017a, 2017b).

2.1.1 Cervical cancer

Cervical cancer is the fourth most public cancer in women, with about 570,000 new cases in 2018, accounting for 6.6% of all cancers affecting women. About 90% of deaths from cervical cancer happened in low- and middle-outcome countries (Bray et al., 2018; World Health Organization, 2019). Cervical cancer begins in the cells lining the cervical surface of the uterus, it occurs when healthy cells change on the
surface of the cervix and grow out of control, invading tissues and other organs of the body, forming a mass called a tumor (American Cancer Society, 2016d; Pafumi et al., 2011). The infection of the human papillomavirus (HPV), typically HPV-16 and HPV-18, leads to the development of cervical cancer by 20% and 50% of the cases infection (Ghittoni et al., 2015; Goodman, 2015).

American cancer society recorded that 13,170 cases of invasive cervical cancer diagnosed in the United States in 2019. The incidence rates dropped by more than half between 1975 (14.8 per 100,000) and 2015 (6.8 per 100,000). It is expected that the number of deaths of up to 4,250 deaths (American Cancer Society, 2019). In Gaza strip, 21.9 cases of the disease were recorded per 100,000 among females (Palestinian Ministry of Health, 2016).

Cervical cancer malignant and pre-cancerous classified into two main types according to their morphological characteristics under the microscope. Squamous cell carcinoma, which arises from the cells present in the exocervix. Adenocarcinoma which arises in the glandular cells that line the lower birth canal (American Cancer Society, 2016d).

Symptoms of cervical cancer do not appear in the early stages in most cases but they clearly appear in advanced stages of injury. Key symptoms in both stages include abnormal vaginal bleeding that can be heavy or light during the menstrual period, unusual heavy vaginal secretions (foul smelly), pelvic pain, loss of appetite and low weight and pain during urination or urinary incontinence or feces from the vagina (Soumya and Arun Kumar, 2011).

Factors that increase the risk of cervical cancer is: having a family history of cervical cancer, having a personal history of cervical cancer, exposure to diethylstilbestrol, economic status, prolonged use of oral contraceptives (OC), severe diet, age, sex (female), smoking, having a weakened immune system, chlamydia infection and HPV (American Cancer Society, 2017c).

2.1.1.1 Human papillomavirus

Human papillomavirus (HPV) is one of the most important sexually transmitted infection. The frequent infection with high-risk genotypes of HPV is one of the factors necessary for the development of cervical cancer (Roura et al., 2016).
There are two kinds of HPV that are connected with cervical cancer are HPV-16 and HPV-18 (Ghittoni et al., 2015). For the first time in 2007, the World Health Organization (WHO) realized that HPV, especially the HPV-16 virus, maybe a reason for injury to multiple cancers in men (penile and anal), as in women (cervical, vaginal, vulvar and anal). It is possible that several other factors besides the infection of the HPV to induce cervical cancer (e.g. smoking, Immune-suppression, lifestyle and a high range of childbirths) (Giuliano et al., 2015).

2.1.1.2 Immune-suppression

Women with low immune systems suffer from the chance of developing cervical cancer, where the human immunodeficiency virus women suffer from cervical squamous epithelial lesions (SIL) and invasive cervical cancer (ICC). The U.S. centers for disease control and prevention (CDC) showed that invasive cervical cancer was an illness called AIDS in 1993 (Denslow et al., 2014).

2.1.1.3 Exposure to diethylstilbestrol

Diethylstilbestrol (DES) is an industrial estrogen hormone that is prepared for pregnant women to forestall abortion and other gestation complications. Health defects in the uterus appeared in women taking DES drugs are genital mutilation, infertility, and complications of gestation, like abortion, ectopic pregnancy, premature birth, clear-cell adenocarcinomas of vagina or cervix (CCA), in addition to breast cancer (Tournaire et al., 2016). The United States Food and Drug Administration (FDA) prohibited DES describe for pregnant women's in 1971 as a result of reported cases of clear cell glandular carcinoma of the vaginal cell and cervix in young ladies who took their mothers DES (Hilakivi-Clarke, 2014).

2.1.1.4 Smoking

Exposure to smoking may cause the development of cervical HPV infection to malignancy. The international agency for research on cancer (IARC) classified tobacco smoking as a reason for cervical cancer, in particular, the development of both (ICC) and pre-cancer (Ali et al., 2016).
2.1.2 Breast cancer

Breast cancer is the leading cause of cancer death in women by 15.0% (IARC, 2018). Breast cancer may be a heterogeneous illness, consisting of many distinct entities with completely different biological options and clinical behaviors (Weigelt et al., 2010). Breast cancer can start from various places of the breast, but most breast cancers begin in the channels that carry milk to the nipple and called ductal cancers, and also some may occur within the glands that build breast milk known as lobular cancers. When confined to the site of origin, they are classified as non-invasive. But when they are spread to neighboring tissues they are classified as invasive breast cancer (American Cancer Society, 2017d; Weigelt et al., 2010). It estimated that there would be about 2.1 million newly diagnosed female breast cancer cases and 626,679 deaths due to breast cancer globally based on the data from the IARC in 2018. The incidence of breast cancer accounts for about 1 in 4 cases of cancer among females in Australia /New Zealand, Northern Europe, Western Europe, and Southern Europe, requiring complete tissue removal, chemotherapy, radiotherapy, and hormone therapy most of the time (Bray et al., 2018).

The American cancer society estimates that about 268,600 new cases of invasive breast cancer diagnosed in women with 30% cases among females (2,670 in men and 62,930 cases of in situ breast lesions (ductal carcinoma in situ "DCIS" or lobular carcinoma in situ "LCIS" in women) in the United States for 2019 (American Cancer Society, 2019).

Breast cancer in the West Bank was the most common type of cancer, with 503 reported cases in 2017, or 17.2% of all cancers, with an incidence rate of 19.6 per 100,000 population. The proportion of deaths from breast cancer has reached 10%, comes in third place in terms of mortality rate of cancer after lung cancer and colon cancer. Breast cancer was the most common with 498 reported cases in 2017, representing 32.4% of all cancer cases among females, with incidence rate 39.5 per 100,000 female population (Palestinian Ministry of Health, 2017b). While in Gaza strip, the number of cases registered is 684 case, with 20.5% of the total cancer cases, and 36.9% of females’ cancer cases (Palestinian Ministry of Health, 2017a).
2.1.2.1 Types of breast cancer

Breast cancer classified into biological and clinical subgroups depending on histological grade and histological type. The histological grade is an evaluation of the degree of variation and proliferative activity of the tumor. As for the histological type, it refers to the pattern of tumor growth. It is believed that histological types of breast cancer arise from distinct microanatomical structures of the natural breast was identified by Welling's and Jensen, (1973) and Welling's et al. (1975) who proved that most invasive breast cancers arise from the terminal duct-lobular unit regardless of the histological type (Weigelt et al., 2010).

Based on histological type, breast cancer classified into several types as follows:

*Invasive ductal carcinoma* of no special type (IDC-NST) (Carcinoma with osteoclastic and giant cells), *invasive lobular carcinoma* (Classical, Alveolar, Solid and Tubulo-lobular), pure tubular carcinoma, invasive cribriform carcinoma, medullary carcinoma, mucinous carcinoma, and other types. But the most common is the so-called (IDC-NST) (Weigelt et al., 2010).

**Molecular subtypes of breast cancer**

The existence of four molecular subtypes of breast cancer was recognized by the genes expressed by cancer cells (associated with the presence of estrogen receptor "ER", progesterone receptor "PR" and Human epidermal growth factor receptor 2 "HER2") (Weigelt et al., 2010).

These molecular subtypes include luminal (A and B), Triple negative breast cancers (TNBCs)/ basal-like, HER2 and normal breast-like.

**Luminal (A and B): luminal B subtype** is a hormone-receptor positive (estrogen-receptor positive "ER+" and/or progesterone-receptor positive "PR+") subtype, often expressing HER1/ HER2 receptors, the most form of ER + aggressive breast cancer. In this type, Kiel- the clone number in the 96 well plate (Ki-67) used as an alternative sign of proliferation and this subtype shows high levels of Ki-67 protein. **Luminal A subtype** is also known as hormone-receptor- positive (ER+ and/or PR+) but negative for HER2. It shows low levels of Ki-67 protein (Carey et al.,2006; Coates et al., 2015; Creighton, 2012).
Triple-negative breast cancers (TNBCs)/ basal-like: are a hormone-receptor negative (estrogen-receptor-negative "ER-") and progesterone-receptor negative "PR-") and HER2 negative. TNBCs are aggressive tumors of high quality, which is usually resistant to conventional chemotherapy. This subtype of breast cancer account for 10% to 20% of all breast tumors (Mittendorf et al., 2014). These tumors are associated with the age of the younger patient, and these are more common in African American women, especially among premenopausal women. Reports indicated a vital role of BRCA1 and BRCA2 (breast cancer susceptibility 1 and 2) play in the development of TNBCs subtype (Dai et al., 2015).

HER2: subtype is a hormone-receptor-negative (ER-and PR-) and it is positive to HER2. It shows high levels of Ki-67 protein (Russo, 2016). They are tumors identified using gene expression array. HER2 has a bad diagnosis, but it is sensitive to anthracycline and taxane-based neoadjuvant chemotherapy, with a completely satisfactory response far superior to luminal tumors (Dai et al., 2015).

Normal breast-like: it is hormone-receptor-positive (ER+ and/or PR+) and HER2 negative with low levels of the protein Ki-67. Approximately 7.8% of all breast cancer cases are represented in a lymph-node negative cohort (Dai et al., 2015).

2.1.2.2 Signs and symptoms of breast cancer

Symptoms do not usually appear in early breast cancer patients. The most common symptom could be a lump or mass within the breast. Other symptoms may include persistent changes in the breast, such as thickening, swelling, deformation, tenderness, skin irritation, redness, nipple deformities or spontaneous nipple discharge (American Cancer Society, 2019).

The most important factors that increase the incidence rate on this type of cancer are the age of patient for more than 40 years, the history of mammary gland disease, late procreation (35 years later) and women's age at menopause. Although many risk factors identified that increase breast cancer incidence, in 75-80% of women there is no risk factor. This means that it is difficult to explain the pathological causes of this disease accurately (Kamińska et al., 2015).

Factors that increase the risk of breast cancer is: having a family history of breast cancer, having a personal history of breast cancer, exposure to DES, economic
status, prolonged use of OC, severe diet, age, sex (female), certain inherited genes, not breastfeeding and not having children (American Cancer Society, 2017a).

Family susceptibility to incidence breast cancer is one of the most important factors that increase the risk of developing this type of tumor. Several studies carried out in previous years, through which the genes whose function disorder was associated with an increased risk of malignant breast cancers, most notably the BRCA1 and BRCA2 genes that perform the function of tumor suppressor genes in the cell. In normal cells, this gene helps in creating proteins that repair damaged DNA. If mutations occur within the BRCA1 and BRCA2 genes, these may lead to the change of the coding sequence; this alteration might result in genetic syndromes referred to as HBC-SS (Hereditary Breast Cancer Site Specific) or HBOC (Hereditary Breast Ovarian Cancer) syndrome that seems in the form of breast and/or ovaries cancer (Kamińska et al., 2015). Genetic syndromes caused by BRCA1 or BRCA2 gene mutations attached to several clinical signs, which may need complex molecular analysis in the patient. Thus, women with one of these mutations are at risk of infected breast cancer at a small age (below 45 years). This risk is also affected by the number of family members who have contracted this type of cancer (Kamińska et al., 2015). In addition, the high endogenous estrogen level is a well-defined risk factor that contributes to a high rate of breast cancer incidence. A study published in 2002 confirmed a strong relationship between increased concentrations of sex hormones in postmenopausal women and a higher risk of breast cancer (Key et al., 2002). Other studies have confirmed the relationship between estrogen and androgen in the high risk of breast cancer before menopause and may have a role in the development of the disease after menopause. However, the link between OC and the risk of breast cancer is still controversial. Some studies have shown that women using hormonal contraceptives, which take them orally, increase their risk of developing breast cancer by 24% compared to women who did not use these drugs (Kamińska et al., 2015; Roura et al., 2014).

2.2 Cancer treatments

There are different types of treatment for breast and cervical cancer patients. The choice of treatment depends on the kind of cancer as well as the phase of the
disease. Current treatments used for breast and cervical cancer include surgery, radiation, chemotherapy, immunotherapy, and targeted therapy (Zaman et al., 2016).

2.2.1 Surgery

Surgery is a common practice for treating breast and cervical cancer and has been used for centuries. This treatment used for patients with early-stage breast and cervical cancer or for prolonging the patient's life (Bunn et al., 2014; Rydzewska et al., 2014). There are several types of surgery used to treat cervical cancer, including cryosurgery, laser surgery, conization surgery, and hysterectomy surgery (Rydzewska et al., 2014). In addition to some types of surgery that are specifically used to treat breast cancer are (1) breast-conserving surgery (lumpectomy), which is the process of removing the part of the breast that contains only cancer. (2) Mastectomy is a process in which the breast completely is removal, in addition to all tissues of the breast and tissues nearby (American Cancer Society, 2016a).

There are other procedures used to treat cervical cancer, including a traditional procedure called radical hysterectomy (RH). Used in the treatment of cervical cancer patients in the early stages which is associated with postoperative diseases like bladder dysfunction, sexual dysfunction, and colorectal motility disorders (Long et al., 2014). It is a process in which the uterus is removed, fallopian tubes, ovary, cervix, lymph nodes in the pelvis and the upper part of the vagina (Soumya and Arun Kumar, 2011).

All surgeries of breast and cervical cancer surgery exposure risk of complications such as pain or tenderness, temporary swelling, a scar formed at the site of surgery and this scar changes the shape of the breast, the abundance of watery brown secretions, bleeding excessive, urinary or intestinal systems damage in addition to infertility (Bunn et al., 2014; Rydzewska et al., 2014). Incidence rates for breast cancer surgical site infection are documented at 3% to 15%, which is higher than average for clean surgery procedures (Bunn et al., 2014). However, surgical treatment is still the best option to be applied to breast and cervical cancer patients, especially in the early stages of the disease (Rydzewska et al., 2014).
2.2.2 Radiotherapy

Radiation therapy is the mainstay of treatment for breast and cervical cancer and is one of the important indispensable treatments, particularly if the tumor is prevalent to different parts of the body or within the early phases of the unwellness (American Cancer Society, 2017b; Biswal et al., 2011). Adjuvant radiotherapy is given in the case of negative diagnostic factors for the patient or after surgery to help prevent cancer from returning again (Lammerink et al., 2012).

Radiotherapy uses high-energy waves such as X-rays. Besides the use of waves, it is possible to use radioactive molecules that placed inside the patient's body in order to destroy DNA inside cancer cells, which leads to the inability of cells to divide and thus cause death. This treatment can affect normal cells and lead to their destruction. However, most normal cells can recover and return to work properly (Baskar et al., 2012).

Two main types of radiotherapy were used for treatment: external radiation therapy and internal radiation therapy (Baskar et al., 2012).

**External radiation therapy:** also known as external beam radiation (EBRT) uses an external source of high-energy radiation (x-ray), the most common type of radiation therapy in women with cervical and breast cancer. This type of treatment works to direct the radiation on the site of the tumor to eliminate it. They are very resemble to regular x-rays but with a stronger dose and the process is not painful but may cause some side effects which may include fatigue, dyspepsia, diarrhea or loose stools, nausea and vomiting, skin changes, swelling of the breast, radiation cystitis, vaginal pain, menstrual changes, makes the breast becomes smaller, redness and bruising in the treatment site, and low blood counts, which can cause: anemia and leukopenia (American Cancer Society, 2016c). When radiation therapy is used as a major treatment for cervical cancer, external beam radiation and chemotherapy are combined (called concurrent chemo-radiation) (American Cancer Society, 2016c). Primary chemoradiotherapy is used to treat locally advanced stage patients (Lammerink et al., 2012). In addition, it is possible to use EBRT to treat areas of cancer spread or as a major treatment for patients with cervical cancer without the need to use chemotherapy with it (American Cancer Society, 2016c).
Internal radiation therapy (also known as brachytherapy): brachytherapy is used with advanced stages of the disease for patients with predictable cure rates of 30-90% depending on the stage (Jadon et al., 2014). For this treatment, a radioactive source is put inside the woman’s vagina or into the breast tissue for a short time (small shards of radioactive metal implanted in the body close to the cancer mass) (Baskar et al., 2012; Soumya and Arun Kumar, 2011). The use of this treatment is determined based on the size of the tumor and its location and other factors (Baskar et al., 2012). The most common aspect effects are the irritation of the vagina. It may do become red, discharge, breast pain and damage to fatty tissue in the breast. And can cause side effects similar to the side effects of EBRT (American Cancer Society, 2016c).

Radiation therapy for breast and cervical cancer in the early stages for patients reduces the rates of recurrence and death from these diseases (Darby et al., 2013). For breast cancer, radiation therapy has the ability to get rid of tumor foci located in locoregional tissue (such as the chest wall or regional lymph nodes) that, if untreated will cause the return of the disease and death from this carcinoma. Doctors prefer to give radiation therapy to women who have undergone mastectomy, especially for those with four or more positive axillary lymph nodes. In addition, it given to women who underwent surgery that keeps the shape of the breast to prevent the recurrence of the disease. Also to prevent the prevalent of the disease to other organs of the body (McGale et al., 2014).

Intensity-modulated radiotherapy (IMRT) is an example of advanced radiotherapy techniques that may greatly benefit cervical cancer patients by reducing late toxicity and potentiating dose escalation. It is necessary to look at the extent and patterns of internal organ movement before applying IMRT to cervical cancer. For many reasons are: the acute dose gradients around the planning target volume (PTV) with IMRT planning, as the interior movement of organs during treatment, may cause geographic loss of target and non-essential organs at risk (OAR) in high dose areas (Jadon et al., 2014).

2.2.3 Chemotherapy

Chemotherapy is an anti-cancer treatment given either by injection into the vein or by oral administration. In addition, it given to the patient before surgery or
after surgery (Senkus et al., 2015). Chemotherapy used as a methodological approach for cancer treatment and is particularly important for patients with advanced stages of cancer (if the tumor is bigger, or has prevalence to tissues around the tumor), at the same time women can get radiotherapy (chemoradiation) (Rydzewska et al., 2014; Senkus et al., 2015). It recommended giving chemotherapy to triple-negative, HER2-positive breast cancers and in high-risk luminal HER2-negative tumors. It is most useful in women with a type of ER-negative tumors (Senkus et al., 2015).

The most common types of chemotherapeutics are (1) Alkylating agents and related compounds such as cisplatin. (2) Antimetabolites such as methotrexate. (3) Anti-tumor antibiotics such as anthracyclines. (4) Topoisomerase inhibitors. (5) Mitotic inhibitors. (6) Corticosteroids (Corticoid) (Mohammed et al., 2016).

Chemotherapeutics that mostly used to treat breast and cervical cancer include cisplatin, carboplatin, paclitaxel (Taxol), topotecan, gemcitabine (Gemzar) and anthracyclines, such as doxorubicin (Adriamycin). Drugs are usually collected with each other and given to the patient (Senkus et al., 2015; Soumya and Arun Kumar, 2011). Other drugs may be applied against these types of cancers like docetaxel (Taxotere), ifosfamide (Ifex), 5-fluorouracil (5-FU), irinotecan (Camptosar, CPT-11), and bevacizumab (Avastin) (American Cancer Society, 2016b; Senkus et al., 2015). Chemotherapy drugs work to kill fast-growing cancer cells in addition to damaging some normal cells that divide and grow rapidly, causing some side effects depending on the type and dose of the drugs and the length of time it is treated (Soumya and Arun Kumar, 2011). Side effects can include nausea, vomiting, anorexia, loss of hair, mouth sores, fatigue (tiredness), and problems with low blood counts (American Cancer Society, 2016b; Minotti et al., 2004).

Actually, neoadjuvant chemotherapy reduces the overall size of the tumor, extends the survival period of 5 years and reduces the rate of recurrence of the disease. However, the resistance of tumor cells for chemotherapy affects on clinical application and efficacy of treatments (Pariente et al., 2016). Thus, new-targeted therapeutic approaches to reversing the resistance of tumor cells to drugs for cervical and breast cancer treatment had to be developed (Minotti et al., 2004; Pariente et al., 2016).
2.2.3.1 Cisplatin-based chemotherapy

Cisplatin-based chemotherapy (CRT) was considered a standard treatment for patients with locally progressive cervical cancer (Nogueira- Rodrigues et al., 2014). However, chemotherapy remains an option for patients with recurrent disease, especially after surgical treatment and radiation therapy (American Cancer Society, 2016b). Cisplatin is a well-known treatment used as an anticancer drug that is often used to treat various human malignancies (Soumya and Arun Kumar, 2011).

Cisplatin activated as soon as it enters the cell. In the cytoplasm, chloride atoms are broken onto cisplatin by water molecules. Cisplatin related with the N7 reactive center on purine residues and so will spoil DNA found in cancer cells thus cisplatin mostly stimulates cell death by apoptosis. The genotoxic stress resulting from cisplatin activates multiple signal transfer methods, which can be involved in apoptosis or chemical resistance (Dasari and Bernard Tchounwou, 2014).

Cisplatin treatment problems occur because cisplatin reacted with DNA, which results toxic effects on the cells, leading to the occurrence of toxic side effects including nephrotoxicity, hepatotoxicity, and cardiotoxicity (Dasari and Bernard Tchounwou, 2014). Many cases of heart diseases resulting from the use of cisplatin have been reported in Yousef et al., study (Yousef et al., 2009). These reports include changes in electrocardiographic (ECG), arrhythmias, myocarditis, cardiomyopathy, and congestive heart failure (Dasari and Bernard Tchounwou, 2014).

There are several reasons for cisplatin resistance as a defect in the MAPK/ERK kinase kinase (MEKK)/ c-Jun N-terminal kinase (MEKKI/JNK) pathway blunting the activity of caspases 3 and the enzyme dihydrodiol dehydrogenase (DDH1) is upstream of cisplatin-induced mitochondrial membrane depolarization and activation of JNK and p38 in the cisplatin-resistant cells. It also observed that transcription factor "Nuclear transcription factor Y subunit alpha (NF-Y)" has a role in multiplying transcriptional activity of the DDH1 gene in the cisplatin-sensitive cells but not in the cisplatin-resistant cells next cisplatin treatment. However, it is not possible to know how reactive oxygen species (ROS) scavenger works well because her method of work is more complicated (Chen et al., 2015).
2.2.3.2 Anthracyclines

Anthracycline is one of another drug that act as anti-tumor antibiotics. It interferes with DNA replication enzymes and works throughout all stages of the cell, such as doxorubicin (Minotti et al., 2004).

Doxorubicin (DOX), like any other toxic agent, activates tumor protein-53 – DNA (p53-DNA) binding, which in turn plays a role in apoptosis. DOX works to destroy DNA in cancer cells in several steps. The first step involves the introduction of DOX into cancer cells by simple diffusion and is strongly associated with proteasomes in the cytoplasm. The second step DOX is linked with a 20S proteasomal subunit and forming a DOX-proteasome complex that passes into the nucleus through the nuclear pores. In the last step, DOX separated from the proteasome and goes to bind with DNA in the nucleus. Because of the possible chemical and therapeutic effects of DOX-proteasome interactions, it will help to increase anthracycline targeting in the nucleus and the accumulation of undegraded proteins that indicate apoptosis (Minotti et al., 2004).

2.2.3.3 Triazole

Triazoles are heterocyclic compounds with a chemical structure comprising one or more five-membered rings that contain three nitrogen atoms and two carbon atoms at non-adjacent positions (Mohammed et al., 2016). It is a white to pale yellow crystalline solid with a distinctive weak smell, it is soluble in water and alcohol, dissolves at 120 ° C and boils at 260 ° C (Kharb et al., 2011).

Triazole is one of the most heterocyclic compounds that exhibit significant pharmacological activity because it is played a vital role in the metabolism of all living cells, and considerable biological actions as antimigraine, antimycotic, antiallergic, herbicidal and anticancer (Celik et al., 2018; Saini et al., 2013). It is an aromatic compound dissolved in all organic solvents. Triazole was first synthesized by Fisher in 1878 (Maddila et al., 2013). In 1885, the triazole turned into first given to the carbon-nitrogen ring system C2N3H3 by Bladin. There are two kinds of triazole, 1,2,3-triazole (v-triazoles) and 1,2,4-triazole(s-triazoles) (Mohammed et al., 2016). Each of the two types owns two tautomers, which vary from each other by the way nitrogens linked to the hydrogens (Figure 2.1).
Figure (2.1): Illustration of the two classes of triazoles, 1,2,3 triazoles (v-triazoles) and 1,2,4-triazoles (s-triazoles) (Mohammed et al., 2016).

The compound 1, 2, 3-triazole and its derivatives was used as anti-bacterial (Tan et al., 2016), anti-fungal (Dai et al., 2015), anti-oxidant (Shaikh et al., 2016), anti-malarial (Raj et al., 2013) and anti-leishmaniasis drugs (Gontijo et al., 2015). While compound 1, 2, 4-triazole and its derivatives were used in pharmaceutical formulations more than the use of compound 1, 2, 3-triazole were of great importance in the treatment of a large number of diseases such as epilepsy (Kücükgüzel et al., 2004), hypertension (Kakefuda et al., 2002) and cancer (Yadagiri et al., 2015) and Alzheimer's (Wang et al., 2018). Both types of triazole derivatives (1,2,3-triazole and 1,2,4-triazole) were used in the manufacture of dyes and in photography, in addition to being used as insecticides, anticonvulsant (Shneine and Alaraji, 2016) and it also has been reported as inhibitors of glycogen synthase kinase-3 (Tantray et al., 2016) and the antagonist for gamma-amino-butyric acid (GABA) receptors (Giraudo et al., 2018).

The triazoles compounds and their derivatives played a vital and biological role in the manufacture and development of medical chemicals. Because of these massive effects from the use of triazole compounds, work on the design of new triazole derivatives has become a trend of medicinal chemists (Pokhodylo et al., 2013).

There are several reasons that can lead to the emergence of a wide spectrum from biological activities for compounds that have triazole rings (Li et al., 2012). One of these reasons is the electron-rich aromatic structure that binds easily to a variety of
enzymes and receptors via weak interaction such as hydrogen bond (Kulabaş et al., 2016).

### 2.2.3.3.1 Previous studies

The triazole compounds have demonstrated their ability to show different drug activities e.g. antifungal, antibacterial, anti-inflammatory antiviral and anticancer activities.

Sun et al. manufactured seventeen novel 1,2,4-triazole derivatives containing pyridine intensity under the microwave helper condition to investigate their ability to perform a biological activity against Stemphylium lycopersici (Enjoji) Yamamoto, Fusarium oxysporum. sp. cucumebrium, and Botrytis cinerea in vivo. The results showed that some compounds showed excellent fungicidal activity (Sun et al., 2014).

Sekhar et al., in 2018 described how to produce a group of new categories of methylthio linked pyrimidinyl under conventional and ultrasound irradiation ways. The results revealed that the compound 12c and 12f showed effective antibacterial activity against Pseudomonas aeruginosa whereas the compounds 13c and 13f showed noticeable antifungal activity against Aspergillus niger (Madhu Sekhar et al., 2018).

In 2014, a series of diaryl-1,2,4-triazole and hydroxamic acid or N-hydroxyurea were produced in order to evaluate them as a novel anti-inflammatory agent. Results showed that compounds (8c - 8e), 10e, 10k, 10m and 15e acted as a potent COX-2 inhibitory activity. In addition, the compound 15e showed strong anti-inflammatory and oral analgesic activity and 15e showed optimal dual cyclooxygenase/5-lipoxygenase (COX-2/5-LOX) inhibitory activities in vitro. Finally, the molecular modeling study revealed that 15e were associated with the active sites of COX-2 and 5-LOX by hydro-phobic interactions and stabilizes of binding formation with one or more hydrogen bonds. Thus, it was concluded that compound 15e may be considered an anti-inflammatory agent, by Jiang et al (Jiang et al., 2014).

De Lourdes et al. created three novel nucleosides of 1,2,3-triazole analogs successfully. The results showed that the chemical composition of the 1,2,3-triazole analogs could be important prototypes for the development of novel antiretroviral medicine and anti-influenza medicine (De Lourdes et al., 2014).
Most likely, probably attribute the anti-cancer properties of the triazole derivatives due to their proximity to the vital anti-cancer targets such as tumor necrosis factor (TNFα), anti-apoptotic bio-complex Bcl-XL-BH3. This makes the triazole drugs can be used in the discovery of a beneficial treatment against cancer (Kharb et al., 2011).

Bózsity et al. have described the mechanism of action of d-secoestrone-triazole (D-SET) on three cervical cancer cell lines (HeLa "HPV 18+", SiHa "HPV 16+", C-33 A "HPV negative"). The results showed that D-SET is a potent antiproliferation factor against HPV 16+ and HPV negative cell lines, with effective inhibitory activity against HPV 16+ cells and it has been shown that the D-SET inhibits the migration of HeLa cells after 24 hours of incubation and prevents its spread to tissues surrounding the cervix (Bózsity et al., 2017).

Nagarsenkar et al. have synthesized groups of triazole-linked glycoconjugates (29 compounds). The new compounds were assessed on DU145 (prostate cancer), HeLa (cervical cancer), A549 (lung cancer) and MCF-7 (breast cancer) cell lines. The results indicated that compounds 5f (indole derivative) and E-9b (oxindole derivative) showed notable cytotoxic activity against DU145 cells using Sulforhodamine B (SRB) assay. As well as its ability to cell cycle arrest through the accumulation of cells in the sub-G1 phase, which prevented the growth and proliferation of DU145 cells using cell cycle analysis. Thus, these compounds can be potential drugs that will be used as an anticancer (Nagarsenkar et al., 2016).

In 2017, three new compounds manufactured from substituted indolyl-triazoles. The ability of these compounds to act as an antiproliferative activity against cell lines HEPG-2 (liver cancer) and MCF-7(breast cancer) examined. The results showed that these compounds had the ability to act as anticancer agents against the cell lines HEPG-2 and MCF-7 (Boraei et al., 2017).

2.3 Anti-tumor activity of triazole compounds

Triazole has an important anti-tumor activity in many types of cancer cells, but there have been few reports that show how these compounds begin their activity against the tumor. Triazole compounds showed anticancer effects, stimulating apoptosis and cell cycle arrest (Obchoei et al., 2016).
2.3.1 Apoptotic pathway

There are two main pathways for apoptosis, the external pathway, and the internal pathway, as well as another pathway called granzyme B, a pathway that includes T-cell mediated cytotoxicity. In addition, there are other paths to activate the caspase that is not well known (Goldar et al., 2015).

Extrinsic apoptosis process

The extrinsic path of apoptosis begins when an extracellular signal such as the tumor necrosis factor (TNF) attached to the extracellular domain of cell receptors (death receptors). Because of the correlation of the death signal with the death receptors, the adaptor proteins such as Fas-associated death domain (FADD) will be associated with the specific death domain (DD) of the death receptor by the FADD death domain. Adaptor proteins contain an area of protein interaction domain called Death Effector Domain (DED). Procaspase-8 contains DED and therefore Procaspase-8 will bind to the adaptor protein (FADD) through DED. At this time, a death-inducing signaling complex (DISC) was developed, triggering an automatic catalyst for procaspase-8, and because of the inactive procaspase-8 bind with the receptor, it will cut the inactive part of procaspase-8 and convert it to active caspase-8 Which in turn activates executioner procaspases (3, 6 and 7), leading to occurrence external apoptosis (Goldar et al., 2015) see (Figure 2.2).

Intrinsic apoptosis process

The destruction of the DNA activates the tumor-suppressor protein p53 that activates BCL-2 Associated x (BAX), BCL-2 homologous antagonist/ killer (BAK), Phorbol-12-myristate-13-acetate-induced protein 1 (NOXA), and others. At the same time, it inhibited the anti-apoptotic Bcl-2 family and prevented the cells from surviving (i.e. preventing their transcription). The association of the p53 protein with the pro-apoptotic BH3-only proteins will trigger the cytochrome c release from the mitochondria to the cytoplasm. The role of BAX and BAK begins after apoptosis activated and its role is to activate and insert these stimuli into the mitochondrial membrane, which causes the cytochrome c to be emitted to the cytoplasm. At the same time, the second mitochondria-derived activator of caspase/direct IAP-binding protein with low PI (Smac/DIABLO) and high-temperature requirement protein A2
(Omi/HtrA2) proteins will be released to the cytoplasm, which highlights its role in providing an additional mechanism to activate the caspase. After the launch of the cytochrome c will react with apoptosis protease-activating factor 1 (Apaf1) and this reaction, leads to the composition of a compound called apoptosome. The apoptosome compound acts to activate caspase-9 which in turn activates executioner caspases (3,6and7), resulting in intrinsic apoptosis (Goldar et al., 2015) see (Figure 2.2).

Figure (2.2): Schematic Diagram of the Intrinsic, Extrinsic Pathways of Apoptosis (Goldar et al., 2015).

Research on a group of triazole compounds revealed that they have potent anti-cancer activity on different cancer cell lines, and results showed these compounds to contribute to apoptosis through destroying the membrane mitochondria and activating of caspases −9, -3 (Khan et al., 2016). In addition, researchers who designed triazole-piperazine hybrids compounds showed that it has anticancer agents
against six cancer cell lines. These compounds showed has the ability to stimulate apoptosis, and stop G2/M from the cell cycle (Mishra et al., 2017) see Appendix 1.

2.3.2 Cell cycle arrest

Another suggested mechanism of action for triazole complexes is cell cycle arrest is to stop cell division. A recent study on several 1,2,4-triazole compounds revealed that it had different effects on cell cycle status in different cancer cell lines, treatment ovarian cancer cell lines (SKOV3) with the triazoles compounds induces cells cycle arrest in G2-M phase (Ghanaat et al., 2019). Moreover, another study conducted on Carboxyamido-triazole compound in breast cancer cell lines (MCF-7) proved that the compound induced G2/M cell cycle arrest through increased p21 protein (Guo et al., 2006) see Appendix 1.
Chapter Three
Materials and Methods
3. Materials and Methods

3.1 Materials

3.1.1 Chemicals and Reagents

The chemicals and reagents used in this study summarized in Table 3.1.

Table 3.1: Chemicals and reagents that used in the study.

<table>
<thead>
<tr>
<th>#</th>
<th>Reagents</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DMEM– Dulbecco's Modified Eagle Medium</td>
<td>Biological Industries</td>
</tr>
<tr>
<td>2</td>
<td>RPMI1640-Roswell Park Memorial Institute medium media</td>
<td>Biological Industries</td>
</tr>
<tr>
<td>3</td>
<td>Trypsin</td>
<td>Biological Industries</td>
</tr>
<tr>
<td>4</td>
<td>Phosphate Buffer Saline</td>
<td>AppliChem, Germany</td>
</tr>
<tr>
<td>5</td>
<td>Fetal Bovine Serum (FBS)</td>
<td>Biological Industries</td>
</tr>
<tr>
<td>6</td>
<td>Primary Antibody</td>
<td>Santa cruz, USA</td>
</tr>
<tr>
<td>7</td>
<td>Secondary Antibody</td>
<td>Santa cruz, USA</td>
</tr>
<tr>
<td>8</td>
<td>MTT Kit</td>
<td>Sigma, USA</td>
</tr>
<tr>
<td>9</td>
<td>Trypan Blue</td>
<td>Biological Industries, OPT</td>
</tr>
<tr>
<td>10</td>
<td>Western Blot Reagents</td>
<td>AppliChem, Germany</td>
</tr>
<tr>
<td>11</td>
<td>Ethylene diamine tetra acetic acid (EDTA)</td>
<td>AppliChem, Germany</td>
</tr>
<tr>
<td>12</td>
<td>Dimethyl sulfoxide (DMSO)</td>
<td>AppliChem, Germany</td>
</tr>
<tr>
<td>13</td>
<td>Giemsa stain</td>
<td>Sigma, USA</td>
</tr>
<tr>
<td>14</td>
<td>New Triazole compound (14a)</td>
<td>Department of chemistry – The Islamic university of Gaza</td>
</tr>
</tbody>
</table>
3.1.2 Disposables

Table 3.2: The major disposables used.

<table>
<thead>
<tr>
<th>#</th>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Culture Flask</td>
<td>Intron</td>
</tr>
<tr>
<td>2</td>
<td>96 well plate</td>
<td>Intron</td>
</tr>
<tr>
<td>3</td>
<td>6 well plate</td>
<td>Intron</td>
</tr>
<tr>
<td>4</td>
<td>Petri dish (6cm)</td>
<td>Intron</td>
</tr>
<tr>
<td>5</td>
<td>Pipettes</td>
<td>Intron</td>
</tr>
<tr>
<td>6</td>
<td>Falcon Centrifuge Tube</td>
<td>Intron</td>
</tr>
<tr>
<td>7</td>
<td>Eppendorf</td>
<td>Intron</td>
</tr>
</tbody>
</table>

3.1.3 Equipment

Table 3.3: Major Equipment’s used in this study.

<table>
<thead>
<tr>
<th>#</th>
<th>Instrument</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Centrifuge</td>
<td>Centurion scientific Ltd</td>
</tr>
<tr>
<td>2</td>
<td>Safety cabinet</td>
<td>Biological Safety Cabinet (NB-602 WS/WSL)</td>
</tr>
<tr>
<td>3</td>
<td>Western Blot Unit</td>
<td>BIO RADMMini- protean @ tetra system</td>
</tr>
<tr>
<td>4</td>
<td>Micropipettes</td>
<td>Scilogex (100-1000µM)</td>
</tr>
<tr>
<td>5</td>
<td>Dispenser</td>
<td>JENCONS SELPETTE</td>
</tr>
<tr>
<td>6</td>
<td>CO₂ Incubator</td>
<td>Nb-203x1</td>
</tr>
<tr>
<td>7</td>
<td>Elisa Reader</td>
<td>VMax® Kinetic ELISA Microplate Reader with Softmax® Pro Software</td>
</tr>
<tr>
<td>8</td>
<td>Inverted microscope</td>
<td>Motic- at3ie- tension</td>
</tr>
</tbody>
</table>
3.1.4 Cell lines

Table 3.4: Cell lines used in this study.

<table>
<thead>
<tr>
<th>#</th>
<th>Cell lines</th>
<th>sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-</td>
<td>Human breast adenocarcinoma (ER+) called (MCF7)</td>
<td>Faculty of health sciences, Islamic University of Gaza</td>
</tr>
<tr>
<td>2-</td>
<td>Uterine Cervical adenocarcinoma (HeLa)</td>
<td>Birzeit University of Palestine</td>
</tr>
</tbody>
</table>

3.2 Methodology

3.2.1 Study design

An experimental *in vitro* study cell culture.

3.3 Methods

3.3.1 Cell culture maintenance

The MCF-7 human breast adenocarcinoma (estrogen receptor positive) cells kind gifts of Faculty of health sciences, Islamic University of Gaza and Hela cells from Birzeit University of Palestine. MCF-7 cell line was preserved in the Dulbecco's Modified Eagle Medium (DMEM) and the Hela cell line was preserved in Roswell Park Memorial Institute medium media (RPMI 1640). All media were complemented with 10% fetal bovine serum, 100 U/mL penicillin and 100μg/mL streptomycin. Cells conserved at 37°C in a 5% CO₂ and 95% air-humidified incubator. The media was replaced every 2-3 days (Aliwaini et al., 2015; Wang et al., 2016).

3.3.2 Treatments

A panel of triazoles based compounds synthesized in the laboratory of chemistry department, to act as anticancer agents against the uterus cervical and breast cancer cell line. The manufactured compounds were composite (14a) (Figure 3.1). The compound 14a dissolved in Dimethylsulfoxide (DMSO) or Dimethylformamide (DMF) (at 100 °C) to give 10mM. The compound stored at RT for no more than 7 days. Dilutions of the triazole derivative compound in the
appropriate media to get the final concentration for each cell line (Figure 3.2). Vehicle-treated cells incubated in normal media with DMF or DMSO (the vehicle in which triazole-compound was dissolved in).

Figure (3.1): Structural formula of the Triazole compound (14a). 3-Acetyl-4-benzoylamino-1-(4-chlorophenyl)-1,2,4-triazaspiro[4.6]undec-2-ene (14a) (C23H25ClN4O2).
3.3.3 Cell morphology

Cells plated at suitable numbers in order to obtain 60-70% confluency on the days of treatment. After treating the cells with triazole compound (14a), the morphological changes were monitored and photographed using an inverted light microscope (Olympus 1X71, USA) with camera (Zeiss Axio Cam, Germany) respectively. The morphological changes photographed using a light microscope.

3.3.4 Cytotoxicity assays [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide "MTT"]

To determine the cytotoxic effect of the indicated compound, HeLa and MCF-7 cells were seeded, separately (4000-6000 HeLa cells/well), (8000-1,000,000 MCF-7 cells/well) in quadruplicate in a 96-well plate in 100µL culture media and treated after 24 hours with a range of the indicated concentrations (0.0–100µM) of triazole.
compound (14a) or vehicle for 48 hours. Cell viability was determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instructions (Roche, USA). Briefly, 10μL of MTT solution added to each well and incubated for 4 hours at 37°C. This followed by addition of 100μL solubilization buffer (10% SDS in 0.01 M HCl) and incubated overnight at 37°C. The absorbance was read at (595nm) by using Automated ELIZA reader (CF-fiocchetti, Italy). Cell viability of treated cells calculated after taking in reference to the untreated control cells (Hela and MCF-7 cell lines) using the formula. Cell viability percent was taken after calculated the average of quadruplicate reading of absorbance (Shindikar et al., 2016; Martinho et al., 2017).

\[
\text{Viability} \, (\%) = \frac{\text{Sample Abs}}{\text{Control Abs}} \times 100
\]

Two dimensions chart drawn for different triazole compound concentrations on X-axis and percent of cell viability on Y- axis. Then IC₅₀ calculated by using linear regression of chart formula \(Y = a \times X + b\), IC₅₀ value equal X value estimated when Y value equal 50.

Three separate experiments performed to determine the concentration of 14a required for killing 50% of the cells (IC₅₀). The IC₅₀ values were calculated from the linear equation from Microsoft office excel as describe before (Aliwaini et al., 2015; Wang et al., 2016).

### 3.3.5 Viability assay /Trypan blue assay

Trypan blue exclusion technique used to study the cytotoxic effect of the indicated compound in HeLa, and MCF-7cell lines. To determine the ability of the triazole compound (14a) to induce cell death in cancer cells, these cells were seeded, in duplicate in a 6-well plate in order to obtain 60-70% confluency and treated in a second day with the indicated concentrations of specific compound or vehicles. Cell viability assessed by the trypan blue assay after 24 and 48 hours of treatment and results were analyzed by Excel Microsoft office 2010. Total cells and viable cells counted by trypan blue exclusion as follows. The medium was disposed and then the cells harvested by trypsinization, placed in the center of the centrifuge for 5 minutes and then washed with phosphate-buffered saline (PBS). The cells then diluted at a 1:9 ratio of the volume of 0.4% trypan blue stain (Biological Industries, Occupied
Palestinian Territory "OPT"). After 5 min incubation, the cells counted and analyzed under a light microscope (Olympus CX L, Tokyo, Japan) with a cell-counting chamber. The unstained (viable) cells and the blue-stained (dead) cells were counted separately and the total numbers of living and dead cells were calculated (Yamasaki et al., 2007). The percentage of cell viability calculated using the following equation:

\[
\% \text{ cell viability} = \frac{\text{total viable cells (unstained)}}{\text{total cells (stained + unstained)}} \times 100
\]

3.3.6 *In vitro* cell migration assay

*In vitro* scratch motility assay: Cells grown to 100 % confluence in 6cm tissue culture dishes. A linear wound performed by scratching the monolayer using a sterile 100μl pipette tip. To remove cell debris, the growth medium replaced after the cells washed one times with media, and the plates incubated at 37 °C in 5% CO₂, after added treatment. Several markings made along the edges of the scratch line, which used as reference points. The wound width measured for the Hela cells line at the time of the scratching (0 hr.) and thereafter at 6, 12, 24 hr. after scratching. The images taken using a microscope and Image J software used to measure the wound width. The difference in width represents the distance migrated in μm (Liu et al., 2016).

3.3.7 Clonogenic survival assay

Hela cells were pre-cultured in six well plates, treated with 30μM and 60μM of triazole compound (14a) for 24 hours, re-plated at low density (500 and 1000), and incubated for 14 days. Following 14 days of growth, surviving cells fixed in methanol for 5 min at room temperature and stained with 10% Giemsa stain (Sigma, USA) in deionized water for 20 min at room temperature. Stained colonies washed one time in distilled water. Percentage change in surviving colonies is calculated by dividing the number of colonies calculated in the sample by the number of colonies calculated in the control multiplied by 100% (Aliwaini et al., 2015).
3.3.8 Western blot analysis

Cell cycle arrest and apoptosis examined by western blotting, using primary antibodies determined for apoptosis and cell cycle arrest proteins such as P53, P21, cyclin D1, PARP, tubulin, and caspases8.

SDS-PAGE and immunoblotting, cells plated at 6×10^5 in 6cm dishes and treated with 14a for 48 hr. Cells analyzed in whole cell lysis buffer (0.5 M Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 1% b-mercaptoethanol and 0.02% bromophenol blue) and samples boiled for 10 min. Proteins resolved by SDS/PAGE (8–12% gels) as required and transferred to Hybond ECL membranes (Amersham Biosciences). Membranes blocked for 1 hr. at room temperature with PBS containing 5% non-fat dry milk and probed with appropriate primary antibodies at 4°C with shaking. Membranes washed in PBS containing 0.1% Tween 20 (PBS/T) and incubated with donkey anti-goat (Santa Cruz Biotechnology, CA, and USA), goat anti-mouse or goat anti-rabbit IgG peroxidase-conjugated secondary antibodies (1:5000) (BioRad, Hercules, CA, USA). The membranes were incubated with primary antibodies against p53, p21, PARP, Cyclin D1, tubulin, and caspases8 (Santa Cruz, CA, USA) in blocking solution at room temperature with shaking for 1 hour. After the primary antibody incubation, the membranes were incubated with appropriate HRP-conjugated secondary antibodies (1:5000) (Biorad) and antibody-reactive proteins were visualized using the chemiluminescence reaction (ECL) detection system (Thermo Scientific, Hudson, NH, USA) (Aliwaini et al., 2013; Yamasaki et al., 2007).

3.3.9 Statistical analysis

Data presented are mean ± SEM (Standard error of the means) of appropriate replicates. Statistical significance assessed between the groups using the Student’s t-test.
Chapter Four

Results
4. Results

This experiments were *in vitro* study cell culture revealed the anti-cancer properties of newly synthesized triazole based compound (14a) as a promising anticancer agent against breast and cervical cancer cell lines.

4.1 Anti-cancer activities of Triazole compound (14a) on the cervical cancer cell line (Hela).

4.1.1 *The antiproliferative effect of Triazole compound (14a) on cervical cancer cell line (Hela).*

The anti-proliferative effect of 14a compound on cervical cancer cell line (Hela) determined by MTT assay. Hela cells were treated using different concentrations of the compound 14a (0.0µM–100µM). The results showed that 14a exerts a pronounced anti-proliferative effect on Hela cells with an IC₅₀ value of 54.6µM. Figure (4.1a) reveals that 14a induced very strong cytotoxic effects at 60µM. While low concentrations of 14a (10µM–30µM) showed a reasonable cytotoxic effect on Hela cells, approximately 55% of cervical cancer cells were killed at 60µM of 14a (*Figure 4.1a*). Moreover, 14a at 30µM and 60µM induced clear apoptotic morphological changes such as shrinked, rounded and flattened cells (*Figure 4.1b*). These results showed that 14a has a significant cytotoxic effect on Hela cells.
Figure (4.1 a): 14a induces cytotoxicity on cervical cancer cells (HeLa). HeLa cells plated in 96-well plates and after 24 hours, the cells treated with accumulative concentrations of 14a compound (0-100μM). Cell viability assessed by (MTT) assay after 48 hours of treatment. Results show the mean percentage of cell growth ± STD (bars) of at least three experiments conducted in quadruplicates.
Figure (4.1 b): 14a induces apoptotic morphological changes on cervical cancer cells (Hela). Hela cells treated with 30μM and 60μM of 14a compound. After 48 hours of the treatment, cells were visualized and photos were taken under 4X, 10 X. Pictures showed differences in cells morphology between control (0.0μM) and 14a treated cells.
4.1.2 Effect of Triazole compound (14a) on the viability of the cervical cancer cell line (Hela).

The effect of 14a on the cell viability of Hela cells measured by trypan blue assay. Hela cells were treated by different concentrations of the compound 14a (0.0μM, 30μM, and 60μM) for 24 and 48 hours. The results showed that the cell viability of Hela cells decreased in a dose- and time-dependent manner. For example, 14a killed 5% of Hela cells at 30μM after 24 hours of treatment and 75% after 48 hours of treatment. Importantly, at 60μM, 14a killed 10% of the Hela cells after 24 hours of treatment and 85% after 48 hours, as shown in (Figure 4.2). These results suggest that the toxicity of 14a might be via activating a certain type of cell death.

![Figure 4.2: 14a inhibits cervical cancer cell viability (Hela).](image)

Figure (4.2): 14a inhibits cervical cancer cell viability (Hela). Hela cells plated in 6-well plates and after 24 hours, cells treated with 0.0μM, 30μM and 60μM of compound 14a or its vehicle. Cell viability assessed by trypan blue assay after 24 and 48 hours of the treatment. Results represent the mean percentage ± SEM of control of at least two experiments performed in twice replicates.
4.1.3 Effect of Triazole compound (14a) on cervical cancer cells migration (Hela).

The possible anti-migration ability of 14a, was evaluated by scratch motility assay. A slight reduction in cell migration was observed of Hela cell line exposed to 30μM of 14a for 0, 6, 12 and 24 hours (Figure 4.3a). Importantly, the anti-migratory effect more pronounced at 12 hours where untreated cells showed an 85% closure of the scratch while treated cells showed 73% closure. While untreated cells showed a 99% closure of the scratch after 24 hours while the treated cells showed an 88% closure of the scratch. Figure 4.3b showed the wound healing distance in the control and the treated Hela cells at different time points (0, 6, 12 and 24 hours) of treatment. These results showed that the 14a compound has a minor anti-migration effect on Hela cells.

Figure (4.3a): 14a slightly inhibits the migration ability of cervical cancer cells (Hela). Cells grown to 90-100% confluence and a linear wound created through the cell monolayer. Cell motility was assayed at the indicated times (0, 6, 12, 24) hours after the addition of either vehicle (control) or 14a (30μM). These results represent the mean percentage ± SEM of control cells (treated with vehicle) of three experiments conducted in quadruplicate.
Figure (4.3 b): Illustrates the wound healing distance of cervical cancer cells (Hela). Hela cells treated with 30μM of the 14a compound. At specific time points, cells photographed using (10x; Inverted microscope) and the area of the wound measured and expressed relative to zero time. Pictures showed the wound healing distance at different time points in control and treated Hela cells.
4.1.4 Effect of Triazole compound (14a) on colony formation ability of cervical cancer cells (Hela).

Cell survival rate of Hela cells treated with compound 14a measured by clonogenic assay. Hela cells treated with 30μM and 60μM of 14a compound for 24 hours, re-plated at low density (500 and 1000 cells) and incubated for 14 days. The results showed that while untreated Hela cells survived at both cell concentrations, the survival rate of Hela cells treated with 14a decreased significantly (Figure 4.4a). Indeed, 14a reduced Hela cells survival rate more than 90% at 60μM and 70% at 30μM in 1000 cells concentration. Interestingly, in 500 cells concentration, the compound 14a reduced Hela cells survival rate 100% at 60μM and over 80% at 30μM. The pictures in Figure 4.4b showed that both concentrations of compound 14a decreased the ability of treated Hela to form colonies.
Figure (4.4): Inhibition effect of 14a compound on cell survival rate of cervical cancer cells (Hela). Hela cells were treated with 30μM and 60μM of 14a compounds for 24 hours and then re-plated at low density (500 and 1000 cells) and incubated for 14 days. (a) Results show the mean percentage ± SEM of untreated cells and represent the pooled results of at least two experiments performed. (b) The pictures show the number of colonies formed in control and treated Hela cells.
4.1.5 Anticancer effect of 14a, Molecular mechanism of action.

Hela cells treated with 30μM and 60μM of 14a for 48 hours to investigate how 14a induces its anticancer effect. Protein lysate analyzed by western blotting for key proteins of cell cycle arrest and apoptosis.

4.1.5.1 Triazole compound (14a) induces cell cycle arrest on cervical cancer cells (Hela).

In this part, we analyzed the levels of cell cycle proteins P21, P53, and cyclin D1 by the western blotting technique.

The results showed that there was a remarkable increase in the level of p53 protein in a concentration-dependent manner in Hela cells treated with compound 14a after 48 hours. Consequently, P21 protein appeared clearly at 60μm concentration. However, no band observed in the untreated cells (0.0μm) and the treated cells (30μm) for protein p21 as shown in (Figure 4.5).

In addition, the level of cyclin D1 (a checkpoint protein) was also increased when Hela cells were treated with compound 60μm of 14a (Figure 4.5). In summary, these results showed that compound 14a has the ability to induce the cell cycle arrest in Hela cells.
Figure (4.5): Effect of 14a on the level of p53, p21 and cyclin D1 in cervical cancer cells (Hela). Cells treated with increasing concentrations (0, 30, 60µM) of 14a and incubated for 48 hours. Western blotting performed to detect the levels of P53, P21, and cyclin D1. Tubulin used as a loading control.
4.1.5.2 Triazole compound (14a) induces extrinsic apoptosis

In the results of the trypan blue test, death in Hela cells appeared after 48 hours of treatment. To know the exact mechanism in which the compound 14a works. A western blotting technique applied to examine the level of key proteins responsible for the apoptosis.

The results showed a very low level of PARP cleavage in the untreated cells, which indicates that the cells did not die by apoptosis. In contrast, PARP cleavage obviously increased in response to 14a treatment (Figure 4.6).

In addition, the results showed the presence of active caspases 8 (18 kDa and 10 kDa) in the 14a treated cells as shown in (Figure 4.7), indicating that compound 14a has the ability to stimulate the extrinsic apoptosis pathway.

![Image of western blot result showing cleavage of PARP and Tubulin with increasing concentrations of 14a](image)

Figure (4.6): 14a induces PARP cleavage in cervical cancer cells (Hela). Hela cells treated with increasing concentrations (0, 30, 60µM) 14a for 48 hours and protein extracts were separated by (8%) SDS gel and analyzed by western blotting to estimate the level of cleaved PARP and Tubulin.
Figure (4.7): 14a activates caspases 8 in cervical cancer cells (Hela). Hela cells treated with increasing concentrations (0, 30, 60µM) 14a for 48 hours and protein extracts were separated by (12%) SDS gel and western blotting performed to estimate the level of caspases 8 and Tubulin.
4.2 Anti-cancer activities of Triazole compound (14a) on the breast cancer cell line (MCF-7).

4.2.1 The antiproliferation effect of Triazole compound (14a) on breast cancer cell line (MCF-7).

Cell cytotoxicity was determined using the MTT assay. The breast cancer cell line treated with a range (0.0µM –100µM) of the 14a compound for 48 hours. The results obtained showed that compound 14a has a cytotoxic effect with IC$_{50}$ (equal 110µM) in the breast cancer cell line. Figure (4.8a) showed that 14a exerts potent cytotoxic effects specifically on 60µM where it killed nearly 25% of MCF-7 cells while on 100µM, it killed 45% of breast cancer cells. Furthermore, 14a at 60µM and 100µM induced clear apoptotic morphological changes such as shrunked, rounded and flattened cells (Figure 4.8b). These results showed that 14a has a significant cytotoxic effect on MCF-7 cells.
Figure (4.8a): 14a induces cytotoxicity on breast cancer cells (MCF-7). MCF-7 cells plated in 96-well plates and after 24 hours, the cells treated with increasing concentrations of 14a compound (0-100μM). Cell viability assessed by (MTT) assay after 48 hours of treatment. Results show the mean percentage of cell growth ± STD (bars) of at least three experiments conducted in quadruplicate.
Figure (4.8b): 14a induces apoptotic morphological changes on breast cancer cells (MCF-7). MCF-7 cells treated with 60μM and 100μM of 14a compound. After 48 hours of the treatment, cells were visualized and photos were taken under 4X, 10X. Pictures showed differences in cells morphology between control (0.0μM) and 14a treated cells.
4.2.2 **Effect of Triazole compound (14a) on the viability of the breast cancer cell line (MCF-7).**

The effect of 14a on the cell viability of MCF-7 cells measured by trypan blue assay. The cell viability was determined after 24 and 48 hours of 14a (0.0μM, 60μM, and 100μM) treatment. Results showed that the cell viability of MCF-7 cells not significantly reduced in a dose and time-dependent manner (Figure 4.9). For example, 14a killed around 21% of MCF-7 cells at 60μM and 25% at 100μM after 24 hours of treatment. However, at 60μM and 100μM of 14a killed around 5% and 30% of MCF-7 cells, respectively after 48 hours of the treatment (Figure 4.9). These results showed that 14a has a moderate inhibitory effect on MCF-7 cells in a dose-dependent manner.

![Figure 4.9: 14a inhibits breast cancer cell viability (MCF-7). MCF-7 cells plated in 6-well plates and after 24 hours, cells treated with 0.0μM, 60μM, and 100μM of compound 14a or its vehicle. Cell viability assessed by trypan blue assay after 24 and 48 hours of the treatment. Results represent the mean percentage ± SEM of control of at least two experiments performed in twice replicates.](image-url)
Chapter Five
Discussion
5. Discussion

Cancer remains one of the most deadly diseases in the world, where its incidence in women increasing worldwide (Bray et al., 2018). Breast and cervical cancers remain the most common dangerous threats facing women (Aliwaini et al., 2015; Roura et al., 2016). Chemotherapy is one of the most important and effective medications used against these cancers. However, it's using is limited due to its side effects and drug resistance. Therefore, there is an urgent need to find new and innovative therapies based on the current knowledge of cancer biology and the phenotype of these cancers. Several triazole compounds have been shown to exert promising activities against cancer cell lines (Hou et al., 2011).

This study aimed to examine the cytotoxic activity of a new triazole compound (14a) against cervical (HeLa) and breast (MCF-7) cancer cell lines. The results showed that this compound might be more effective in the treatment of cervical cancer cells than breast cancer cells. Triazole (14a) compound inhibits the ability of cervical cancer cells to migrate in vitro. This study provides several lines of evidence that the compound 14a induce apoptotic cell death in cancer cells.

5.1 Anti-proliferative activity of Triazole compound (14a) on cervical and breast cancer cell lines

The antigrowth activity of the newly synthesized triazole compound 14a was examined against two cancer cell lines (Hela and MCF-7 cells). These cells treated with different concentrations of the triazole 14a compound (0.0-100µM). The cytotoxicity and viability results illustrated in figures 4.1-4.9, showed that 14a compound had more obvious effect on Hela cells compared to MCF-7 cells with an IC$_{50}$ value of 54.6µM and 110µM, respectively. In addition, 14a compound showed a strong anti-survival effect on Hela cells using clonogenic assay (Figure 4.4).

Hou et al., in 2011 have manufactured a series of 1,2,4-triazole derivatives containing 1,4-benzodioxan. Anti-proliferation activities were evaluated for all manufactured derivatives (5a-5q) against a range of human cancer cell lines (Hou et al., 2011). The researcher has observed that among all these compounds, the Hela cell line was sensitive to 5f and 5m with an IC$_{50}$ value of 15.03µM and 18.60µM, respectively, which is less than the value that appeared in our study. The most
important characteristic of these two compounds is that they contain "Cl" and "F" that helps to bind to DNA and damage it. However, 5i and 5j compounds showed ineffective activity against Hela cell line. This might be due to the fact that these compounds contained the methyl group, which is considered electron donor groups (Hou et al., 2011).

Benci et al., in 2012 have synthesized a group of novel 1,2,4-triazole, 4,5-dicyanoimidazole, and purine coumarin derivatives (Benci et al., 2012). The inhibitory activities of these compounds (3-18) evaluated against a group of human cancer cell lines and normal human fibroblasts. All compounds (except compounds 6 and 10) whose cytotoxic activity was evaluated against Hela cells were shown to have an IC₅₀>100μM, while the compounds 6 and 10 had IC₅₀ values of 35μM and 33μM, respectively (Benci et al., 2012). The value of IC₅₀ for most compounds was greater than the value that appeared in our study except for the two compounds 6 and 10 whose IC₅₀ values were slightly lower than the IC₅₀ of 14a. As the compounds 10 and 6 are different in structure from the other compounds mentioned in the study. Where compound 6 contains 1,2,4-triazole-3-carboxamide and compound 10 contains 2-amino-6-chloroquine (The presence of carboxamide prevents the formation of new DNA and chloropurine helps the compound to bind to DNA and destroy it) (Benci et al., 2012).

The cytotoxicity of new compounds synthesized from a 1,2, 3 triazole against three different types of cancer cell lines (HeLa, MCF-7 and DU145) was determined by MTT assay. Hela cells showed resistance against two compounds of this series. They are called 6g and 6k with an IC₅₀>100μM, although these two compounds are strongly active against DU145 with an IC₅₀ value of 18.21μM and 13.9μM, respectively. The compounds 6i and 6j demonstrated a strong toxic effect on Hela cells with an IC₅₀ value of 50.7μM and 52.4μM, respectively. This might be based on the length of the alkyl chain, the longer the chain, the greater the cytotoxicity of these compounds (Kumar et al., 2012). These results in agreement with our results in compound 14a on HeLa cell lines.

Senwar et al., in 2015 have manufactured a series of new Spirooxindole-derived morpholine-fused-1,2,3-triazoles. The anti-proliferation activities of these compounds (6a-t) evaluated against a group of human cancer cell lines especially
cervical cancer (Hela). Doxorubicin and 5-fluorouracil were used as controls. Compound 6s showed notable cytotoxicity against the Hela cancer cell line, with an IC\textsubscript{50} value <10μM. While 6p and 6r showed stronger cellular toxicity than compound 6s with an IC\textsubscript{50} value of 12.02μM and 22.27μM, respectively. The main characteristic of compound 6p is that it contains "Br", which affects the oxindole core. In addition to that compound 6r contains "cyano", which has a similar effect as "Br" (Senwar et al., 2015). The composition of compound 6p corresponds to the chemical composition of our compound in that it contains one element of the halogen group. The remaining compounds showed values of IC\textsubscript{50} less than the value obtained in this study with triazole compound 14a.

Ma et al. worked to create a group of novel 1,2,3-triazole–pyrimidine–urea hybrids (Ma et al., 2015). Anti-cancer activities were assessed for all compounds (10-41) against the MCF-7 cancer cell line and other human cell lines. The results showed that the compounds (14, 27, 35 and 37) only had a strong effect on the MCF-7 cancer cell line with IC\textsubscript{50}s of $8.62 \pm 1.41$, $8.14 \pm 0.76$, $6.12 \pm 0.59$ and $3.11 \pm 0.34\mu$M, respectively. These compounds contain a trifluoromethyl group at the 3-position on the phenyl group which represent a strong inhibitor. Based on the results of the study it was revealed that the location of a substituent on the phenyl group has a significant effect on the compound cytotoxicity. While the other compounds had a much stronger effect than these compounds on other cancer cell lines, it requires concentrations higher than the concentrations used in this study to inhibit MCF-7 cancer cell line (Ma et al., 2015). This is similar to what we obtained from MCF-7 cells results.

A study carried out in 2016 described the anticancer activity of Indole-2-carboxylic acid derived mono and bis 1,4-disubstituted 1,2,3-triazoles on a group of cancer cell lines, including the MCF-7 cancer cell line. All compounds (I\textsuperscript{1}–I\textsuperscript{12}) showed moderate to good effects against MCF-7 cancer cell line with a value of IC\textsubscript{50} less than the value shown in our study on 14a. However, only one compound showed a strong effect against the MCF-7 cancer cell line compared to Cisplatin called I\textsuperscript{12} (IC\textsubscript{50}= 13.26 $\pm$ 2.344 μM). The reason why it is considered better than other compounds is that it contains fluorine which helps to destroy DNA in addition to it indole, which is heterocyclic compounds that acts as bioactive molecules (Narsimha et al., 2016).
A recent study published in 2019, showed the ability of triazole compound (NMK-T-057) to inhibit the formation of colonies of cancer cells (Das et al., 2019). In this study, NMK-T-057 was tested on different types of breast cancer. Cells were treated at different concentrations of NMK-T-057 (0-10µM). Interestingly, this compound has a high ability to reduce the survival rate of cells and to inhibit the formation of colonies, especially on breast cancer cells MCF-7 at a concentration of 5µM. The distinguished characteristic of this compound is that it contains the indole ring system, which has been linked with a five-member heterocyclic triazole. The result of this association is the formation of strong anticancer properties including its ability to inhibit the γ-secretase–mediated activation of Notch-signaling (Das et al., 2019).

5.2 Triazole compound (14a) slightly inhibits migration ability of cervical cancer cells (Hela)

There are a number of hallmarks through which cancer is confirmed, including the preservation of proliferative signals, evasion of growth inhibitors, resistance to cell death, enabling immortality, motivating angiogenesis, and activating invasion and metastasis (Hanahan and Weinberg, 2011). Malignancy is the spread of cancer cells to important organs in the body, the most threatening event to the lives of cancer patients. For this reason, there have been many studies conducted on different groups of cancer cells that attempt to prove the importance of preventing the migration of cancer cells from their original place (Wu and Zhou, 2010).

Our results showed that triazole (14a) inhibits the ability of Hela cancer cells to migrate in vitro after 24 hours of treatment. However, the ability of compound 14a to prevent the migration of Hela cells was low. The anti-migratory effect of triazoles also confirmed by other studies (Yu et al., 2016). For example, a study published in 2016 about compound 5a derived from triazole compound, which was tested on a group of cancer cells. In all the cancer cells tested, this compound showed its ability to inhibit the migration of gastric carcinoma "MGC-803", especially after 24-hour of treatment. However, it was noted that this compound at low concentration (2µM) works to prevent cell migration slightly (Yu et al., 2016). Despite the ability of the compound 5a to prevent the migration of cancer cells. The main mechanism for preventing migration was unclear and needed further investigations. This compound
contains an ester group in addition to the presence of a terminal carboxylic amide group and this is useful in inhibiting the activity of important proteins in cancer cells (Yu et al., 2016).

The results of our study showed that the compound 14a has a minor anti-migration effect of Hela cells compared to the study mentioned above.

5.3 Mechanism of action of Triazole compound (14a) on cervical cancer cell line (Hela).

Western blot assays were performed to determine the potential effect of triazole 14a on biological process and the data suggested that triazole 14a can induce cell cycle arrest and extrinsic apoptosis of Hela cells as shown in (Figures 4.5-4.7).

5.3.1 Triazole compound (14a) induces cell cycle arrest in cervical cancer cells (Hela)

Several recent studies have suggested that cell cycle arrest as a potential mechanism of action of triazole compounds against many types of cancer cells (Tokala et al., 2018; Zhao et al., 2016).

The results of our study showed that treatment of Hela cancer cells with 14a stimulates cell cycle arrest. Many protein markers to the cell cycle arrest such as high levels of P53, P21, and cyclin D1 accumulated in Hela cells after 48 hour of 14a treatment.

The increase of cyclin-dependent kinase inhibitors p21 is usually considered as a cell cycle arrest marker (Jang et al., 2017). Its increasing level was observed after 48 hour of 14a (60μM) treatment. This is supported by the emerging level of cyclin D1, which is also considered as an acceptable marker to cell cycle arrest at stage G1 (Benzeno et al., 2004). These results together confirm that the G1 cell cycle arrest is one mechanism induced by 14a in Hela cells.

A study examined the effect of new 3-alkylsulfanyl-4-amino-1,2,4-triazole derivatives (8d) on the cell cycle progress of Hela cells. The results showed that this compound stimulates the cell cycle arrest significantly in the G2/M phase in a dose-
dependent manner. The greater the concentration, the greater the ability of the compound to induce cell cycle arrest after 12 hours of treatment (Zhao et al., 2016).

Several studies have shown the ability of triazole compounds to stimulate cell cycle arrest in many cancer cell types. Among these cells are breast cancer cells (MCF-7) (Tokala et al., 2018). Compound 5t used against MCF-7 cell and results showed that this compound was able to increase the rate of G0/G1 arrest at different concentrations (4 and 8μM). This is due to the ability of compound 5t to disrupt the cytoskeleton of MCF-7 cancer cells. Chemically, this compound contains the element fluorine (F), which binds to the DNA and works to destroy it, which leads to the cell cycle arrest (Tokala et al., 2018). This compound is similar to ours in that it contains one element of the halogen group.

Our obtained results confirm the ability of triazoles to induce cell cycle arrest in cancer cells.

5.3.2 Triazole compound (14a) induces extrinsic apoptosis in cervical cancer cells (Hela)

The ability of 14a compound was tested to induce cell death in Hela cancer cells. The trypan blue results showed that 14a had the ability to induce Hela cell death in a time- and concentration-dependent manner and that after 48 hours it kills 85% of Hela cells at a concentration of 60μM. Different markers of intrinsic and extrinsic apoptosis were tested by western blotting to determine the type of cell death caused by this compound.

Cleaved PARP is one of the important signs of apoptosis, indicating that the cell will not consume more ATP for DNA repair (Chaitanya et al., 2010). The results showed a marked increase in cleaved PARP, in response to 14a treatment (Figure 4.6). The most important marker of extrinsic apoptosis is the active caspase 8 (Diatlova et al., 2018). Results showed a clear increase in cleaved caspase 8 in response to 14a treatment indicating that the type of apoptosis is an extrinsic (Figure 4.7).

Our results are in partial agreement with several studies in this field. A study conducted by Kulabaş et al., (2016) on a series of novel 2-(4H-1,2,4-triazole-3-ylthio) acetamide derivatives showed that two compounds (18 and 25) were working to
increase the activation of the intrinsic caspase pathway in the prostate cancer cell lines and reduced the levels of Bcl-2 (B-cell lymphoma 2) and to increase the activation of caspase-3 (Kulabaş et al., 2016). In addition, there is evidence to support chemotherapy docetaxel as a treatment that stimulates apoptosis by disrupting Bcl-2 protein and increasing caspase 3/7 activity in a dose-dependent method. The compound 19 stimulated the activation of caspase-8, and -3 in cancer lung cells, and this confirms that this compound stimulates the external apoptosis paths in the cancer cells. These compounds contain acetamide, which has the ability to activate the anti-cancer properties in addition to containing elements of the halogen groups "Cl and Br". This is similar to the structure of our chemical compound, which has an element of the halogen group "Cl". This enhanced its ability to bind to cancer cells and to induce significant cellular toxicity (Kulabaş et al., 2016).

The results of our study was in agreement with the molecular mechanism of different triazoles (5b, 5e, and 5j) was clarified by western blot analysis (Milošev et al., 2017). These compounds studied in a range of cancer cell lines (HeLa, K562, HL-60, A549, and MRC-5). The results indicated that Bax expression was largely regulated in K562 cells treated with compounds (5b, 5e, and 5j). Increasing levels of active caspase 8 protein in response to triazoles treatment. This means that the compound 1,2,4-triazole-3-thione has the ability to stimulate both intrinsic and extrinsic apoptosis of cancer cells (Milošev et al., 2017).

A recent study showed that the 1,2,4-triazole-Chalcone hybrids (24, 25 and 27) have the ability to activate caspase-3,-8 and -9 in lung cancer cells (Ahmed et al., 2018). This confirms the ability of these compounds to induce both extrinsic and intrinsic apoptosis in cancer cells. Although these compounds are different in chemical composition, they have similar effect to our compound.
Chapter Six
Conclusion and Recommendation
6. Conclusions and Recommendations

6.1 Conclusions

The compound 14a is a promising anti-cancer compound as evident by the followings:

1. Compound 14a has significant cytotoxic effect against cervical cancer cells (Hela) and breast cancer cells (MCF-7).
2. Anticancer activity of 14a compound includes the induction of both cell cycle arrest at G1 phase and extrinsic apoptosis in cervical cancer cells (Hela).
3. Compound 14a has the ability to prevent the formation of colonies of the cervical cancer cells (Hela).
4. Compound 14a inhibits the ability of cervical cancer cells (Hela) to migrate in vitro after 12 and 24 hours.

6.2 Recommendations

Further studies are needed to

1- Investigate the effect of compound 14a on the other cancer cell lines.
2- Test the possible cytotoxic effect of 14a on normal cells.
3- Study the anticancer effect of 14a and its mechanism of action in vivo.
References
References


Tournaire, M., Epelboin, S., Devouche, E., Viot, G., Le Bidois, J., Cabau, A., …&


Appendix 1:

A diagram illustrating the mechanism by which triazole compound 14a exerts its anticancer activity on Hela cells.
Appendix 2:

Helsinki Committee
For Ethical Approval

Date: 2019/04/01
Number: PHRC/HC/495/19

Name: Ghadeer Salem Ramadan Idhair

We would like to inform you that the committee had discussed the proposal of your study about:

Anticancer properties of newly synthesized Triazoles (14a) against cervical and breast cancer cell lines

The committee has decided to approve the above mentioned research. Approval number PHRC/HC/495/19 in its meeting on 2019/04/01

General Conditions:-
1. Valid for 2 years from the date of approval.
2. It is necessary to notify the committee of any change in the approved study protocol.
3. The committee appreciates receiving a copy of your final research when completed.

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Member

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Translation:

نفيذكم عملاً بأن اللجنة قد ناقشت مفترج دراستكم:

وقل قد قررت الموافقة على البحث المذكور عليه

وبالم الخارج المذكور عليه