In Vitro Evaluation of Some 1, 2,4-Triazole against Breast, Colorectal and Cervical Cancer Cell Lines

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In Vitro Evaluation of some 1, 2,4-Triazole against Breast, Colorectal, and Cervical Cancer Cell Lines

Declaration

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 نتيجة الحكم على أطروحة ماجستير

بناءً على موافقة عمادة البحث العلمي والدراسات العليا بالجامعة الإسلامية بغزة على تشكيل لجنة الحكم على أطروحة الباحثة/ تغريد عبد القادر محمد أبوسلطان ليل درجة الماجستير في كلية العلوم/برنامج العلوم الحياتية/علم الحيوان وموضوعها:

دراسة تأثير مركبات التريازول علي خلايا سرطان الثدي والقولون وعنق الرحم

In Vitro Evaluation of Some 1, 2,4-Triazole against Breast, Colorectal and Cervical Cancer Cell Lines

وبعد المناقشة التي تمت اليوم الأربعاء 24 ذو الحجة 1439هـ الموافق 05/09/2018م الساعة الحادية عشرة صباحاً، في قاعة مبنى طبيبة اجتمعت لجنة الحكم على الأطروحة والمكونة من:

- د. صائب حسين العويني
- د. كمال عبد الكطول
- د. مازن مدحت الزهارنة
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- د. مشروفا ورئيس
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وبعد المداولة أوصت اللجنة بمنح الباحثة درجة الماجستير في كلية العلوم/برنامج العلوم الحياتية/علم الحيوان.

واللجنة إذ تمنحها هذه الدرجة فإنها توحي بها بتقوى الالتحاق ولزوم طاعات وأن تسخر علمها في خدمة دينها ووطنها.

وكل عام وانتم بخير،

أ.د. مازن إسماعيل هنية
الموضوع: استلام النسخة الإلكترونية لرسالة علمية

قامت إدارة المكتبات بالجامعة الإسلامية باستلام النسخة الإلكترونية من رسالة الطالب، ناصر عبد الله محمد، رقم جامعى: 79/000214.

رقم جامعى: 79/000214

قسم: العلوم الإنسانية والدراسات الإسلامية: العلوم

وتم إطلاع عليها، ومطابقتها بالنسخة الورقية للرسالة نفسها، ضمن المحددات المبينة أدناه:

- تم إجراء جميع التعديلات التي طلبتها لجنة المناقشة.
- تم توقيع المشرف والمشرفين على النسخة الورقية لاعتمادها كنسخة معدلة ونهائية.
- تم وضع ختم "عمادة الدراسات العليا" على النسخة الورقية لاعتماد توقيع المشرف والمشرفين.
- وجد جميع فصول الرسالة مجمعة في ملف (PDF) وآخر (WORD).
- وجد فهرس الرسالة، والملخصين باللغتين العربية والإنجليزية بملفات منفصلة (PDF +WORD).
- تطابق النص في كل صفحة ورقية مع النص في كل صفحة تقابلها في الصفحات الإلكترونية.
- تطابق التنسيق في جميع الصفحات (نوع وحجم الخط) بين النسخة الورقية والإلكترونية.

ملحوظة: ستقوم إدارة المكتبات بنشر هذه الرسالة كاملاً بصورة (PDF) على موقع المكتبة الإلكترونية.

واعداً: والتوقيع،

توقيع الطالب

ناصر عبد الله محمد
Abstract

**Introduction:** Cancer is one of the main causes of morbidity and mortality worldwide, with 14 million new cases in 2012. It is expected to rise by 70% during the next two decades. In 2015, it was responsible for 8.8 million deaths. Nearly one in six deaths is attributed to cancer. In 2010, the economic cost of cancer was estimated at 1.16 trillion USA dollars annually.

**Problem:** In spite of the enormous efforts invested to treat cancers, success was limited. This is attributed to late diagnosis; in addition to that, several types of tumors develop resistance to the current chemotherapies. Therefore there is a need to develop more effective therapies to treat this devastating disease.

**Objectives:** This research was conducted to evaluate the anticancer activity of newly synthesized triazole compounds named 14a, 9a, and 11a. Compounds were tested on the breast (MCF-7), cervical (HeLa) and colorectal (Caco-2) cancer cell-lines. Furthermore, determine the mechanism of action of 14a, and 9a, 11a against cervical cancer (HeLa) cell-line.

**Methodology:** The cytotoxic and anti-proliferative effects of triazole compounds were determined in MCF7, HeLa, and CACO-2 cell lines by MTT assay, trypan blue and scratch assay. Western blot was used to explore the anticancer mechanisms induced by 14a, 9a, and 11a.

**Results:** The results revealed that the synthesized Triazole compounds have strong anti-growth effect in time and concentration-dependent manner against all cancer-cell lines in vitro. 14a compound showed potent cytotoxicity against cervical cancer cell-line with IC$_{50}$ values of 54.6 µM and a similar effect was shown on MCF7 with IC$_{50}$ values of 59.8 µM. In addition to that, 14a induced a significant cytotoxic effect against colorectal cancer cells with an IC$_{50}$ of 113 µM. The mechanism by which 14a inhibits cancer cells might be apoptosis as evident by the increasing level of PARP cleavage after 14a treatment on Hela cells. 9a compound showed cytotoxicity against cervical cancer cell-line with IC$_{50}$ values of 64.6 µM and close effect observed when using 11a compound with IC$_{50}$ values of 62.4 µM.

**Conclusions:**

This newly synthesized triazole compound (14a) represents a potential active novel anticancer drug against cervical, breast and colorectal human tumors in vitro.

**Keywords:** triazole compound, Breast cancer, cervical cancer, colorectal cancer, cytotoxicity, and apoptosis.
ملخص الدراسة

المقدمة: السرطان هو السبب الرئيسي للوفيات بالعالم، وهناك 14 مليون حالة جديدة مصابة بالسرطان خلال عام 2012م ومن المتوقع أن يزيد معدل انتشاره 70% خلال العقود القادمة. ووفقاً لـ8.8 مليون شخص بسبب السرطان في عام 2015م وهو ما يعادل واحد لكل سعة حالات وفاة، وقرينة تكلفته الاجتماعية في العام 2010 1.1 مليار دولار أمريكي سنوياً.

المشكلة: على الرغم من الجهود الضخمة المبذولة لعلاج السرطانات كان هناك نهج محدود لأن معظم السرطانات لا يزال يتم تشخيصها في وقت متأخر والعديد من أنواع الأورام تطور مقاومة لعلاج الكيميائي الحالي. ولذلك هناك حاجة لتطوير علاجات أكثر فعالية لعلاج هذا المرض المدمر.

أهداف البحث: تم إجراء هذا البحث لتقديم النشاط المضاد للسرطان من مركبات التريازول الجديدة المسمى (14a). وهذا يشمل تأثيره على خطوط خلايا الثدي وعنق الرحم وسرطان القولون والمستقيم. علاوة على ذلك، يوفر هذا المشروع بعض المؤشرات حول آلية عمل ودراسة تأثير كل من 9a و11a على خلايا عنق الرحم.

منهجية البحث: يعتبر هذا البحث دراسة مقطعية لتقييم خواص العلاج الجديد وسمى (14a) ضد العديد من السرطانات أهمها سرطان الثدي وعنق الرحم وسرطان القولون والمستقيم. وذلك باستخدام العديد من الفحوصات خارج الجسم مثل فحص أم تي تي، وفحص تقييم حيوية الخلايا باستخدام صيغة الMTT، وفحص الهجرة، واستخدام فحص وسترن بموت للقيام بتقييم الآلية التي يعمل بها العلاج.

نتائج البحث: أظهرت نتائجنا أن مركب التريازول له تأثير قوي مضاد للنمو على الطرق التركيز في المختبر ضد جميع خطوط الخلايا السرطانية المستخدمة في الدراسة. كان التأثير السام لمركب 14a ضد خط خلايا سرطان عنق الرحم HeLa مع قيمة IC50 من 54.6 ميكرومول. بالإضافة إلى ذلك، أثرت IC50 من 59.8 ميكرومول على الخلايا السرطان القولون والمستقيم 0.7 مية عم على IC50 B ميركل و CACO-2 مع IC50 من 113 ميكرومول. آلية عمل 14a في تثبيط الخلايا السرطانية يمكن أن تكون موت الخلايا المبرمج كما يُضحى من زيادة مستوى إنقسام PARP بعد العلاج بمركب 14a على خلايا سرطان عنق الرحم، بالإضافة إلى ذلك كان تأثير 9a السام ضد خط خلايا سرطان عنق الرحم IC50 كان قوي مع قيمة IC50 من 64.6 ميكرومول وتأثر 11a مشابه لتأثير 9a على نفس الخلايا مع قيم IC50 62.4 ميكرومول.

خلاصة البحث والتوصيات: يمثل مركب التريازول الجديد (14a) عقارًا فعالًا مؤثرًا مضادًا للسرطان ضد أورام عنق الرحم والثدي والأورام البشرية في المستقيم خارج الجسم.

الكلمات المفتاحية: سرطان الثدي، سرطان القولون، السمية، الموت المبرمج، مركبات التريازول الجديدة.
Dedication

It gives me pleasure to dedicate this thesis to

The sake of Allah, my Creator, and my Master

My great messenger, Mohammed (May Allah bless and grant him)

Pure soul of my father

My mother and my brother AL muhammad who is always supporting me, without

your support I wouldn’t be here today

My brothers Jamal, My sister Ghada and to all my family members

My supervisor who supported me Dr. Saeb Aliwaini.

My friends who encouraged and supported me

This work is also dedicated to

The Palestinian people who have suffered and will be struggling with the

persistence to be a free Palestine.

And mostly

to the pure soul of all

Palestine’s martyrs, prisoners in occupation prisons and resistance hero's men.
Acknowledgment

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❖ I am greatly and deeply indebted to the Master Degree program coordinators at the IUG. This work could not have come to light in its presented structure without the complete support and continuous encouragement of Dr. Saeb Aliwaini my supervisor whose foresight, efforts, meticulous revision and for his gaudiness and supervision helped me to produce this thesis. Thanks to Dr. Kamal Elkahlout my co-supervisor for his helping.
❖ I would like to take the opportunity to express my thanks to those who have played a role in the process of this work. However, I owe my Special thanks for Salssabel Al-Gogo, Sanabel Dawas, Hussam Abu Taem, Noura Ramadan Mariam Ghunaim to the creative staff of the Tissue Culture lab. At the IUG.
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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>Apoptosis protease activating factor 1</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast cancer susceptibility gerel</td>
</tr>
<tr>
<td>BCL-2</td>
<td>B- cell lymphoma-2</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>CRC</td>
<td>Colorectal cancers</td>
</tr>
<tr>
<td>CACO-2</td>
<td>Heterogeneous human epithelial colorectal adenocarcinoma cells</td>
</tr>
<tr>
<td>CDDP</td>
<td>Cisplatin, cis-diamminedichloroplatinum(II)</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribo nucleiac acid</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DSB</td>
<td>Double strand break</td>
</tr>
<tr>
<td>DISC</td>
<td>Death inducing signaling complex</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethyl formamide</td>
</tr>
<tr>
<td>DCIS</td>
<td>Ductal carcinoma in sita</td>
</tr>
<tr>
<td>ELIZA</td>
<td>Enzyme linked immunosorbant assay</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamin tetracetic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>EBRT</td>
<td>External beam radiation therapy</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-Fluro uracil</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GDP-bound</td>
<td>Gunanosine diphosphate- bound</td>
</tr>
<tr>
<td>GAB</td>
<td>Gamma aminobutyric acid</td>
</tr>
<tr>
<td>HER-2</td>
<td>Human epidermal growth factor receptor</td>
</tr>
<tr>
<td>HIV-1</td>
<td>Human immunodeficiency virus-1</td>
</tr>
<tr>
<td>IC50</td>
<td>The half maximal inhibitory concentration</td>
</tr>
<tr>
<td>IMB</td>
<td>Inter- menstrual bleeding</td>
</tr>
</tbody>
</table>
MCF7  Michigan Cancer Foundation-7
MAPK  Mitogen-activated protein kinase
MIC  Minimum inhibitory concentration
MTT  3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
MGC-803  human gastric carcinoma cell line
MOLM13  Myeloid leukemia cell lines MOLM13
mM  Milli molar
µM  Micro molar
NCHS  National center for health statistics
OMM  Outer mitochondrial membrane
PR  Progesterone receptor
PCB  Post-coital bleeding
PMB  Post-menopausal bleeding
PNB  Propagation not biting
PMA  Phorbol myristate acetate
PCD  Programmed cell death
PARP  Poly Adp Ribose Polymerase
PI3K  Phospho-inositide 3-inase signalling pathway
P53  Tumor protein-53
PBS/T  Phosphate buffer salin/ Tween
RNA  Ribonucleic acid
RPMI  Roswell park Memorial institute medium medical
SEM  Standard error of the means
STD  Standard deviation
TNBC  Triple-negative breast cancer
TNF  Tumor necrosis factor
THP-1  human monocyte; acute monocytic leukemia
US  United state
UK  United Kingdom
U87MG  a human primary glioblastoma cell line
WHO  World health organization
Chapter 1

Introduction
Chapter 1
Introduction

1.1 Overview

Cancer is among one of the threatening diseases that imposes fatal dangers to human lives around the world (Kumbhare et al, 2015). Estimations indicated that in 2013 as total as 13% of mortality around the world caused by cancer. This situation going to be worse by the year 2030 where 13.0 million deaths are expected to occur in the world. (American Cancer Society, 2016). Around 550.000 deaths befallen annually by cancer which pointed cancer as the second death reason in the world after cardiovascular diseases (Akhtar et al, 2016). As a heterogeneous disease, cancer may attack different types of human organs and tissues. By looking at the incidence of cancer we found that lung, prostate, stomach, colorectal and esophagus are the most common cancer types among men while breast, lung, stomach, colorectal and cervical cancers are the most common types among women (Ministry of Health, 2014). As the most frequent cancer type among women around the world, breast cancer expects to attack one out of eight women. Unfortunately, a limited success obtained by the present-day treatment approaches (Ahmed Malki, 2016). Regarding colorectal cancer (CRC), it is found that it resulted from the anomalous multiplication of colon and rectum cells. These malignant cells have the capacity to assault and blow out into other tissues and organs of the human body. CRC symptoms include the presence of blood in feces accompanied with abnormal movement of the bowl, fatigue and losing weight (Sagt, Qassim, & Ibrahim, 2016). CRC occupied the third position among the most frequent cancer types in the USA between men and women. CRC incidence and mortality were decreased during the last few decades. This decrease may be attributed to changes in risk factors of the disease like lowering smoking rates, decrease in red-meat utilization and enhanced usage of aspirin (Siegel et al., 2017).

When looking at cervical cancer we find it in the second order in occurrence frequency and killer for women in the world. Spreading of cervical cancer incidences found to associate with poverty and development. It is estimated that around 80% of cases are in low-income populations where the highest incidences occurred due to
infection diseases (Africa, Melanesia, Latin America, Caribbean, South Central Asia, and South East Asia) (González Martín, 2007).

External carcinoma agents (e.g., smoking, some pathogens, and bad nutrition) and interior factors (e.g., immune deviations, gene mutation, and improper hormone activity) may contribute to cancer occurrence. Carcinogenic agents may work as combined factors or in a sequenced pattern. A long period, ten years and more, may pass from exposure to carcinogens and detection of the disease. Treatment of the cancer disease goes over several routs including radiotherapy, surgical operations, using chemical agents, hormonal treatment, induction of immune system and targeted treatment (American Cancer Society, 2015).

The mortality rates resulted from cancer still high in spite of advancement in treatment methods and diagnosis technology (Dizon et al. 2016). Diagnosis is the first action in cancer management activities. This includes assessing the pathological and clinical situation. As diagnosis in confirmed, it is vital to evaluate treatment options and protocols of prognosis for application of the appropriate ones. The treatment protocols of cancer may be applied as single or in a combined program (Global Cancer Facts & Figures, 2012).

The present used treatments committed to harmful side effects and in many situations, they are costly and not readily available. If taken surgery as an example, high amounts of cortisol liberated after operation leading to compromising of immunity with increase possibility of cancer recurrence (Aliwaini, Kichaoui, & Ayesh, 2016).

When regarding chemotherapy, it is familiar that chemical treatment imposes very harsh side effects including inhibiting multiplication of bone marrow stem cells causing depression of immune system. Treatment with radiation is widely used around the world but it imposes sever problems for many functions in the body including lymphocytes which are the most danger-exposed. Radiotherapy leads to long T-cell depression. Many side complications associated with present-day therapies including necrosis of bone, fibrosis for lungs, de-vascularization of skin, formation of ulcers, nausea, vomiting and kidney damage (Desantis et al., 2014).
Huge works done in the battle against cancer including research and development of new drugs and treatments but unlikely success in this war was limited. The limitation in success in cancer treatment attributed to the fact that diagnosis of the disease still going too late and many tumor types able to develop resistance against the drugs (Aliwaini, 2014). It is vital to continue search and developing new therapeutic agents to gain the battle against this fatal disease.

Triazoles together with derivatives have vital importance in chemistry of medicine. They are used to derive various types of heterocyclic chemicals. These chemicals have many uses, to treat infections from viruses, bacteria, fungi, and tuberculosis, in addition, to treat convulsing, depression, inflammation, and cancer. Reports pointed their use as inhibitors for glycogen synthase kinase-3, antagonists of GABA receptors, agonists for muscarine receptors, and neuroleptic. They also showed anti-HIV-1, cytotoxic, antihistaminic, and anti-proliferative activities. Due to the vast use of Triazoles, produce new novel derivatives is a prospective trend in the field of chemistry of medicine (Pokhodylo, Shyyka, & Matiychuk, 2013).

Triazoles may be classified into two classes including v-Triazoles (1, 2, 3, Triazoles) and s-Triazoles (1, 2, 4-triazoles) (Kamel & Megally Abdo, 2014a).

Due to their biological effects, chemical synthesis of s-Triazoles (1, 2, 4-triazoles) and derivation of fused heterocyclic forms drawn big efforts by the researchers (Kamel & Megally Abdo, 2014).

In this work, we examined triazole compounds prepared by Dr. Adel Awadalalah and his colleagues in the Islamic University Labs to determine which of them has an anticancer effect.

1.2 Objectives of the study

1.2.1 General objective

This work aimed to estimate the anticancer properties of a group of novel Triazoles compounds against breast, colorectal and cervical cancer cell-line.

1.2.2 Specific objectives

1. To inspect anti-proliferative activities of novel Triazoles compounds and identify the most effective one.
2. To examine the anti-migration activities of novel Triazoles compounds and identify the most effective one.
3. Explore the mechanism by which these compounds exert their effects.

1.3 Significance of the study

Despite intensive work by scientists and physicians to develop effective and safe treatments against breast cancer, incidence frequency of breast cancer still increasing due to late diagnosis of cancer in addition to developing resistance by cancer cells against different treatments. From here, discovering new and safe anticancer compounds from a well-known anti-cancer triazole family could be a good point. Furthermore, since Gaza strip suffers from cancer in particular due to poor diagnosis and siege that prevents access to cancer therapies, it is important to note that the manufacture of these compounds was made by Palestinian hands that may contribute to solving a big global problem such as cancer.

1.4 Limitations:
1. The arrival of materials delayed.
2. Difficult of importing the cell lines from outside Palestine due to the siege on Gaza Strip.
Chapter 2

Literature Review
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Literature Review

2.1 Cancer

Cancer, as commonly known is out controlled growth of cells which may be accompanied with the offensive spreading of the malignant cells to other tissues and organs, a manner named metastasis (Wei et al., 2010). As one of the main reasons for mortality in the world, it affects greatly the personal life as well as the whole society (Ma et al., 2015). It described in the second order as incidence of disease after cardiovascular ones (Kumbhare et al., 2015a). Cancer distribution enjoyed global spreading with different malignant types where dominating types changing constantly particularly when consider developing countries. Until 1975, around 51% of incidences found in low and mid-income countries. By 2007, this ratio increased to 55% and it expected to jump to 61% in 2050. When looking over cancer types like lung cancer, breast cancer, colorectal and prostate, they became worldwide distributed in spite in the past they were restricted to industrialized countries in the West (Thun, DeLancey, Center, Jemal, & Ward, 2010).

2.1.1 How cancer rises

The development of the tumor started as some of resting cells gained a genetic mutation which causes an abnormal elevation in their tendency to proliferate. The altered cell multiplies very quickly forming an enlarged mass in a stage called hyperplasia (Weinberg, 1996). In addition to excessive cell proliferation, cells appear unusual in shape, orientation and new tissue said to exhibit dysplasia (Hanahan & Weinberg, 2000). The tumor cells continue showing the unusual shape and abnormal growth behavior. If the tumor cells did not pass across tissues, they are named in-situ cancer and may stay in place indeterminately. When the genetic deviation induces tumor cells to pass the local area to neighbor tissues and invade new places, cancer cells may spread through blood and lymph and in such case, the formed mass regarded as malignant and the invading cells are able to form new cancer masses (metastases). In such a situation, invading cells may become fatal when reaching vital organs (Brodersen, Schwartz, & Woloshin, 2014).
The American cancer society and the National Center for Health Statistics (NCHS) stated that around 1,685,210 annual incidences equivalent to 4,600 new cancer diagnoses each day in 2016 have recorded (Siegel, Miller, & Jemal, 2016). Those statistics expected around 595,690 deaths in America equivalent to about 1,600 deaths per day (Siegel et al., 2016).

On the other hand, the results of the cancer data analysis in Gaza Strip for the years 2009-2014 showed that the total number of cancer patients has reached 7069 patients registered within the monitoring center of tumors in Gaza Strip (Ministry of Health, 2016).

According to survey studies, incidences and mortality rates are elevated regarding various cancer kinds. Fighting against cancer still going on and still needs more scientific and clinical research (Siegel et al., 2016).

In this work, we evaluated the effect of new triazole compounds against three types of cancer, breast, colorectal and cervical cancer.

2.2 Breast cancer

Like other types of cancer, breast cancer takes place when breast cells gain abnormal growth forming a tumor mass. Mostly breast cancer takes place among women, but men can gain the disease too (American Cancer Society, 2016). From 1975 to 1979, frequency of breast cancer was very low among men with less than 1% in USA. This ratio elevated slightly up to 1.3% during 2010-2014 (American Cancer Society, 2017). The occurrence of cancer in breast takes place in various parts of breast's tissues but in most cases positioned in the milk ducts that conveying milk to the nipple. In some cases, it deployed in the mammary glands while other breast cancer types can take place but they are less common. These types are named lymphoma and sarcoma and in fact not regarded as real breast cancer (Hunt, Green, & Buchholz, 2012).
Cancer cells from breast can invade the lymph system where they deployed in the lymph nodes through the lymph vessels and form malignant masses. Those cells can spread to other parts of the body through lymph fluid (Hüsemann et al., 2008).

On a global level, breast cancer regarded as the most frequent type of cancers with the estimation that about one woman out of eight women may gain this dangerous disease. In spite of this evaluation, limited achievements wined by using major therapeutic approaches (Ahmed Malki, 2016).
According to the estimation of the American Cancer Society's regarding breast cancer in the United States for the year 2016:

- Expectations indicated more than 246,660 new cases will be revealed among women.
- Regarding breast cancer among men, estimations said that around 2,600 new diagnosed cases have revealed.
- New 61,000 non-invasive in situ ductal (DCIS) carcinoma expected to diagnosis as the earliest form of breast cancer.
- Mortality rates among infected women expected to be around 40,450 women (American Cancer Society, 2016).

Closely related observations seen in Palestine indicated that breast cancer found as the most prevalent cancer type among women. The number of registered cases figured 1283 cases representing 18.5% of the total cancer cases, and 31.3% of females’ cancer cases (Ministry of Health, 2014).

In Gaza strip, breast cancer ranked the first, with 427 reported cases and formed 17.8% from all reported cases. (Ministry of Health, 2016).

### 2.2.1 Molecular subtypes of Breast Cancers

Five principle subtypes of breast cancer determined according to the genes expressed by the cancer cells. These molecular subtypes include luminal A, luminal B, **Triple-negative/basal-like**, HER2-enriched and **Normal-like**.

**Luminal A** subtype is known as hormone-receptor-positive (estrogen-receptor and/or progesterone-receptor positive). This subtype is negative for a human epidermal growth factor receptor 2 (HER2). Luminal A subtype owns low levels of Ki-67 protein, which used as a cellular marker for proliferation. Ki-67 aids in controlling the fastness of cells growth. This subtype of breast cancer is inferior and has a tendency to grow slowly with the finest prognosis.

**Luminal B** subtype is a hormone-receptor positive (estrogen-receptor and/or progesterone-receptor positive). It may HER2 positive or HER2 negative. This subtype of breast cancer has high levels of Ki-67 protein. It characterized with slight fast growth compared to the subtype luminal A. It has a somewhat worse prognosis.
**Triple-negative/basal-like (TNBC)** subtype which is described as hormone-receptor-negative (estrogen-receptor and progesterone-receptor negative). It is also HER2 negative. TNBC more frequent among women suffering mutations in human tumor suppressor (BRCA1) gene. With no clear reasons, this subtype of breast cancer is more frequent in younger and African-American women. This type represented around 10-20% among diagnosed cases. Since this type is deficient in the required receptors, treatments commonly used to fight breast cancers like hormonal therapies and drugs targeting estrogen, progesterone, and HER-2 are not useful. Reports indicated a vital role of BRCA1 and BRCA2 in developing TNBC subtype.

**HER2-enriched** subtype known as hormone-receptor-negative (estrogen-receptor and progesterone-receptor negative) and it is positive to HER2. HER2-enriched subtype has growth tendency faster than laminal subtype A and B with a possible worse prognosis. Generally, successful treatment of this subtype is more achievable with therapies targeted HER2 protein like Herceptin (trastuzumab), Perjeta (pertuzumab), Tykerb (lapatinib), and Kadcyla (T-DM1 or ado-trastuzumab emtansine).

**Normal-like** subtype resembles luminal a case. It is hormone-receptor positive (estrogen-receptor and/or progesterone-receptor positive) but negative to HER2 with low levels of the protein Ki-67. In spite, it has a good prognosis but remains somewhat worse than luminal A prognosis (Dai, X., Li, T., Bai, Z., Yang, Y., Liu, X., Zhan, J., & Shi, B. 2015).

### 2.2.2 MCF-7 Cell-Line

The MCF-7 cell-line is epithelial cells achieved in the year 1970 from adenocarcinoma a caucasian patient woman with 69-years. This cell-line used widely in works aiming investigations of breast cancer (Cooper, 2012). The name MCF-7 was on the honor of the Michigan Cancer Foundation (MCF). MCF-7 found to be the most studied human cancer cell-line around the world (Lee, Oesterreich, & Davidson, 2015). Through 45 years and till now, this cell line occupied the first position as the most used cancer cell line by many research groups in the field of cancer research (Comşa, Cîmpean, & Raica, 2015).

MCF-7 cell-line in used extensively around the world in works for estrogen receptor (ER)-positive breast cancer cell experiments and many sub-clones, which
have been established, represent different classes of ER-positive tumors with varying nuclear receptor expression levels (Comşa et al., 2015).

2.2.3 Symptoms of breast cancer

Symptoms of advanced breast cancer may include bone pain, breast pain or discomfort, skin ulcers, swelling of one arm (next to breast with cancer) and weight loss (Burgess, Hunter, & Ramirez, 2001).

2.2.4 Treatment of breast cancer
i) Surgery

Eradication of breast tumor depends on injury or non-injury lymph nodes in the axillary. This process aims to remove the tumor completely and check lymph nodes in the armpit to make sure that the tumor has not yet spread. If the tumor has spread, the physician needs to know the number of nodes which hit the tumor (Fallowfield, Hall, Maguire, Baum, & A’Hern, 1994).

ii) Radiation therapy

Radiation therapy regarded as an integral measure for breast cancer treatment. (Tabár, Fagerberg, Day, Duffy, & Kitchin, 1992). It reduces the incidence of recurrence by 50-75% (Cuzick et al., 1987).

iii) Chemotherapy

Chemotherapy is the best after radiation therapy and uses many of the drugs that differ in terms of their impacts on breast cancer and the treatment of each particular period to show its impact on breast cancer (Layeequr Rahman & Pruthi, 2012).

Cytotoxic drugs are chemotherapeutics that functions in cell death or preventing of cell growth. This action generally gained by suppressing microtubule role, protein activity, or synthesis of DNA. The cytotoxicity of chemotherapeutics may proceed as cell cycle-dependent activity causing repression of growth of the cells at precise stages during the cell-cycle (Layeequr Rahman & Pruthi, 2012)
2.2.5 Breast cancer chemotherapies

Chemotherapeutic drugs used to treat breast cancer include:

i) Anthracyclines: This type of therapeutics comprises doxorubicin (Adriamycin) and epirubicin (Ellence) (Sparano et al., 2008).

Mechanism of Anthracyclines

It inhibits synthesis of DNA and RNA by inserting its molecules in between base pairs of nucleic acids (DNA/RNA). This intercalation caused termination of replication process of the nucleic acid leading to suppress the growth of the cancer cells. Insertion of the anthracyclines molecules between base pairs suppresses the topoisomerase II enzyme and decline relaxation of the DNA causing a blockade of both transcription and replication. This followed by iron-dependent generation of free oxygen-radicals that destroy the biomolecules (DNA, proteins and cell membranes). Subsequently induction of histone ejection out of chromatin going on causing deregulation of DNA damage response (Pang et al., 2013).

ii) Taxanes: It is such type of cancer medicines comprises docetaxel (Taxotere) and paclitaxel (Taxol). These therapeutics usually applied in combination with other drugs as carboplatin, cyclophosphamide (Cytoxan), and fluorouracil (5-FU) (Sparano et al., 2008).

Mechanism of Taxanes

Texans activity takes place through interruption the function of microtubules, which play a vital role during cell division. On the molecular levels, taxans act to stabilize the GDP-bound tubulin during microtubule formation. This stabilization caused suppression of cell division process. Stabilization of the GDP-bound tubulin suppresses de-polymerization of the microtubules leading to abortion of cell division (Hagiwara & Sunada, 2004).

2.3 Uterine cervical cancer

This tumor begins as abnormal growth of the cells lining the uterine cervix (Global Cancer Facts & Figures, 2012). In most cases, the disease starts to appear in the cells of the transformation zone of the cervix tissue where gradual change takes place in these cells giving precancerous form, which developed later to the malignant tumor. Both the malignant and the pre-cancerous forms are classified according to
their morphological characters under the microscope. adenocarcinoma of the squamous cells regarded as the main types of cervix cancer (American Cancer Society, 2016).

Figure (2.3): Illustration of the anatomical position of the cervix (American Cancer Society, 2016).

Cervical cancer is seen as the second major frequent form of cancer and representing the sixth main cause of mortality among cancer-related death cases in women around the world. The incidence of cervical-cancer reached up to ~16 women per 100,000 annually and ~0.008% predicted to acquire the disease (Hu & Zhao, 2013). In 2014 the WHO projected that more than a million women have cervix cancer around the world. Estimations of WHO pointed that more than 80% of cases found among women in low and middle-income states. It is important to say that many of those women do not access preventive, curative or palliative health care services. One of the leading causes of cervical cancer is long-term infection with human papillomavirus (HPV). In 2015 the American Cancer Society estimated that more than 12,900 women diagnosed with the invasive cervical cancer (American Cancer Society, 2016).

In the Gaza Strip, around 177 cervical cancer cases are registered, with 4.6% of the total females’ cases (Ministry of Health, 2016).
2.3.1 HeLa cell line

HeLa cell-line considered as the oldest and most used cell-line in cancer lab researches around the world. This cell-line is universally inhabitant in tissue culture labs where due to its rapid growth pattern regarded as a contaminant when culturing less robust cell-lines. HeLa cell-line obtained on February 1951 from Henrietta Lacks. She was a patient died from cervical cancer in October 1951 (Tweedale, 2002). HeLa cell-line showed characterized durability and productivity. This permits widespread application in research labs (Lucey, Nelson-Rees, & Hutchins, 2009).

2.3.2 Symptoms of cervical cancer

The symptoms related to cervical cancer mostly communal and ambiguous. They comprise inter-menstrual bleeding (IMB), post-coital bleeding (PCB), postmenopausal bleeding (PMB), the anomalous shape of the cervix (suspicion of malignancy) blood-stained discharge from vagina and pain in the pelvis area (Scottish Intercollegiate Guidelines Network, 2008).

2.3.3 Treatment of cervical cancer

Surgery, radiotherapy, chemical therapeutics, and hormonal treatments are the common routs followed for treating cervical cancer. Type of treatment protocol associated with the stage of the disease (American Cancer Society, 2017).

i. Surgery

Surgery treatment rout for cervical cancer includes cryosurgery, laser surgery, cervical conization, and hysterectomy. Cryosurgery utilized direct application of liquid nitrogen-cooled metal. The cooled probe cause the killing of the cancer cells by freezing. This operation may be done in the physician office. Following cryosurgery, the patient may have watery brown discharge, which continues for a couple of weeks. This treatment rout is valid to treat carcinoma in situ of the cervix (phase 0) but it is not valid for invasive cancer type (Rydzewska, Tierney, Vale, & Symonds, 2010).

Laser surgery methods operated by focusing a laser beam, which works on burning off and vaporization of the malignant cells and abstract little pieces of the tissue for studying. This operation may do in the physician office as cryosurgery. This operation needs for local anesthesia. Like cryosurgery, this treatment method
applied for the treatment of cervical carcinoma in situ (stage 0) and is not valid as treatment route for invasive cancer (Rydzewska et al., 2010).

Cervical conization surgery used to remove a cone-shaped part of the cervical tissue, hence the name conization. The operation curried out by using either surgical or laser knife or application of electrically heated thin wire. (Rydzewska et al., 2010).

Hysterectomy surgery involves removal of the uterus as a whole with leaving the organs and tissues next to it intact. Hysterectomy surgery does not remove the vagina and lymph nodes of the pelvic area. Both ovaries and fallopian tubes left without removing if there is no medical reason to remove them (American Cancer Society, 2016).

ii. Radiotherapy

Treatment with radiotherapy exploits high-energy radiations including x-rays or radioactive particles for killing cancer cells. This method of treatment may exploit external beam radiation therapy (EPRT), brachytherapy, radioisotope therapy. EPRT utilizes an external source of high-energy x-ray radiation. Treatment by x-ray resemble having regular x-ray image but with stronger dose and for longer exposing period. The treatment process is not painful by itself but may cause some side effects. Using EPRT as principle treatment route for cervical cancer mostly combined with chemotherapeutics (concurrent chemoradiation). The chemotherapy mostly administered as low doses of cisplatin or other types of chemical drugs. Radiation treatment protocol administered five days weekly and completed for 6-7 weeks. EBRT may apply alone for fighting spread cancer regions or as principle medication with patients who are intolerable for chemoradiation treatment.

In radiotherapy implants (brachytherapy) small shards of radioactive metal implanted in the body close to the cancer mass. When using radiotherapy injection (radioisotopic therapy), a radioactive liquid administered orally or intravenously. Radiotherapies usually gave in the hospital and leaving after finishing the process. In some cases, the patient may be hospitalized for some days especially when administering implants or radioisotopes (American Cancer Society, 2016).
iii. **Chemotherapy**

Chemotherapeutic protocols utilize anti-cancer medications, which may be administered orally or intravenously. Drugs passed to the blood stream from where they passed and killed cancer cells in most body areas. Chemotherapy protocols depend on the cyclic administration program form of treatment period followed with the recovery period. Chemotherapeutics that mostly used for the treatment of cervical cancer include Cisplatin, Carboplatin Paclitaxel (Taxol), Topotecan, Gemcitabine (Gemzar). Usually, drugs used in a combined manner. Other drugs may be applied against cervical cancer like docetaxel (Taxotere), ifosfamide (Ifex), 5- fluorouracil (5-FU), irinotecan (Camptosar, CPT-11), mitomycin and bevacizumab (Avastin) (American Cancer Society, 2016).

### 2.4 Colorectal cancer (CRC)

CRC is a type of cancer that attacks rectum or colon. According to the site of assault, it called colon or rectal cancers (Rodriguez-Salas et al., 2017). Mostly, CRC initiated as extra growing of inner lining cells of either colon or rectum titled as polyps. Some polyps may develop to malignant tumor through a period of many years. Developing of any polyp to the malignant form related to the type of the polyp (Schreuders et al., 2015).

![Figure 2.4: Illustration of the normal colon and rectum](image)

*(American Cancer Society colorectal cancer, 2016).*
CRC accounted as the third most incident cancer type in Europe and North America (Siegel et al., 2017). Estimations indicated the presence of 95,270 cases of colon cancer and 39,220 patients of rectal cancer in 2016. Observations pointed a decline in mortality rate related to CRC since 1980 in men and in women since 1947. Reports indicated an overall decline in mortality by around 49% in the period 1976 to 2012. This tendency reflected progressing in the premature diagnosis and better treatment strategies in addition to the decline in the frequency of the disease. The period 2003 to 2012 witnessed an annual decrease in mortality rates by 2.8% (American Cancer Society, 2017).

In Gaza Strip, 709 cases of colorectal cancer registered, with 10.0% of the total both males and females cancer patients (Ministry of Health, 2016).

2.4.1 CACO-2 cell line

This cell-line appeared as unceasing heterogeneous human epithelial colorectal adenocarcinoma tissue. Developing of the cell-line accomplished in Sloan-Kettering Institute for Cancer Research through efforts done by Dr. Jorgen Fogh (Sambuy et al., 2005).

The human intestinal CACO-2 cell line has been extensively used over the last twenty years as a model of the intestinal barrier (Health, 2016). The CACO-2 cell line is originally derived from a colon carcinoma (Sambuy et al., 2005).

2.4.2 Symptoms of colorectal cancer

CRC disease may not show any symptoms, but when this takes place, one or more symptoms may appear including alterations in behavior mode of the bowl as diarrhea, chronic constipation. Patient may suffer rectal bleeding with bloody stool. CRC patient may feel abdominal disquiets like cramps and gases or abdominal pain. In addition to that, patients feel that bowl does not empty entirely. Other symptoms may be noticed as weakness and fatigue in addition to unintentional weight loss (American Cancer Society colorectal cancer, 2016).

2.4.3 Treatment of Colorectal Cancer

Struggling against CRC may follow any of the classical routs of cancer treatment including surgery, chemotherapy, and radiotherapy.
i. Surgery

Surgical removal of colon CRC is the most applied treatment when the tumor, not blowout especially during the early phases of the disease. In colon CRC treatment, it rare to have a durable colostomy (abdominal hole for purging of wastes) and in common it is not needed during treatment CRC rectal cancer. For integrated management of CRC, chemotherapy may be applied solely or combined with radiotherapy either, as neoadjuvant or adjuvant (Cancer facts and figures, 2016; American Cancer Society colorectal cancer, 2016).

ii. Radiation

Radiotherapy of CRC demands high-energy radiations like X-ray or particles that cause destruction for the malignant cells. The effectiveness of radiotherapy may be enhanced by combined chemotherapeutic agents when used with some CRC types. When applying both treatments together, the method attributed to chemoradiation therapy or chemoradiotherapy (cancer facts and figures, 2016).

Types of radiation therapy:
As seen with treatment of other types of cancer, CRC radiotherapy may use external beam radiation therapy (EPRT), brachytherapy, radioisotope therapy.

In most EPRT treatment protocols, treatment process continues for five days each week and this may continue for several weeks depending on the evaluation of each case individually.

When using internal radiation therapy or brachytherapy, a piece of radioactive material located inside the rectum in close to the tumor. This approach allows radiation to pass directly to the tumor without passing to other tissues. This permits fewer occurrences of side effects (American Cancer Society colorectal cancer, 2016).

Chemotherapy

Many drug types are used to treat CRC patients but 5-fluorouracil (5-FU) in among the commonly used drugs. Usually, 5-FU used in combination with vitamin-like drug leucovorin or other drug called levo-lecovorin. Combination found to help the chemotherapeutics to work better. Capecitabine (Xeloda) in the form of pills dosed orally to the patients where they are converted into 5-FU when passed to the tumor.
Other commonly used drugs for the treatment of CRC include Irinotecan (Camptosar), Oxaliplatin (Eloxatin), Trifluridine and tipiracil (Lonsurf). They formulated as combined drugs, which dosed as oral pills.

In most treatment protocols, two drugs are used to enhance the effectiveness of the treatment process. In some cases, chemotherapeutics dosed with the targeted therapy (American Cancer Society colorectal cancer, 2016).

2.5 Triazole compounds

Historical

In 1885, Baldwin reported the synthesis of carbon-nitrogen heterocyclic ring system (C₂N₃H₃) and described its derivatives giving the term Triazoles. Fischer's work revealed that cyanogen reacts with phenylhydrazine to produce dicyanophenyhydrazine. Even though the description of Bildin was not precisely correct he achieved full admission as the founder and inventor of such ring structure (Potts, 1961).

Triazoles are crystalline structures with a pale-yellow color having a weak distinguishing aroma. They are soluble in water and alcohol. They have acidic nature attributed to three nitrogen atoms within the rings (Mohite PB & Bhaskar, 2015). Presence of the nitrogen atoms within the heterogeneous 5-ring system identified the Triazoles which sorted into two classes, 1,2,3 Triazoles (v-triazoles) and 1,2,4-triazoles (s-triazoles) (Potts, 1961). Both v- and s-triazoles are very important heterocycle chemicals having qualified natural and synthetic members (Asif, 2014). Each of the two types owns two tautomers, which varies from each other by the way nitrogen’s linked to the hydrogens (see Figure 2.5).
Figure (2.5): Illustration of the two classes of Triazoles, 1,2,3-triazoles (v-triazoles) and 1,2,4-triazoles (s-triazoles) (Clark, 2011; Havaldar & Patil, 2008).

1, 2, 3-Triazoles can be divided into three groups depending on the position of the substituent at the nitrogen atom. The third isomer 3, officially called as 3(1) H-1, 2, 3-triazole, acquired in occasional incidents (see Figure 2.6).

Figure (2.6): Illustrations of the three representative classes of the 1, 2, 3 triazoles (Clark, 2011; Havaldar & Patil, 2008).

The Triazoles having unaltered nitrogen atom within the ring attracted great attention as key compounds for the derivation of new materials with wide application potentials (Berlinck, 2007). In spite that 1, 2, 4-triazoles are familiar for more than a
century, studying their potentials stated just at the beginnings of 1970's (Haasnoot, 2000). Knowledge about Triazoles derivatives and their functions and characteristics steadily increased largely since the 1980's (Haasnoot, 2000). The 1,2,4-triazoles and the derivatives produced for various applications as for example the best known Amixol (3-aminotriazole) which widely applied as herbicide (Potts, 1961). Substitutions of the 1, 2, 4-triazoles found beneficial uses as bioactive compounds. They applied as fungicides, insecticides, anti-microbes, herbicides, and anti-parasites of animals. Some derivatives of Triazoles may apply to sensitize tumors for radiotherapies. Both types of Triazoles derivatives (1, 2, 3-triazoles and 1, 2, 4-triazoles) found applications in dyes industry and in photography arts. Derivation of polymers from Triazoles represents a key application for this heterocyclic compounds (Ferwanah, 2003a).

Triazoles together with their derivatives played a vital role in manufacturing and developing medicinal chemicals. Such chemicals may be introduced as antivirals, anti-bacterial, anti-tuberculosis, antidepressant, anticonvulsant, anti-inflammatory, anticancer, etc. Some reports pointed for their effects as glycogen synthase kinase-3 inhibitors, an antagonist for gamma-aminobutyric acid (GABA) receptors and muscarine receptors agonists. They found uses as neuroleptics and exhibited activities as anti-HIV-1, cytotoxin, antihistamine, and anti-proliferation. Because of huge potential effects of triazole compounds, searching and designing new and novel derivatives became a general trend in medicinal chemistry works (Pokhodylo, Shyyka, & Matiychuk, 2013).

2.5.1 The biological activity of Triazoles
The biological activities of Triazoles were investigated by many workers.

i. Antifungal

In 2013, Kocyigit-Kaymakcioglu et al. evaluated the biological activity of Triazoles derivatives including antifungal properties against plant pathogens. They found that Phomopis species showed the highest sensitivity when treated with these compounds. Compounds designated 1b, 1c, 3a and 4e showed selective activity when dosed to Phomopsis obscurants and the compounds signed as 1b and 4e displayed a comparable level of action when treating Phomopsis viticola (P. viticola) (Kocyigit-Kaymakcioglu et al., 2013).
A new triazole compound called viconazol addressed by Pearson et al, (2003) as treatment material. They exhibited very good antifungal activity when experimented in *vitro* against many types of yeasts and molds. This azole compound addressed as the second generation of antifungal treatment. Dosing of the compound may be introduced intravenously or orally. The oral formula showed brilliant bioavailability. Voriconazol accepted as a treatment against aggressive aspergillosis as well as recalcitrant infections by Pseudallescheria/Scedosporium and Fusarium species. Voriconazol in expected to be the best choice for struggling against serious fungal infections caused by filamentous fungi (Pearson et al., 2003).

Resistance against Triazoles studied by Resendiz Sharpe et al, (2018) They found that spread in the six continents *Aspergillus fumigatus* in spite presence of such phenomena still unknown in many countries. In most countries, resistance mutations against Triazoles found connected with the dominated environment but it still unknown whether the resistant traits evolved locally or emerged as migrant traits. Emerging resistance against Triazoles by some infectious fungi traits attracted working groups as the ISHAM/ECMM Aspergillus Resistance Surveillance to study the issue aiming to solve problems emerged from treatment failure in some cases. They concluded that treatment failure attributed in many cases to the delaying of diagnosis or restriction of treatments opportunities. Establishing of the group aimed to assist surveillance works and enhance global cooperation in this field. It is vital to define the resistance epidemiology on a global level since there is lacking in information about the issue (Resendiz Sharpe et al., 2018).

### ii. Antimicrobial

In 2013, researchers described how the synthesis of three series of new 1, 2, 4-triazole and benzoxazole derivatives containing substituted pyrazole moiety (11aed, 12aed, and 13aed). The results revealed that the compound 11c having a 2,5-dichlorothiophene substituent on pyrazole moiety and a triazole ring showed significant analgesic and antimicrobial activity (Vijesh, Isloor, Shetty, Sundershan, & Fun, 2013)

Al-Abdullah et al, in 2014 produced a triazole compound formally named 5-(1-adamantyl)-4-phenyl-1, 2, 4-triazoline-3-thione (compound 5). This compound
included formaldehyde and piperazines substitutions. The antimicrobial activity of the compound examined with group of gram-positive and gram-negative bacteria in addition to the infectious fungus Candida albicans. Around thirteen structures of that synthesis showed strong antibacterial effect (Al-Abdullah et al., 2014).

Other novel triazole compounds designed and created by Gupta & Jain, (2015) showed antimicrobial effects. The prepared compounds were in the form of Schiff bases built on 4-(benzylideneamino)-5-phenyl-4H-1,2,4-triazole-3-thiol skeleton. Production of the compound achieved by heating the thiocarbohydrazide and substituted benzoic acid. Then the reactants treated with substituted benzaldehyde. Around seventeen compounds derived from this reaction and surveyed for antimicrobial action (Gupta & Jain, 2015).

iii. Anti-inflammatory

In 2009, Moise et al have synthesized thiosemicarbazides derivatives (1, 3, 4-thiadiazole and 1, 2, 4-triazole). The derivatives contained phenylalanine moiety. The synthesized derivatives were examined for anti-inflammatory properties and showed promising results (Moise et al., 2009).

Palaska, Şahin, Kelicen, Durlu, & Altinok, (2002) have synthesized novel Sixteen 1-(2-naphthoxyacetyl)-4-substituted-3-thiosemicarbazide, 2-(2-naphthoxyethyl)-5-substitutedamino-1,3,4-oxadiazole, 2-(2-naphthoxyethyl)-5-substitutedamino-1,3,4-thiadiazole and 5-(2-naphthoxyethyl)-4-substituted-1,2,4-triazole-3-thione derivatives have been prepared and evaluated as orally active anti-inflammatory agents with reduced side effects (Palaska, Şahin, Kelicen, Durlu, & Altinok, 2002).

Haider et al, (2013) have synthesized a library of benzoxazolinone based 1, 2, 3-triazoles using click chemistry approach and screened them for their in vitro and in vivo anti-inflammatory activity. The compound 1 exhibited potent in vivo anti-inflammatory activity; the compound 2 exhibited significant TNF-α inhibitory activity (Haider et al., 2013).
iv. Anticancer activities

Developing of anticancer compounds derived from Triazoles was one of the most significant efforts done by the researchers in the field of medicinal chemistry.

Kumbhare et al, (2015) have synthesized a group of innovative triazole-linked N-(pyrimidine-2yl) benzo[d] thiazol-2-amine 5a-k. The compounds were assessed for anticancer capacity by using different cancer cell-lines (breast MCF-7, lung A549 and skin A375). The synthesized chemicals were examined for apoptosis in addition to their capacity to suppress some vital proteins (NF-kB, Survivin, CYP1A1, and ERK1/2) for survival and propagation of the cancer cells. MCF-7 cells were treated with compounds (5b, 5g, 5h, and 5i) at 2µM for 24h. The apoptotic aspect of these compounds further evidenced by an increase in the activity of caspase-9 in MCF-7 cells. Hence, these small molecules have the potential to control both the cell proliferation as well as the invasion process in highly malignant breast cancers (Kumbhare et al., 2015).

Ma et al, (2015) designed and created a series of novel 1, 2, 3-triazole-pyrimidine hybrids. They examined the compounds for their anticancer activity against four selected cancer cell-lines (MGC-803, EC-109, MCF-7 and B16F10). By using MTT assay method and compared with the well-known anticancer drug 5-FU, it was shown that most of the synthesized compounds exhibited moderate to good activity against all the cancer cell lines selected. Compound 17 showed excellent anticancer activity with single-digit micro molar IC$_{50}$ values ranging from 1.42 to 6.52µM. Further mechanistic studies revealed that compound 17 could obviously inhibit the proliferation of esophageal carcinoma (EC-109) cancer cells by inducing apoptosis and arresting the cell cycle at G2/M phase (Ma et al., 2015).

A group of 1, 2, 3-triazoles having heterocyclic moieties evaluated for anticancer ability. The derivatives produced the starting chemicals by using regular synthetic protocols. Antitumor capacity of the derived chemicals examined by using NCI60 cell line belongs the National Institute of Cancer. Observations revealed that some of the derivatives showed slight activity against cancer cell-lines. Only one compound exerted moderate antitumor capacity against melanoma, colon, and breast
cancer cell lines. Antitumor effectiveness calculated on the base of GI50 (Growth inhibition of 50%) level (Pokhodylo et al., 2013).

In 2018, El-Sherief et al., examined innovative synthesized chemicals having 1, 2, 4-triazole skeleton for antitumor capacity against a group of cancer cell lines. They assessed the effectiveness of the derivatives by using MTT assay. The derived chemicals signed as 8a, 8b, 8c, 8d, 10b, 10e, and 10g. They showed notable anti-proliferative action in contradiction of the examined cell lines. These derivatives showed lowest IC50 values with MTT assessment when regarded the three well-known antitumor targets (EGFR, BRAF, and Tubulin). Observations showed that 8c and 8d materials have nearly the same suppressing ability toward BRAF. They revealed strong suppression capacity against cells proliferation in addition to powerful inhibition of tubulins. 8c compound exhibited the best inhibition activity against EGFR with IC50=3.6µM (El-Sherief, Youssif, Bukhari, Abdel-Aziz, & Abdel-Rahman, 2018).

Rearranged derivatives of 1, 2, 4 triazoles nominated 8c; 10b, c; 13a, c and 14 screened for their antitumor capacity. 8c 13a and 13c compounds exhibited notable activity against leukemia, ovarian, renal and lung cancers. 8c exhibited GI50 of 0.70 µM and 0.07µM against leukemia cell-lines (CCRF-CEM and RPMI- 8226), 0.02 µM against ovarian cancer cell-line (OVCAR-3) and 0.60µM against kidney cancer cell-line (CARKI-1) (Al-Soud, Al-Masoudi, & Ferwanah, 2003).

A group of innovative derivatives of 3, 6-disubstituted 1, 2, 4-triazolo-[3, 4-b]-1, 3, 4-thiadiazoleinvented by Charitos et al, in 2016. Many of those derivatives showed themselves as potent cytotoxic and cytostatic antineoplastic agents. In their work, researchers underlined strategy of combining two chemicals (1, 2, 4-triazole & 1,3,4 thiadiazole) having pharmacological compatibility in one frame (Charitos et al., 2016).

In 2017, Bębenek et al produced a group of new Triazoles. They synthesized them through 1, 3-dipolar cycloaddition reaction occured between alkyne products of betulin and organic azides. 1H and 13C NMR, IR, and high-resolution mass spectrometry (HR-MS) analysis used to identify the molecular structures of products. The workers screened the products named compounds 5a–k and 6a–h for cytotoxic
activity against human cancer cell-lines (T47D, MCF-7, SNB-19, Colo-829, and C-32). The compound bistriazole 6b showed encouraging IC50 value (0.05 µM) when used against human ductal carcinoma T47D. The activity was higher than cisplatin with 500-double (Bębenek et al., 2017).

Molecular docking strategy used to design and synthesize 5- mercapto-1, 2, 4-triazoles. The derivatives examined for their anti-proliferative capacity targeting PI3K/ AKT pathway. This method produced three structures having high favorability for housing the active binding site of the PI3K protein revealing their capacity as potent inhibitors for the PI3K protein. The anti-proliferative capacity of the synthesized compounds examined by using cancer cell lines signed as A375, B164A5, MDA-MB-231 and A549 in addition normal human keratinocytes (HaCaT). Results confirmed with in vitro Alomar Blue assay. The synthesized compounds showed antitumor effects against breast cancer cell-line (MDA-MB-231). It also showed low toxicity against the normal cell-line (HaCaT) (Mioc et al., 2017).

A group of innovative compounds derived from coupling of oleanolic acid with 1, 2, 3-triazoles. The derived compounds showed excellent antitumor activity when examined with some cancer cell-lines. The derivative signed as 3t showed the most potent suppression activity against HT1080 cell-line. Pharmacological trails pointed apoptotic capacity of the 3t derivative against HT1080 cell-line (H. Wang et al., 2010).

Different alkyl halides used to alkylate and alter 1, 2-dihydro- 5-(1H-indol-2-yr)-1, 2, 4-triazole-3-thione. Alterations produced three groups of indolyl-triazoles derivatives. Capacity of these derivatives as anti-proliferative agents examined on HEPG-2 and MCF-7 cancer cell-lines. Observations revealed that a derivative signed as 2a showed itself as the most potent anticancer agent with IC50 equal to 3.58µg/mL against the HepG-2 cell-line and IC50 equivalent to 4.53µg/mL when used with MCF-7. The derivative signed as 7 showed the lowest activity with IC50>100 µg/mL in comparison to the drug doxorubucin (IC50 4.0 µg/mL) which was used as a standard. Mode of interaction of theses derivatives with tyrosine kinases (Akt, PI3,
and EGFR) revealed by using molecular docking simulation to expect their means of effectiveness (Boraei, Gomaa, El Ashry, & Duerkop, 2017).

In 2014, Kamal and Megaly derived a group of triazoles (N-substituted-3-mercaptop-1,2,4-triazoles named 3a,b and 7aed; triazole [1,3,4] thiadiazines named 5a,b and triazole [1,3,4] thiadiazoles named 4aed, 6 and 8aed). The derived chemicals were screened for their anticancer effectiveness by using six human cancer cell-lines in addition to normal fibroblasts. The derivatives signed as 3a, b, 5a and 8bed showed good cytotoxic effects against major of cell-lines. 4c derivative showed excellent cytotoxicity against the gastric cancer-cell line with IC$_{50}$ 25nM. The 4c showed very less cytotoxicity when applied to the normal fibroblast cells (WI38) with IC$_{50}$ around 10,000nM (Kamel and Megally Abdo 2014).

2.6 Mechanisms of anti-tumor activity of triazole compounds

The anti-cancer effects exerted by triazole compounds attack DNA to do DSB then inducing arrests of the cell cycle and apoptotic cell death (Berlinck, 2007). Indeed, there are little reports that contribute to the understanding of how they beginning their anti-tumor activity, also there are two pathways that have been regarded. One of these mechanisms explained the induction of apoptosis (Kamel & Megally Abdo, 2014).

2.6.1 Cell death

The chemotherapeutic approaches objective in killing the tumor cells by inducing programmed cell death (Notte, Leclere, and Michiels, 2011; Ropolo et al., 2012). However, suggested that they also induce other forms of cell death (no apoptotic death) as autophagy, necrosis and mitotic catastrophe (Notte, Leclere, and Michiels, 2011).

2.6.2 Apoptotic pathway

“Apoptosis” means leaves falling from a tree that is a Greek word. Also, it identified as the programmed cell death (PCD) naturally occurring, apoptosis responsible for maintaining tissue homeostasis (Okada and Mak, 2004) (Hotchkiss et al., 2009). The cell shrinkage, cell membrane blabbing, chromatin condensation, and nucleosomal fragmentation all there are considered morphological characteristics of apoptotic cell death. Apoptosis has two basic pathways according to its caspase
dependency. The mechanism of caspase dependent apoptosis includes a: receptor-mediated (extrinsic) or extracellular activated pathway and b: mitochondria-mediated (intrinsic) pathway (Figure 2.7) (Okada and Mak, 2004) (Fulda and Debatin, 2006; Kaushal et al., 2008). Apoptosis has been represented the main mechanism of chemotherapy -induced death of the cells. However many cancers activate other death pathways, such necrosis due to defective in the apoptotic pathway.

2.6.2.1 Intrinsic apoptosis

The intracellular signals as hypoxia, DNA damage stimulate the mitochondrial mediated apoptosis or intrinsic apoptotic pathway (Ghatage et al., 2012). The BCL-2 family proteins including the anti-apoptotic subfamily members (BCL-2, Bcl-xl, Bcl-w, Mcl-1, and Bf1-1/A1) in addition to pro-apoptotic subfamily members seen as mediators for intrinsic apoptosis which lacks the (BH4) domain and separated into two groups (Bax, Bok, Bak group and BH3 domain-only proteins group counting Bid, Bad, Bim and PUMA) (Yan et al., 2008). Bax and Bak proteins translocated from the cytoplasm to the OMM which regarded as the main pro-apoptotic proteins (Martinou and Youle, 2011). Normally, they are found as inactive monomeric protein in the cytosol attached to the intracellular membranes. Their oligomerization of the mitochondrial outer membrane allows efflux of apoptogenic factors as cytochrome c to the cytoplasm (Yan et al., 2008). Then the cytochrome c interacts with apoptosis protease activating factor 1 (Apaf-1) to excite apoptosome assembly. Consequently, the apoptosome activates caspase 9 and then activates downstream effector caspases such as caspase 3 and caspase 7. As a result a proteolytic cascade begins to degrade of different intracellular structures and finally to cell death (Lakhani et al., 2006; S. Yuan et al., 2013). Inhibit apoptotic proteins family (IAP); including X-linked IAP (XIAP), c-IAP1, and c-IAP2, can bind this caspase-3, caspase-7, and caspase-9 to inhibit the active sites. Smac/DIABLO and Omi/HtrA2 released from mitochondria can bind these IAPs to prevent their inhibition of the activated caspases (Du, Fang, Li, Li, & Wang, 2000; Verhagen et al., 2000). There are low levels of Bax/Bak in the outer mitochondrial membrane (OMM) of the normal cells but it has been observed that Bax moves from the cytoplasm to the mitochondria continuously (Edlich et al., 2011). The BCL-2/Bcl-XL anti-apoptotic proteins are present to retro-translocate Bax to the cytoplasm. The responsibility of
sending Bax/Bak to the mitochondria and of triggering changes in its conformation on several factors as p53 and BH3-only proteins, thus preventing MOMP (Cory and Adams, 2002; Kuwana et al., 2005).

**Figure (2.7):** Regulation of caspase-dependent apoptotic pathways.

As shown in Figure (2.7), the black color represents of Pro-apoptotic pathways and the blue color represents the anti-apoptotic (survival) pathways. Depending on the binding a ligand to a death receptor, then the extrinsic apoptotic death is exciting. Adapter proteins FADD and TRADD lead to activate of caspase 2 and caspase 8. The active caspase 8 initiates extrinsic apoptotic pathway directly (type 1) with activating executioner caspases (3 and 7) or targeting the extrinsic apoptotic pathway (type 2) then it/activates the intrinsic apoptosis including cleavage of Bid to induce cytochrome c release from inner mitochondria. The intrinsic (mitochondrial) apoptotic pathway can be excited through different cellular stresses that result in cytochrome c release from the mitochondria, to form apoptosome particles and activation of caspase 9. Active caspase 9 then activates
the executioner caspases (3 and 7) which called the common pathway then destruction the cell to particles, finally macrophage process occurs by macrophages (Benn and Woolf, 2004).

2.6.2.2 Extrinsic apoptosis process

When cell death ligands bind (as APO 21 or TRIAL) to cell death receptors as tumor necrosis factor (TNF) family, CD95L (FasL/Apo-1), DR3, DR4, DR5, and DR6 initiated the extrinsic apoptotic pathway (Peter and Krammer, 2003). This collection of proteins (FADD, receptor, and caspases) induce structural changes in the domains of intracellular death receptors to be active (Fulda and Debatin, 2006). Afterward, the apoptotic proteins were induced by active death receptors to produce a death-inducing signaling complex (DISC) which initiate extrinsic apoptosis, leads to their auto proteolytic cleavage by activated a caspase cascade (Cory and Adams, 2002) (Donepudi; Boatright., 2003). The initiator procaspases 2 or 8/10 were activated to excite the extrinsic apoptotic pathway irreversibly (Scaffidi et al., 1998) (Ghatage et al., 2012). It appears that caspase 8, not caspase 2 is involved in response to treatment with cisplatin of a various cancer cell lines such as melanoma, ovarian carcinoma, lung cancers and osteosarcoma (Bagnoli et al., 2007; Mirmohammadsadegh et al., 2007; Paul et al., 2012).
Chapter 3
Methodology
Chapter 3
Materials and Methods

3.1 Materials

3.1.1 Chemicals and Reagents
The chemicals and reagents used in this study are summarized in Table 3.1.

Table (3.1): Chemicals and reagents that were used in this 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>Dimethyl sulfoxide(DMSO)</td>
<td>AppliChem- Germany</td>
</tr>
<tr>
<td>3</td>
<td>Dimethylformamide (DMF)</td>
<td>AppliChem- Germany</td>
</tr>
<tr>
<td>4</td>
<td>Ethylene diamine tetra etic acid (EDTA)</td>
<td>AppliChem- Germany</td>
</tr>
<tr>
<td>5</td>
<td>Fetal Bovine Serum</td>
<td>Biological Industries</td>
</tr>
<tr>
<td>6</td>
<td>MTT Kit</td>
<td>Sigma USA</td>
</tr>
<tr>
<td>7</td>
<td>Novel Triazoles compounds</td>
<td>The compounds are provided by Prof. Dr. Adel Awad Allah from the department of chemistry – The Islamic University of Gaza</td>
</tr>
<tr>
<td>8</td>
<td>Phosphate Buffer Saline</td>
<td>AppliChem- Germany</td>
</tr>
<tr>
<td>9</td>
<td>Primary Antibody</td>
<td>Santa cruz USA</td>
</tr>
<tr>
<td>10</td>
<td>RPMI1640-Roswell Park Memorial Institute</td>
<td>Biological Industries</td>
</tr>
<tr>
<td>11</td>
<td>Secondary Antibody</td>
<td>Santa Cruz USA</td>
</tr>
<tr>
<td>12</td>
<td>Trypsin</td>
<td>Biological Industries</td>
</tr>
<tr>
<td>13</td>
<td>Trypan Blue</td>
<td>Biological Industries</td>
</tr>
<tr>
<td>14</td>
<td>Western Blot Reagents</td>
<td>AppliChem- Germany</td>
</tr>
</tbody>
</table>
3.1.2 Disposables

**Table (3.2):** Major Disposables will be used in this study.

<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>24 well plate</td>
<td>Intron</td>
</tr>
<tr>
<td>4</td>
<td>6 well plate</td>
<td>Intron</td>
</tr>
<tr>
<td>5</td>
<td>Petri dish (6cm)</td>
<td>Intron</td>
</tr>
<tr>
<td>6</td>
<td>Pipettes</td>
<td>Intron</td>
</tr>
<tr>
<td>7</td>
<td>Falcon Centrifuge Tube</td>
<td>Intron</td>
</tr>
</tbody>
</table>

3.1.3 Equipment

**Table (3.3):** Major Equipment 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>Mini Trans-Blot® Cell and Criterion™ Blotter</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>Olympus 1X71, USA</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>Dr. Mazen ALzaharna, faculty of health sciences, Islamic University of Gaza</td>
</tr>
<tr>
<td>2</td>
<td>Human colorectal adenocarcinoma called (Caco-2)</td>
<td>Dr. Mazen ALzaharna, faculty of health sciences, Islamic University of Gaza</td>
</tr>
<tr>
<td>3</td>
<td>Uterine Cervical adenocarcinoma (HeLa)</td>
<td>Dr. Johnny Stiban, Birzeit University of Palestine</td>
</tr>
</tbody>
</table>
3.2 Methodology

3.2.1 Study design
An Experimental in vitro study cell culture.

3.2.2 Setting
Labs of Biology and Biotechnology department at the Islamic University of Gaza.

3.3 Methods

3.3.1 Cell culture Maintenance

The MCF-7 human breast adenocarcinoma (estrogen receptor positive) cells and CACO-2 (human colorectal adenocarcinoma) kind gifts of Dr. Mazen ALzaharna, faculty of health sciences, Islamic University of Gaza and Hela cells from Dr. Johnny Stiban, Birzeit University of Palestine. MCF-7 and CACO-2 were maintained in DMEM medium and HeLa (Cervical cancer cells) were maintained in RPMI 1640 medium. All media were supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100μg/mL streptomycin. Cells were maintained at 37°C in a 5% CO₂ and 95% air-humidified incubator. Media was replaced every 2-3 days (Aliwaini et al., 2015; H. C. Wang et al., 2016).

3.3.2 Treatments

3.3.3 Triazole-based compounds treatments

Several triazole-based compounds were synthesized in the laboratory headed by Prof Adel Awadalal, labelled (14a, 13a, 11a, 9a, 6a, 4a, 12d, 11d, 11c, and 11b) (Figure 3.1), which showed expected to have anticancer activity against the uterus cervical cancer cell line, breast cancer cell line and colorectal cancer cell line in vitro. The compound 14a was dissolved in DMSO or DMF (at 100 °C) to give 10μM. The compounds 9a and 11a dissolved in heated DMF (at 100 °C) to give a final concentration of 10 μM. All compounds were stored at room temperature for no more than 7 days. At use and in order to get the final concentration, subsequent dilutions in the appropriate media for each cell line were prepared as shown in (Figure 3.2). Vehicle-treated cells were incubated in normal media with DMF or DMSO (the vehicle in which triazole-compounds were dissolved in).
Figure (3.1): Structural formulas of the Triazole compounds

(A) 3-Acetyl-4-benzoylamino-1-(4-chlorophenyl)-1,2,4-triazaspiro[4.6]undec-2-ene (14a) \((C_{23}H_{25}ClN_4O_2)\).

(B) Acetyl-4-benzoylamino-1-(4-chlorophenyl)-8-methyl-1,2,4-triaza-spiro[4.5]dec-2-ene (9a) \((C_{23}H_{25}ClN_4O_2)\).

(C) Acetyl-4-benzoylamino-1-(4-chlorophenyl)-8-methyl-1,2,4,8-tetra-azaspiro[4.5] dec-2-ene (11a) \((C_{22}H_{24}ClN_5O_2)\) (Ferwanah, 2003).
Figure (3.2): Dilutions of the triazole derivatives compounds in the appropriate media to get the final concentration.

3.3.4 Cell morphology

Cells were plated at suitable numbers in order to obtain 60-70% confluency on the 2 days of treatment. After treating the cells with the BA-compounds the morphological changes were monitored and photographed using an inverted light microscope (Olympus 1X71, USA) and camera (Zeiss Axio Cam, Germany) respectively. Any morphological changes were photographed using a light microscope.
3.3.5 Cytotoxicity assays (MTT)

To determine the cytotoxic effect of the indicated compounds HeLa, MCF-7 and CACO-2 cells were seeded respectively at (3000-6000 HeLa cells/well), (7000-9000 MCF-7 cells/well), (10,000-12,000 CACO-2 cells/well) in quadruplicate in a 96-well plate in 100µL culture media and treated after two days with a range of the indicated concentrations (0.0 – 100µM) of Triazole compounds 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, 10 µL of MTT solution was added to each well and incubated for 4 hours at 37°C. This was followed by the addition of 100µL solubilization buffer (10% SDS in 0.01 N 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 was calculated after taking in reference to the untreated control cells using the formula. Cell viability percent was taken after calculated the average of quadruplicate reading of absorbance.

(Behray et al., 2016; Deshpande, 2016; Martinho et al., 2017)

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

To draw the two dimensions chart for different Triazole compounds concentrations on X-axis and percent of cell viability on Y-axis. Then was calculated IC50 by using linear regression chart formula (Y = a * X + b), IC50 value equal X value is estimated when Y value equal 50

Three separate experiments were performed to determine the concentration of 14a, 9a, and 11a required for killing 50% of the cells (IC50). The IC50 values were calculated by linear equation from Microsoft office excel as described before (Aliwaini et al., 2015; Wang et al., 2016).

3.3.6 Trypan-blue dye exclusion viability assay

Trypan blue exclusion technique (Strober, 2015) was used to study the cytotoxic effect of the indicated compounds in HeLa, MCF-7 and CACO-2 cell lines. Cells were seeded at density of (200,000- 300,000 HeLa cells/well), (600,000-900,000 MCF-7 cells/well) and (1400, 000 000- 1600,000 CACO-2 cells/well) induplicate in a 6-well plate. Plated cells were treated after 24 h the medium was
replaced with a range of the indicated concentrations of specific compound or vehicle for 48 hours. Afterward, the medium was discarded and the cells were harvested by trypsinization and washed twice with (PBS). A volume of 0.4% trypan-blue stain (Biological Industries Israel Beit Haemek) that is equal to the residual PBS was then added. After 5 min incubation, the cells were 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. Negative control cells were incubated with media without a vehicle and treated the same way.

The percentage of cell viability was calculated using the following equation:

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

3.3.7 Counting using a hemocytometer

Cells were seeded in duplicate in a 6-well plate at a density of 4X 10^4 Cells/well and treated as described above. Afterward, the cells were stained with Trypan blue dye collected by trypsinization to analyzed and counted using a haemocytometer at 2-3 day intervals.

3.3.8 In vitro cell migration assay

Cells were grown to 100 % confluence in 35 mm tissue culture dishes. A linear wound was made by scratching through the monolayer using a sterile 200µl pipette tip. To remove cell debris, the growth medium was replaced after the cells were washed 2–3 times with phosphate-buffered saline (PBS), then the plates were incubated at 37 °C in 5% CO₂, after adding the treatment. Several markings were made along the edges of the scratch line, which were used as reference points, and the wound widths measured at the time of the scratching (0h) and thereafter at 24h, 48h and 72h intervals after scratching. Pictures were taken using a phase contrast microscope and Image J software was used to measure the wound width. The difference in width represents the distance migrated in µm.

3.3.9 Western blot analysis

Cells washed twice with ice-cold PBS and collected by scraping with a 1 ml plunger. Whole cell extracts were prepared using 2X Laemmli sample buffer, boiled for 10 min and stored at -20°C. The protein concentration for each cell extract
determined using the BCA Protein Assay kit (Pierce, USA), with bovine serum albumin as the standard. Equal amounts of protein loaded in each lane and resolved on 6-15% SDS-PAGE gels, then transferred electrophoretically to a Hybond ECL nitrocellulose membrane (Amersham Biosciences, USA). Membranes blocked for 1 hour at room temperature with PBS containing 5% non-fat dry milk and probed with appropriate primary antibodies O/N 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) in blocking solution at room temperature with shaking for 1 hour. Membranes again washed in PBS/T and visualised by enhanced chemiluminescence (Pierce, USA). The following dilutions of these antibodies and dilutions used: 1:1000 rabbit polyclonal anti-PARP1/2 (sc-7150), (Santa Cruz, California, USA), 1:5000 rabbit polyclonal anti-p38 (M0800) (Sigma, St. Louis, MO, USA) or the following antibodies which obtained from Cell Signaling (Boston, MA, USA): 1: 1000 rabbit polyclonal. If necessary, the expression of these proteins was quantified as the densitometry value analyzed by UN-SCAN-IT gel 6.1 software and normalized to the appropriate loading control.

(Aliwaini, Swarts, Blanckenberg, Mapolie, & Prince, 2013; Yamasaki et al., 2008).

3.3.10 Statistical analysis

Data presented are mean ± SEM (Standard error of the means) of appropriate replicates. Statistical significance was assessed between the groups using the Student’s t-test.
Chapter 4

Results
Chapter 4

Results

4.1 Introduction

Cancer is one of the big health problems worldwide, affecting the lives of people of different ages, and races (Kumbhare et al., 2015). There are many current therapies including surgery radiotherapy, hormone therapy, immune therapy, targeted therapy, and chemotherapy. They are insufficient at providing a cure, prolonging survival and improving the quality of life (American Cancer Society 2016). The main reason for the failure of anticancer therapies is tumor resistance to several groups of drugs (Ministry of Health, 2014). So efforts for overcoming these limitations have focused on identifying novel chemotherapeutic agents and understanding the mechanisms by which they function. Of the suggested therapies 1, 2, 4 triazole still attracts the attention of chemists, biologists, technologists, and other specialists. The 1,2,4-Triazole compounds are considered interesting heterocycles since they possess important pharmacological activities such as antifungal, antiviral activities and anti-inflammatory (Shi, Veerendra, Shi, & Poojary, 2003).

The following part of this thesis focuses on the anti-cancer properties of novel Triazole based compounds as promising anticancer agents against different cancer cell lines.

4.2 Screening of a group of Triazole compounds in cervical cancer cell lines.

A panel of Triazole compounds was synthesized by the laboratory headed by Prof. Dr. Awad Allah. This panel included (4a, 6a, 14a, 11c, 11d, 13a, 9a, 11a, 12a, 11b) (see annex1) the compounds first screened for possible cytotoxic effects in HeLa cervical cancer cell lines. Cells treated with (15µM) of each compound or vehicle for 48 hours. Cell viability determined using the MTT assay (see Figure 4.1) screening for cytotoxic effects of these compounds on HeLa cells achieved by dissolving each compound in DMSO at 100°C. Effects of the triazole compounds tracked after 48 hours. When comparing the screened compounds, 14a, 9a, 11a showed cytotoxic effects but 14a was the strongest among the others against the cervical cancer cell-line and therefore was chosen for further study.
4.3 The antiproliferative effect of Triazole 14a on the cervical cancer cell line (HeLa).

The anti-proliferative effect of 14a compound assayed by determination of its IC$_{50}$ using MTT method. HeLa cell cultures treated with a range of concentration of the compound 14a (0.0 µM – 100 µM). The results obtained showed the strongest cytotoxic effects at 50 µM and 60 µM (see Figure 4.2).

The IC$_{50}$ value was 54.6 µM as calculated by the equation described in materials and methods (see section 3.3.5). Importantly, while low concentrations of 14a (10 µM – 30 µM) showed little any cytotoxic effect in Hela cells, On the other hand nearly 55% of cervical cancer cells were killed at 40 µM 14a (Figure 4.2).
Figure (4.2): 14a induces cytotoxicity in cervical cancer cells.

(a) Results show the mean percentage of cell growth rate ± STD (bars) of at least three experiments performed in quadruplicate.

(b) Morphology of healthy untreated Hela cells after 48h under the light inverted microscope at magnification 40X.

(c) The morphological changes of treated Hela cells with 50µM of 14a as seen under a light inverted microscope at magnification 40X. It shows fragmented cells and dead cells.

4.4 Effect of Triazole compound (14a) on cervical cancer cell line viability.

The effect of 14a on the cell viability of Hela cells was measured by trypan blue assay. Cells were treated by different concentrations of 14a (0.0µM, 50µM &100µM) for 48 hours. Results showed that the cell viability of Hela cells decreased in a dose-dependent manner. For example, 14a killed 55% of Hela cells at 50µM and 80% at 100µM at 48 hours as shown in (Figure 4.3a).

Although trypan blue assay fundamentally measures cell death rate, cell proliferation rate by cell counting can also be evaluated by this assay. In support of MTT data, trypan blue results show that 14a exerts anti-proliferation effects on Hela cells by
killing cancer cells and decreasing its number (Figure 4.3b). While 50μM of 14a decreased 45% of Hela cells proliferation, it reduced 65% at 100μM. Taken together these findings show that 14a has a significant inhibitory effect on HeLa cells viability and proliferation rate in a dose-dependent manner.

![Image](image.jpg)

Figure (4.3): 14a inhibits cancer cell viability and proliferation.

Effect of 14a on cell viability of cervical cancer cell. Cancer cell line was plated in 6-well plates and after 24 hours, cells were treated with 0.0μM, 50μM, and 100μM of 14a or vehicle. Cell viability assessed by the trypan blue assay after 48 hours of the treatment. Results represent the mean percentage ± SEM of control of at least two experiments performed in twice replicate (a) show the percent viability of HeLa cancer cell. (b) Show the anti-proliferation effect of 14a on HeLa cancer cell.

4.5 Migration inhibition effect of 14a on human cervical cancer cells.

For further exploration of the anti-tumor activity of 14a, a scratch motility assay performed and reduction in cell migration observed for HeLa cells exposed to 25μM of 14a for 0h, 3h, 6h, 24h, 48h and 72h (Figure 4.5). Results show that 14a has no anti-migration effect on HeLa cells.
Figure (4.4): 14a Inhibits migration ability of cervical cancer cells.

(a) 14a inhibits the migratory ability of cervical cancer cells in an in vitro scratch assay. Cells were grown to 100% confluence and a linear wound created through the cell monolayer. Cell motility assayed at the indicated times after addition of either vehicle (control) or 14a (25μM) for 48 hours.

(b) Specified time points (x-axis) cells were photographed using (10x; Inverted microscope) and the area migrated was measured and expressed relative to zero time (y-axis).
4.6 The anti-proliferative effect of Triazole 9a on cervical cancer cell line.

Cervical cancer cells treated with a range (0.0µM to 50µM) of the 9a compound for 48 hours. Cell cytotoxicity was determined using the MTT assay. However, it was not possible to calculate the concentration that inhibits the cell growth by 50% (IC\(_{50}\)) from the experiment; therefore, it was calculated from a linear equation by Excel Microsoft office 2010 which explained in chapter materials and methods, section 3.3.5 (Figure 4.5). The results obtained showed that 9a has cytotoxic effects with IC\(_{50}\) (equal to 64.6µM) in the cervical cancer cells. On one hand, low concentrations of 9a (10µM – 20µM) showed a little cytotoxic effect in HeLa cells, On the other hand, nearly 30% of cervical cancer cells were killed at 30µM 9a.

![Figure (4.5): 9a induces cytotoxicity in cervical cancer cells.](image)

Results show the mean percentage of cell growth rate ± STD (bars) of at least three experiments performed in quadruplicate.
4.7 Effect of Triazole compound (9a) on viability and proliferative activity of the cervical cancer cell line (HeLa).

The effect of 9a on the cell viability of HeLa cells was measured by trypan blue assay. The cell viability was determined after incubation for 48 hours with 9a at different concentrations (0.0μM, 10μM, 20μM, 30μM, 40μM, and 50μM) using this assay. Results show that the cell viability of HeLa cells was not changed in a dose-dependent manner as shown in (Figure 4.6a). Although trypan blue assay fundamentally measures cell death rate, cell proliferation rate by cell counting can also be evaluated by this assay. In support of MTT data, trypan blue results show that 9a exerts anti-proliferation effects on HeLa cells by killing cancer cells and decreasing its number (Figure 4.6b). While 30μM of 9a decreases 25% of HeLa cells proliferation, it reduces 30% at 40μM. Taken together these results show that 9a has a little effect on HeLa cells viability and proliferation rate in a dose-dependent manner.

![Image](48)

Figure (4.6): Reduction of HeLa cells viability in response to 14a.

Effect of 9a on cell viability of cervical cancer cell. Cancer cell line was plated in 6-well plates and after 24 hours, cells were treated with 0.0μM, 10μM, 20μM, 30μM, 40μM, and 50μM of 9a or vehicle. Cell viability was assessed by the trypan blue assay after 48 hours of the treatment. Results represent the mean percentage ± SEM of control of at least two experiments performed in twice replicate. (a) show the percent viability of Hela cancer cell. (b) show the anti-proliferation effect of 9a on Hela cancer cell.
4.8 Migration inhibition effect of 9a on human cervical cancer cells.

For further exploration of the anti-tumor activity of 9a, a scratch motility assay performed and a significant reduction in cell migration observed for Hela cells exposed to 25μM of 9a for 0h, 24h, 48h and 72h (Figure 4.7). Importantly, the anti-migratory effect was more obvious at 48 hours where the untreated cells showed 70% closure of the scratch while the treated cells showed 40% closure while after 72 hours, the untreated cells showed 98% closure of the scratch while the treated cells showed 60% closure of the scratch. In conclusion, 9a compound has a potent anti-migratory effect on HeLa cells.

![Figure 4.7: 9a inhibits migration ability of cervical cancer cells.](image-url)

Figure (4.7): 9a Inhibits migration ability of cervical cancer cells.
(a) 9a inhibits the migratory ability of cervical cancer cells in an in vitro scratch assay. Cells were grown to 100% confluence and a linear wound created through the cell monolayer. Cell motility assayed at the indicated times (0, 24, 48, 72) hours after addition of either vehicle (control) or 9a (25μM).

(b) Specified time points (x-axis) cells were photographed using (10xs; Inverted microscope) and the area migrated measured and expressed in relative to zero time (y-axis).
4.9 The ant proliferative effect of Triazole 11a on the cervical cancer cell line (HeLa).

Cervical cancer cells treated with a range (0.0 µM to 50 µM) of the 11a compound for 48 hours. Cell cytotoxicity was determined using the MTT assay. However, it was not possible to calculate the concentration that inhibits the cell growth by 50% (IC\(_{50}\)) from the experiment; therefore, it was calculated from a linear equation by Excel Microsoft office 2010 which explained in chapter materials and methods, section 3.3.5 (Figure 4.8). The results obtained showed that 11a has cytotoxic effects with IC\(_{50}\) (equal to 62.4 µM) in the cervical cancer cells. On the one hand, low concentrations of 11a (10 µM – 20 µM) showed a little cytotoxic effect in HeLa cells, On the other hand, nearly 40% of cervical cancer cells were killed at 30 µM 11a.

Figure (4.8): 11a induces cytotoxicity on cervical cancer cells.

Results show the mean percentage of cell growth rate ± STD (bars) of at least three experiments performed in quadruplicate.
4.10 Effect of Triazole 11a compound on viability and proliferative activity of the cervical cancer cell line (HeLa).

The effect of 11a on the cell viability of HeLa cells was measured by trypan blue assay. The cell viability was determined after incubation for 48 hours with 11a at different concentrations using this assay. Results show that the cell viability of Hela cells was not changed in a dose-dependent manner as shown in (Figure 4.9a). Although trypan blue assay especially measures cell death rate, cell proliferation rate by cell counting can be also evaluated by this assay. In support of MTT data, Trypan blue results exhibit that 11a has little anti-proliferation effects on HeLa cells by killing cancer cells and decreasing its number (Figure 4.9b). While 40μM of 11a decreases 10% of HeLa cells proliferation, taken together these findings show that 11a has no effect on HeLa cells viability and proliferation rate in a dose-dependent manner.

Figure (4.9): Reduction of HeLa cells viability in response to 11a.

Effect of 11a on cell viability of cervical cancer cell. Cancer cell line was plated in 6-well plates and after 24 hours, cells were treated with 0.0μM, 10μM, 20μM, 40μM, 60μM and 80μM of 11a or vehicle. Cell viability was assessed by the trypan blue assay after 48 hours of the treatment. Results represent the mean percentage ± SEM of control of at least two experiments performed in twice replicate. (a) show the percent viability of HeLa cancer cell. (b) show the anti-proliferation effect of 11a on HeLa cancer cell.
4.11 11a inhibits migration of human cervical cancer cells.

To further explore the anti-tumor activity of 11a, a scratch motility assay was performed and reduction in cell migration was observed for HeLa cells exposed to 25μM of 11a for 0h, 24h, 48h and 72h (Figure 4.10). Results show that 11a has anti-migration effect on HeLa cells after 72 hours. Importantly, the anti-migratory effect was more obvious at 72 hours where the untreated cells showed 100% closure of the scratch while the treated cells showed 75% closure.

![Figure 4.10](image)

**Figure (4.10):** 11a Inhibits migration ability of cervical cancer cells.

(a) 11a inhibits the migratory ability of cervical cancer cells in an in vitro scratch assay. Cells were grown to 100% confluence and a linear wound created through the cell
monolayer. Cell motility was assayed at the indicated times after addition of either vehicle (control) or 11a (25μM) for 48 hours.

(b) Specified time points (x-axis) cells were photographed using (10x; Inverted microscope) and the area migrated was measured and expressed relative to zero time (y-axis).

4.12 14a displays cytotoxic activity against breast cancer cells (MCF-7).

Breast cancer cell line was treated with a range (0.0μM to100μM) of the 14a compound for 48 hours. Cell cytotoxicity was determined using the MTT assay, and the concentration that inhibits cell growth by 50% (IC₅₀) was calculated from the linear equation by Excel Microsoft office 2010 in materials and methods (see section 3.3.5) (Figure 4.11). The results obtained showed that 14a has strong cytotoxic effects with IC₅₀ (equal 59.8μM) in the breast cancer cells. 14a exerts potent cytotoxic effects specifically on 50μM concentration where it killed nearly 55% of MCF-7 cells while on 60μM, it killed 40% of breast cancer cells compared to the untreated cells.

![Figure 4.11](image)

Figure (4.11): 14a induces cytotoxicity in breast cancer cells.

(a) Results show the mean percentage of cell growth rate ± STD (bars) of at least three experiments performed in quadruplicate.
(b) Morphology of healthy untreated MCF-7 cells after 48h under an inverted microscope at magnification 40X.

(c) The morphological changes of treated MCF-7 cells with 50µM of 14a as seen under an inverted microscope at magnification 40X. It shows fragmented cells and dead cells.

4.13 Effect of Triazole 14a compound on viability and proliferative activity of breast cancer cell line (MCF-7).

The effect of 14a on the cell viability of MCF-7 cells was measured by trypan blue assay. The cell viability was determined after incubation for 48 hours with 14a at different concentrations using this assay. Results show that the cell viability of MCF-7 cells was decreased in a dose-dependent manner. For example, 14a kills around 20% of MCF-7 cells at 50µM and 55% at 100µM at 48 hours as shown in (Figure 4.12a). Although trypan blue assay fundamentally measures cell death rate, cell proliferation rate by cell counting can be also evaluated by this assay. In support of MTT data, trypan blue results show that 14a exerts anti-proliferation effects on MCF-7 cells by killing cancer cells and decreasing its number (Figure 4.12b). While 50µM of 14a decreases 25% of MCF-7 cells proliferation, it reduces 40% at 100µM. Taken together these findings show that 14a has a significant effect on MCF-7 cells viability and proliferation rate in a dose-dependent manner.

![Figure (4.12): Reduction of MCF-7 cells viability in response to 14a.](image-url)
Effect of 14a on cell viability of breast cancer cell. Cancer cell line was plated in 6-well plates and after 24 hours, cells were treated with 0.0μM, 50μM, and 100μM of 14a or vehicle. Cell viability was assessed by the trypan blue assay after 48 hours of the treatment. Results represent the mean percentage ± SEM of control of at least two experiments performed in twice replicate. (a) show the percent viability of MCF-7 cancer cell. (b) show the anti-proliferation effect of 14a on MCF-7 cancer cell.


For further exploration of the anti-tumor activity of 14a, a scratch motility assay performed and a significant reduction in cell migration observed for MCF-7 cells exposed to 25μM of 14a for 0h, 24h, 48h and 72h (Figure 4.13). Importantly, the anti-migratory effect was more obvious at 48 hours where the untreated cells showed 50% closure of the scratch while the treated cells showed 25% closure while after 72 hours, the untreated cells showed 88% closure of the scratch while the treated cells showed 45% closure of the scratch.
Figure (4.13): 14a Inhibits migration ability of breast cancer cells.

(a) 14a inhibits the migratory ability of breast cancer cells in an in vitro scratch assay. Cells were grown to 100% confluence and a linear wound created through the cell monolayer. Cell motility was assayed at the indicated times after addition of either vehicle (control) or 14a (25μM) for 48 hours.

(b) Specified time points (x-axis) cells were photographed using (10x; Inverted microscope) and the area migrated was measured and expressed relative to zero time (y-axis).
4.15 14a exhibits a moderate level of toxicity on colorectal cancer cells 
(CACO-2)

The cytotoxic effect of 14a against CACO-2 colorectal cancer cell line was 
examined using the MTT assay. CACO-2 cells were treated with gradual 
concentrations of 14a (0.0 µM to 100µM) for 48 hours. A dose dependent inhibition 
of cell proliferation was observed only at higher concentrations than 100µM (Figure 
4.14). The concentration of 14a that inhibited 50% of cell growth (IC\text{50}) was 113µM. 
Taken together, these results showed that 14a exerts a cytotoxic effect on the 
colorectal cancer cell line as well as other cell lines (MCF-7 and Hela cell line).

![Graph showing proliferation rate vs concentration of 14a](image)

**Figure (4.14):** 14a induces cytotoxicity of colorectal cancer cells.

(a) Results show the mean percentage of cell growth rate ± STD (bars) of at least three 
experiments performed in quadruplicate.

(b) Morphology of healthy untreated CACO-2 cells after 48h under an inverted microscope 
at magnification 40X.

(c) The morphological changes of treated CACO-2cells with 50µM of 14a as seen under an 
inverted microscope at magnification 40X. It shows fragmented cells and dead cells.
4.16 Effect of Triazole 14a compound on viability and proliferative activity of colorectal cancer cell line (Caco-2).

The effect of 14a on the cell viability of Caco-2 cells was measured by trypan blue assay. The cell viability was determined after incubation for 48 hours with 14a at different concentrations using this assay. Results show that the cell viability of Caco-2 cells was decreased in a dose-dependent manner. For example, 14a kills 15% of Caco-2 cells at 50μM and 50% at 100μM at 48 hours as shown in (Figure 4.15a). Although trypan blue assay fundamentally measures cell death rate, cell proliferation rate by cell counting can be also evaluated by this assay. In support of MTT data, trypan blue results show that 14a exerts anti-proliferation effects on Caco-2 cells by killing cancer cells and decreasing its number (Figure 4.15b). While 50μM of 14a decreases 10% of Caco-2 cells proliferation, it reduces 50% at 100μM. Taken together these findings show that 14a has a significant effect on Caco-2 cells viability and proliferation rate in a dose-dependent manner.

**Figure (4.15):** Reduction of Caco-2 cells viability in response to 14a.

Effect of 14a on cell viability of colorectal cancer cell. Cancer cell line was plated in 6-well plates and after 24 hours, cells were treated with 0.0μM, 50μM, and 100μM of 14a or vehicle. Cell viability was assessed by the trypan blue assay after 48 hours of the treatment. Results represent the mean percentage ± SEM of control of at least two experiments.
performed in twice replicate. (a) show the percent viability of CACO-2 cancer cell. (b) show the anti-proliferation effect of 14a on CACO-2.

4.17 14a inhibits migration of human colorectal cancer cells.

To further explore the anti-tumor activity of 14a, a scratch motility assay was performed and a significant reduction in cell migration was observed for CACO-2 cells exposed to 50μM of 14a for 0h, 24h, 48h, 72h and 96h (Figure 4.16). Results show that 14a has no anti-migration effect on CACO-2 cells.

Figure (4.16): 14a Inhibits migration ability of colorectal cancer cells.

(a) 14a inhibits the migratory ability of colorectal cancer cells in an in vitro scratch assay. Cells were grown to 100% confluence and a linear wound created through the cell
monolayer. Cell motility was assayed at the indicated times after addition of either vehicle (control) or 14a (50µM) for 48 hours.

(b) Specified time points (x-axis) cells were photographed using (10x; Inverted microscope) and the area migrated was measured and expressed relative to zero time (y-axis).

4.18 14a induces Apoptosis

Triazole compounds have been shown to exert cytotoxicity by inducing apoptosis (Demchuk et al., 2014). To investigate the mechanism by which 14a exerts its cytotoxic effect, HeLa cells were plated and treated with 14a (50µM) for 24 and 48 hours. Protein extraction was tested by western blotting to estimate the level of cleaved PARP which is a strong marker of apoptosis. As shown in Figure (4.17) 14a treatment induced high level of PARP cleavage which confirms the apoptotic effect of 14a.

![Figure (4.17): 14a induces apoptosis.](image)

Hela cells were treated with vehicle (DMSO) or 50µM 14a for 24 and 48 hours and protein extracts were separated by (8 %) SDS gel and western blotting to estimate the level of cleaved PARP and p38.
Chapter 5
Discussion
Chapter 5

Discussion

Cancer is a pernicious and lethal disease that records one in six deaths globally. In spite of the progression in cancer diagnosis and treatment, the number of cancer deaths is expected to elevate to reach 13.5 million deaths in 2030 (American Cancer Society, 2012). The main objective of most of cancer research groups is to find anti-cancer drugs that can be more efficient to treat human cancers and to overcome chemotherapy side-effects. In recent years, numerous research has used synthesized triazole compounds which have promising activity against cancer cell lines (Al-Soud et al., 2003). The current study evaluated the cytotoxic effect of Triazole compounds called 9a, 11a, 14a and showed that their inhibitory effect against breast cancer, cervical cancer, and colorectal cancer cell lines. Our results showed that these compounds may be promising initial drugs for the treatment of breast, cervical and colorectal cancers. Furthermore, this study demonstrates that Triazole compounds namely 14a inhibits cell survival and migration capacity of the breast, cervical and colorectal cancer cells.

Similar previous studies reported that Triazole compounds demonstrated significant anti-tumor activity for various cancer cell lines e.g. MCF7, Hela and CACO2 (Pokhodylo et al., 2013; H. Wang et al., 2010). More recently, Al-Masoudi, et al., (2006) Show that the 1, 2, 4-triazole compounds have a significant anticancer activity to different cancer cell lines with low side effects and less kidney toxicity than metal chemotherapies (Al-Masoudi1, I. A, Y. A, Al-Soud2, Al-Salihi3, N. J and Al-Masoudi4, 2006; Vijesh et al., 2013).

5.1 In vitro cytostatic and cytotoxic activity

The 10 novel Triazole compounds (4a, 6a, 14a, 11c, 11d, 13a, 9a, 11a, 12a, and 11b) were tested in vitro against cervical cancer cell line. Results regarding the most active derivatives are presented in Figure 4.1. As it is shown, compound 14a exhibited a very potent cytotoxic effect against cervical cancer cell line. The derivatives 9a and 11a were active but exhibited lower anticancer potency, while the compounds 6a, 11c, and 12a were less active. The derivatives 4a, 11d, 11a, and 13a
were relatively inactive at the concentrations tested in vitro. Therefore, compounds 14a, 9a, 11a were chosen for further investigation in this study.

5.2 Anti-proliferative activity of triazole compounds 14a, 9a, and 11a on three different cancer cell lines

In this study, the antigrowth effect of the newly synthesized triazole compounds (14a, 9a, and 11a) was investigated against uterine cervical adenocarcinoma, estrogen receptor positive human breast cancer and colorectal adenocarcinoma cell lines by the MTT and Trypan blue assays. The anti-proliferation activity was assessed after the treatment with different concentrations (0.0 - 100 µM) of (14a, 9a, and 11a) compounds for 24 and 48 hours by the MTT (Figures 4.2 - 4.4) and the Trypan blue (Figures 4.5 - 4.7) assays. It was found that the 14a triazole compound has anti-proliferative effect in time and dose-dependent manner. According to the dose-response results, the 14a compound had a stronger cytotoxic effect on HeLa cells and on MCF-7 than CACO-2 cells with the IC50 values of 54.6 µM, 59.8µM, and 113µM respectively. Between the two assays used in the study, there was a good correspondence. It was found that the trypan blue assay results were quite similar to the MTT assay results. But 9a had a cytotoxic effect on HeLa cells for 48 hours at an IC50 value of (64.6 µM), as for 11a compound, its effect on HeLa cells was (62.4µM) by trypan assay.

5.3 Antigrowth effect of triazole compounds (14a, 9a, and 11a) against cervical cancer cell lines (Hela).

The antigrowth effect of triazole compounds 14a, 9a and 11a were tested against cervical cancer cell lines (Hela). MTT assay was used and IC50 values were calculated. The obtained IC50 values were 54.6µM, 64.6µM and 62.4µM for 14a, 9a, and 11a respectively. These values were lower than the IC50 values reported in the literature as follows.

Krešimir Benc et al, (2012) had synthesized novel Coumarin Derivatives containing 1,2,4-Triazole, 4,5-Dicyanoimidazole and purine moieties in chemotherapy. Compounds 3–18 were evaluated for their inhibitory activities against human tumor cell lines; Hela (cervical carcinoma). From all evaluated compounds, only (3, 4, 5, 7, 8, 9) contained a 1,2,4-triazole-3-carboxamide, moiety, heterocyclic
constituent of the ribavirin, showed cytostatic activity against Hela cells at IC\textsubscript{50} > 100 μM (Benci et al., 2012).

Jin Lan, Yu et al. (2010) had synthesized a novel 20, 30-dideoxy-20, 30-diethane thionucleosides bearing 1,2,3-triazole residues. The novel nucleosides were evaluated for their cytotoxicity in vitro towards the human cervical cancer cell line (Hela) and compounds 10–12 showed activity against Hela, with IC\textsubscript{50} values ranging from 177.13 μM to 75.69 μM respectively. However, nucleosides 16d–16g were found to be inactive against Hela cell lines at IC\textsubscript{50} > 100 μM (Yu et al., 2010).

Chinna Devi, Gajula and Bhaskara, (2012) Rayani, have synthesized 1, 4-Disubstituted 1, 2, 3-Triazole. Compound 1-(1-Naphthalen-2-yl-ethyl)-4-phenyl-1H-(1,2,3)triazole (B) among other tested ones (A, C, D, E, F, G, H, I and RK), showed the greatest potency on Hela cells with IC\textsubscript{50} values of 126.31 μM. But the compounds (1-(1-Phenyl-ethyl)-4-p-tolyl-1H-[1,2,3]triazole (G) and 1-Benzhydryl-4-phenyl-1H-[1,2,3]triazole(I)) showed activity against it, with IC\textsubscript{50} values ranging from 148.52μM to 148.12μM respectively (Gajula & Rayani, 2012).

Gaofei Wei et al., (2010). have synthesized 1,2,3-triazole-substituted oleanolic acid derivatives. The IC\textsubscript{50} values revealed that most of the conjugates exhibited more potent inhibitory activities against Hela cell line than oleanolic acid. Compound 3o shows high toxicity with IC\textsubscript{50} values at 54.63µM which are similar to the result that we have, but compounds 3d, 3g, 3j, 3k, 3l, 3m, 3n, 3q, 3r, and 3s showed that IC\textsubscript{50} values (131.26μM,109.13μM,122.01μM,75.04μM, 148.36μM,124.36μM, 86.42μM, 74.86μM, 81.3μM,75.04μM respectively) (Wei et al., 2010).

Our study results showed that 14a, 9a and 11a compounds with IC\textsubscript{50} values of (54.6 μM, 64.6 μM, and 62.4μM) respectively have a higher toxic effect on Hela cell than the studies mentioned above.

5.4 Antigrowth effect of the triazole compound (14a) against breast cancer cell lines (MCF-7)

The antigrowth effect of triazole compound 14a was tested against breast cancer cell lines (MCF-7). MTT assay was used and the IC\textsubscript{50} value was calculated. The obtained IC\textsubscript{50} value was 59.8 μM for 14a. The value was comparable to the IC\textsubscript{50} values reported in the literature as follows.
Novel coumarin derivatives containing 1,2,4-triazole, 4,5-dicyanoimidazole, and purine moieties have been synthesized. Compounds 3–18 (They are new compounds coumarin derivatives containing 1,2,4-triazole, 4,5-dicyanoimidazole and purine moiety) evaluated for their inhibitory activities against human tumor cell line MCF-7 (breast epithelial adenocarcinoma, metastatic) and they showed moderate cytostatic activity against MCF-7 cells at IC$_{50}$ > 100µM (Benci et al., 2012).

Moreover, Suressh Maddila et al. (2016) have synthesized a novel 2-(1-(substitutedbenzyl)-1H-tetrazol-5-yl)-3- phenylacrylonitrile derivatives. The most promising compounds in this study (6a, 6c, 6f, 6g, 6k) inhibited proliferation of MCF-7 cell line with IC$_{50}$ values of (88µM, 90µM, 99µM, 98µM, and 87µM respectively). In addition, the other compounds (6d, 6e, 6i) had inhibitory activity against the MCF-7 cell line with IC50 values of (115µM, 144µM, 134µM respectively). On the other hand, our compound 14a had a higher toxic effect on MCF-7 cell with an IC$_{50}$ value of (59.8 µM) compared to the compounds of their study (Maddila et al., 2016).

In addition, Ewa Bębenek et al., (2017) created novel triazole hybrids of botulin. Triazoles 3–5d were tested for their cytotoxic activity against human and cancer cell line MCF-7 (human adenocarcinoma). Triazoles 3–5d compounds inhibit the proliferation of 50% of tumor cells as compared with the control untreated cells. In the same time, Betulin 1, acetylenic derivatives 3–4 and cisplatin were used as a positive control. The resulting IC$_{50}$ values were 102.1µM, 58.8µM respectively (Bębenek et al., 2017). With comparison to our study, we see that 14a (59.8 µM) has similar values to compound 5d in the study (58.8µM).

Recently, Pei-Liang Zhao et al. derived synthetic 3,4-disubstituted-5-(3,4,5 trimethoxyphenyl)-4H-1,2,4-triazoles and novel 5,6-dihydro-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole compound bearing 3,4,5-trimethoxyphenyl moiety. Preliminary bioassay showed that compound 11 had strong cytotoxicity on MCF-7 cell line with the IC50 value of 63.72µM. On one hand, compound 12 showed cytotoxicity on MCF-7 with an IC50 value of 152.59 µM. On the other hand, the compounds 5, 6, 7, 8, 9 and 10 were inactive against MCF-7 cell lines at IC$_{50}$ > 200 µM (Zhao et al., 2012).
Ying-Chao Duan and others, designed and synthesized novel 1,2,3-triazole-dithiocarbamate hybrids. The most promising compounds in this study (13b, 13a, 6d, and 6c) inhibited proliferation of MCF-7 cell line with IC$_{50}$ values of (79.80µM, 90.38µM, 99µM, 93.05µM and 80.38µM respectively) (Duan et al., 2013). While our study results showed that 14a compound with IC$_{50}$ values of 59.8µM indicates the higher toxic effect on MCF-7 cell than the studies mentioned above.

5.5 Antigrowth effect of the triazole compound (14a) against colorectal cancer cells (CACO-2)

14a compound has been tested against CACO-2 and the obtained IC$_{50}$ value was 113µM. The measured concentration was high because CACO-2 cells have higher resistance to the chemical treatment. Consequently, 14a is an ineffective treatment for CACO-2 because of its higher toxicity on the cell lines. However, our result is highly comparable to the 14a IC$_{50}$ values reported in the literature as shown in the following paragraphs.

Pardeep Singh et al., (2012) derived 1,2,3-triazole tethered b-lactam-chalcone bifunctional hybrids. The IC$_{50}$ values, of the examined compounds against the human cell lines, were calculated. Most of the compounds (7a, 7b, 8b, 9b and 11) have shown inactivity against CACO-2 cell line with IC$_{50}$ values of more than 100µM (Singh et al., 2012).

Suresh Maddila et al., derived a novel 2-(1 (substitutedbenzyl)-1H-tetrazol-5-yl)-3- phenylacrylonitrile (6a–k) as anticancer against CACO-2 cancer cell line. MTT assay used to evaluate the antigrowth effect and 5-Fluorouracil used as a positive control, DMSO as a solvent and as a negative control. Compounds named (6d, 6e, 6f, and 6i) showed antigrowth with IC$_{50}$ values of (122µM, 135µM, 100µM, and 199µM) (Maddila et al., 2016).

Pardeep Singh et al. synthesized 1,2,3-Triazole tethered b-lactam-Chalcone bifunctional hybrids. The tested compounds (7a, 7b, 8b and 11) with IC$_{50}$ values of >100µM proved to be inactive against CACO-2 cell line except for 6b and 6c with IC$_{50}$ values of 45.2µM and 79.5µM) (Singh et al., 2012). These compounds were better than 14a with IC$_{50}$ of 113 µM tested in this study. That’s might be attributed to the chemical formation of 14a (3-Acetyl-4-benzoylamino-1-(4-chlorophenyl)-1,2,4-
triazaspiro[4.6]undec-2-ene (C_{23}H_{25}ClN_{4}O_{2}) which made its effectiveness lower than 6b and 6c tested by Singh et al.

In summary, in this study, novel Triazole compounds synthesized by Dr. Abdel-Rahman S. Ferwanah and Dr. Adell Awad Allah and their colleagues (Ferwanah, 2003). Have a potent cytotoxic effect against three different cancer cell lines (Hela, MCF-7, and CACO-2) which were similar to the approved Triazole compounds in previous studies.

5.6 Inhibition of cancer cell lines (MCF-7, Hela and CACO-2) migration by Triazole compounds 14a, 9a and 11a

Triazole compounds 14a, 9a and 11a were tested as migration inhibitor for cell lines (MCF-7, Hela, and CACO-2). In the experiments in vitro Cell migration assay were used in which percentage of wound closer were quantified. 9a compound had a 40% wound closer after 72h on HeLa cell in reference to the control, while 11a had no effect on HeLa cell. 14a had a 45% wound closer after 72h on MCF-7, but it had shown minor effect on Hela and CACO-2 cells. In the following paragraphs, we compare our results to other studies used Triazole as migration inhibitor.

Rabiya Majeed et al, (2013) investigated the 3-O-propargylated betulinic acid and its 1, 2, 3-triazole compounds (7, 13) on THP-1(human monocyte; acute monocytic leukemia) cancer cell lines. Cell cultures treated with 7 and 13 compound were photographed and cell migration was assessed by comparing the gap sizes between the control and the treated wells. Representative photomicrographs of tumor cell migration were taken and the wound got completely healed in the control wells at the lower concentration of 7 and 13 but at higher concentration the numbers of invasive cells that penetrated the respective wound were inhibited and cell migration got significantly stopped due to the inhibitory effect of the 7 and 13 on cell migration and invasion (Majeed et al., 2013).

Chintakunta Praveen Kumara, et al. (2016) synthesized and evaluated pyrazolo-triazole hybrids. They investigated the effect of 17, 23 and 29 derivatives on the migration potential of U87MG cancer cells. Cells migrated to the wound area decreased significantly after treatment with these hybrids in comparison to the control (untreated group) and showed 15 % of migration inhibition of U87MG cancer cells. These results indicate that the migration of U87MG cancer cells (a
human primary glioblastoma cell line) was significantly suppressed by these pyrazolo-triazole hybrids (Praveen Kumar et al., 2016).

Yi-Chao Zheng and colleagues, (2013) investigated triazole–Dithiocarbamate Based Selective Lysine Specific Demethylase 1 (LSD1). The observed Microphoto graphs showed that untreated gastric cancer MGC-803 cells (human gastric carcinoma cell line) filled most of the wounded area in 2 days after scratching the cell monolayer, whereas treatment with indicated doses of compound 26 markedly suppressed repairment of the wound. The inhibitory effect of compound 26 on repopulation of the wounded area was not due to decreased proliferation because the highest concentration (0.25μM) of compound 26 used in this assay cannot inhibit the cell proliferation. Compound 26 also significantly inhibited the cell migration and invasion even at low concentration (0.02μM), so did the siRNA mediated LSD1 knockdown, when compared to the positive control 100μM 2-PCPA. These results indicate that the effect of compound 26 on cell migration and invasion is much more powerful than 2-PCPA (Zheng et al., 2013).

5.7 14a induces apoptosis of HeLa cells.

To determine the mechanistic origin of 14a on apoptosis, HeLa cells were treated with 14a at a concentration of 50μM for 24h and 48h and examined for apoptosis using Western blot analysis. The test examined whether 14a treatment induced apoptosis in a PARP-dependent manner. We found that 14a induces a high level of cleaved PARP (see Figure 4.18), thereby indicating its mechanism via an apoptotic pathway. The results obtained in this study were compared to literature as seen in the next paragraphs.

Ashwini et al. (2015), showed that the western blot analysis increased expression of cleaved caspase-9 and cleaved PARP protein in 3-(4-(4-phenoxyphenyl)-1H-1, 2, 3-triazole-1-yl)benzo[d]isoxazole (PTB). It is a compound that resulted from attached 1,2,3-triazole moiety to the third position of a 1,2-benzisoxazole heterocycle via Copper (I)-catalyzed azide-alkyne cycloaddition (CuAAC) with various alkynes treated AML cells (MOLM13, MOLM14, and MV4-11) compared to control cells (Ashwini et al., 2015). And these data come in agreement with my studies for a similar compound called 14a. This compound showed the strong apoptotic effect on HeLa cell line.
Nagarsenkar et al. (2016), investigated the molecular mechanisms of compound Z-8l on apoptosis, and this compound comprises two core structural elements: (i) 3-benzylidene isatin, and (ii) a substituted 1,2,3-triazole moiety. Moreover, the study checked the expression of Bcl2, Bax, and PARP and cytochrome c by using the western blot method. Results indicated that compound Z-8l treatment led to the dose-dependent increased expression of cleaved PARP and cytochrome c in DU145 cells. Collectively, these results illustrate that compound Z-8l induced apoptosis through apoptosis-related protein expression (Nagarsenkar et al., 2016).

Chen et al. (2013), results showed that 16a–d triggering apoptotic cell death associated with increased levels of Bax and Bad and decreased levels of Bcl-2 and Bcl-xL. In addition, cell treatment with 16a (triazole-pyrrolo[2,1-c][1,4]benzodiazepines ) induced a concentration-dependent increase in Bax and Bad, cleaved both caspase-3 and PARP whereas the levels of Bcl-2 and Bcl-xL reduced (Chen et al., 2013).
Chapter 6
Conclusions and Recommendations
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Conclusions and Recommendations

6.1 Conclusion

1. 14a was the best compound of triazole compounds tested in this study.
2. The sensitivity of Hela cells to compound 14a is better than MCF-7 and CACO-2.
3. The mechanism induced by 14a to inhibit the growth of Hela cells is apoptosis

6.2 Recommendations

Based on what we have shown in this study we recommend testing these compounds:

1. To investigate the effect of 14a on normal cells.
2. To investigate the effect of 14a on other types of cancer cells
3. To study the effect of 14a in vivo.
4. To further understand its mechanism of action.
References
References


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Annexes
Annex (1)

The laboratory headed by Dr, Adel Awad Allah, Islamic university, Gaza, Palestine, has newly synthesized triazole compounds.

Table of Triazoles

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