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Cytotoxic and Antiproliferative Effects of Four Natural Plants Extracts on Colon Cancer Caco-2 Cell Line

تأثير مستخلصات أربعة نباتات طبيعية على حياة وانقسام خلايا سرطان القولون من نوع Caco-2

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إقـــــــــرار

أنا الموقع أدناه مقدم الرسالة التي تحمل العنوان:

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تأثير مستخلصات أربعة نباتات طبيعية على حياة وانقسام خلايا سرطان القولون من نوع Caco-2

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(تاثير مستخلصات أربعة نباتات طبيعية على حياة وانقسام خلايا سرطان القولون من نوعCaco-2)

(Cytotoxic and Antiproliferative Effects of Four Plants Extracts on Colon Cancer CaCo-2 Cell Line)

وبعد المناقشة التي تمت اليوم الاثنين 22 ذو القعدة 1438هـ، الموافق 2017/08/14 الساعة التاسعة صباحاً في قاعة مؤتمرات مبنى طيبة، اجتمعت لجنة الحكم على الأطروحة والمكونة من:

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وبعد المداولة أوصت اللجنة بمنح الباحثة درجة الماجستير في **كلية *العلوم*/** قسم **العلوم الحياتية - تحاليل طبية**.

واللجنة إذ تمنحها هذه الدرجة فإنها توصيها بتقوى الله ولزوم طاعته وأن تسخر علمها في خدمة دينها ووطنها.

والله ولي التوفيق،،،

نائب الرئيس لشئون البحث العلمي والدراسات العليا

أ.د. عبدالرؤوف علي المناعمة

Abstract

Background: Cancer is an aggressive disease that if untreated leads to death. In Palestine, like any other country in the world, cancer is one of the most dangerous health problems affecting individual's life. Conventional treatment protocols are not effective in all cases. Nowadays the aim of many medical research is to discover new safer and more effective anticancer agents from natural materials. The use of plant extracts with potential anticancer therapeutic effects might be particularly significant, especially in Palestine, which is rich in thousands of plant species known for their medical uses.

Objective: The main objective of the present study is to assess the possible cytotoxic and antiproliferative effects of natural crude extracts of four medicinal plants locally available against Caco-2 cell line.

Methodology: Extracts from the dried aerial and seeds parts of the plants were prepared employing 70% ethanol, chloroform and distilled water using Soxhlet apparatus. All extracts were prepared at various concentrations, then tested against Caco-2 cells to screen their cytotoxic and antiproliferative effects by using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT). DAPI stain was used for determining chromatin condensation in cells. The extracts, which gave the best effects, were tested on normal cells (lymphocyte). After that, the two extracts were tested in a combination manner with 5-Fluorouracil (5-FU).

Results: The results of the present study revealed that most crude extracts, except ethanolic, chloroformic and aqueous extracts of *Portulaca oleracea* and aqueous crude extract of *R. officinalis* and *A. sativa,* exhibited concentration dependent antiproliferative effect against Caco-2 cells to various degrees. *R. officinalis* chloroformic extract and *A. sativa* ethanolic extract gave the best antiproliferative effects at 24 h with IC_{50} 77 µg/ml and 67 µg/ml respectively. The results show that the effect of the crude extracts was much lower on normal cells (lymphocytes) as compared to cancer cells (Caco-2 cells). For combination studies, the combination index (CI < 1) was synergistic at all combinations. The dose reduction index is 2.4 and 23 times for *R. officinalis* and 5-FU, respectively. While, the dose reduction index is 1.8 and 14 times for *A. sativa* and 5-FU, respectively.

Conclusions: Among the tested natural plants crude extracts *R. officinalis* chloroformic extract and *A. sativa* ethanolic extract gave the best antiproliferative effects against Caco-2 cells and have low effect on normal lymphocytes. In addition, synergistic activity of these extracts with commercial chemotherapy showed promising results. Further studies are required to determine the active components of these extracts.

ملخص الدراسة

مقدمة: يعتبر السرطان واحداً من أهم الأمراض المسببة للوفاة حول العالم، وهو مصطلح عام يشمل مجموعة كبيرة من الأمراض التي يمكنها أن تصيب كل أجزاء الجسم. ويُشار اليه ايضا بالورم الخبيث المستعصي والذي اذا لم يعالج أدي الى الهلاك. في فلسطين مثل أي بلد آخر في العالم، يعد السرطان أحد أخطر المشاكل الصحية التي تؤثر على حياة الأفراد. ومن الملاحظ ان استراتيجيات العلاج التقليدية المتبعة ليست فعالة في جميع حالات السرطان. لذلك كان الهدف الرئيسي للعديد من البحوث الطبية في وقتنا الحاضر هو اكتشاف علاجات من مصادر طبيعية أكثر أماناً وفاعلية ضد مرض السرطان. قد يكون استخدام المستخلصات النباتية ذات التأثيرات العلاجية المحتملة المضادة للسرطان ذات أهمية عالية، وخاصة في فلسطين و التي تعتبر بيئة غنية بآلاف أنواع النباتات المعروفة منذ زمن بعيد باستخداماتها الطبية.

الهدف: الهدف الرئيسي لهذه الدراسة هو فحص تأثير مستخلصات أربعة نباتات طبيعية خام متاحة محلياً على حياة و انقسام خلايا سرطان القولون من نوع (Caco-2).

منهجية البحث: جهزت المستخلصات النباتية الخام من خلال تجفيف أجزاء النبتة والبذور في الهواء و من ثم استخلاصها بواسطة جهاز السوكسلت من خلال استخدام الإيثانول بتركيز (70%) و الكلوروفورم و الماء المقطر كمذيبات في عملية الاستخلاص. بعد ذلك قمنا بتحضير جميع المستخلصات الطبيعية الخام بتراكيز مختلفة لدراسة تأثيرها على حياة وانقسام خلايا سرطان القولون من نوع (Caco-2) عن طريق استخدام المادة الكيميائية (MTT) وصبغة (DAPI). قمنا بعد ذلك باختيار أفضل مستخلصين وتجربتها على الخلايا الطبيعية (الخلايا الليمفاوية)، و في النهاية تم اختبار اثنين من المستخلصات الخام ودمجها مع العلاج الكيميائي المعروف (5-FU) لدراسة تأثيراتهم التآزرية على الخلايا.

النتائج: بينت نتائج هذه الدراسة أن معظم المستخلصات الخام لديها تأثير مضاد لتكاثر خلايا (Caco-2) عند زيادة تركيزاتها بدرجات متفاوتة، باستثناء المستخلص الإيثانولي و الكلوروفورمي والمائي لنبات البقلة و المستخلص المائي لنبات اكليل الجبل ونبات الشوفان. وكان المستخلص الكلوروفورمي لإكليل الجبل والمستخلص الإيثانولي لنبات الشوفان صاحبا أفضل نتيجة لتثبيط نمو خلايا (Caco-2) خلال 24 ساعة حيث كانت الجرعة اللازمة لتثبيط نمو 50% من الخلايا 77 ميكروجرام/مل و 67 ميكروجرام /مل لكلا النبتتين على التوالي. وكما أظهرت النتائج أيضا أن تأثير المستخلصات الخام على الخلايا الطبيعية (الخلايا الليمفاوية) كان أقل بكثير من تأثيره على الخلايا السرطانية (Caco-2). أما بالنسبة لدراسة تأثير أفضل نبتتين مجتمعة مع العلاج الكيميائي (5-FU) فقد لاحظنا وجود تأثير تآزري في جميع التراكيز المستخدمة حيث أظهرت النتائج أن الجرعة انخفضت الي 2.4 مرة و 23 مرة لكل من اكليل الجبل الكلوروفورمي و العلاج الكيميائي (5-FU) على التوالي بينما انخفضت الجرعة 1.8 مرة لمستخلص الشوفان الكحولي و بنسبة 14 مرة للعلاج الكيميائي (5-FU).

الاستنتاج: من بين المستخلصات الخام الطبيعية التي تم اختبارها وجد أن المستخلص الكلوروفورمي لإكليل الجبل والمستخلص الإيثانولي لنبات الشوفان صاحبا أفضل نتيجة لتثبيط نمو خلايا (Caco-2). و بالإضافة الى ذلك يوجد نشاط تآزري واعد عند دمج المستخلصات النباتية مع الأدوية الكيميائية التجارية. لذلك نوصي بإجراء دراسات أخرى لتحديد المركبات الفعالة لهذه المستخلصات النباتية.

Dedication

I would like to dedicate this work to my father **Dr. Riad Elburai** my precious, invaluable treasure for his kindness and devotion, and for his endless support, who cultivate the real love of knowledge in my heart. I also dedicate this work to my mother **Dr. Izdehar Alwadeia** who carried the pain and troubles to make me stronger, who taught me love and loyalty. For those who own my heart, who left fingerprints of grace on my life, to my brothers and sisters (**Mohammed**, **Ahmed**, **Alaa**, **Israa** and **Asmaa**) I dedicate my modest work. The life could not be pleasant without the presence of my dear aunt **Entesar Alwadeia**, I am glad to dedicate my work to you.

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Finally, I can just say thanks for everything and may Allah give all who stood beside me, all the best in return.

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5-FU	5-Fluorouracil
AJCC	American Joint Committee on Cancer
AMN3	Murine mammary adenocarcinoma cell line
AP	Aerial Parts
ATCC	American Type Culture Collection
BA	Bark
BMI	Body Mass Index
Caco-2	Colorectal Adenocarcinoma
CAM	Complementary and Alternative Medicine
CAP	Capecitabine
CI	Combination Index
CRC	Colorectal cancers cell line
DAPI	4',6-diamidino-2-phenylindole
DLD-1	Colorectal cancer
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DRI	Dose Reduction Index
EDTA	Ethylene Di Amine Tetra Acetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
Fa	Fraction affected
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FOL	Leucovorin Calcium
FR	Fruits
G1	Gap phase 1
G2	Gap phase 2
GISTs	Gastrointestinal Stromal Tumors
HCT116	Colon cancer cell line
IC50	half maximal inhibitory concentration
IR	Infrared concentrator
IRI	Irinotecan Hydrochloride
LE	leaves
M	Metastasized

MCF-7	Breast adenocarcinoma cell line
MTT	(3- (4,5dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)
N	Regional lymph nodes
NA	Not Applicable
NCI-H460	Lung cancer cell line
OD	Optical Density
OX	Oxaliplatin
PBS	Phosphate Buffer Saline
PC3	Human Prostate Cancer cells
RD	Human Rhabdomyosarcoma Cell Lines
RNA	Ribonucleic acid
RT	Roots
SD	seeds
ST	Stem
SW620	Colorectal cancer metastatic site: lymph node
T	Primary Tumor
TAPHM	Traditional Arabic Palestinian Herbal Medicine
TK1	thymidine kinase 1
TM	Traditional medicine
TYMS	thymidylate synthetize
WHO	World Health Organization
XEL	Capecitabine(Xeloda)

Chapter I
Introduction

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Introduction

1.1 Overview

Cancer is one of the most severe and devastating health problems in the world. The disease affects individuals from different sexes, ages, and races and is characterized by uncontrolled and rapid proliferation of transformed cells. Cancer is used as a general term to describe malignant diseases. cancers share common characteristics, formation of a solid mass tumor or propagation throughout the body, where their progress may cause death. Cancer cells propagate through invading their neighboring tissues, and may also progress to other organs through the bloodstream or the lymphatic system (Knudson, 2001). Nowadays, there are many types of cancer, and each type is named according to the organ or tissue in which it originated.

Globally, cancer remains one of the leading causes of mortality and morbidity. Amongst the non-infectious diseases, cancer comes after cardiovascular diseases as the second cause of human death (Kung, Hoyert, Xu, & Murphy, 2008). Cancer is responsible for one in eight deaths worldwide, more than AIDS, tuberculosis, and malaria together (Sener & Grey, 2005).

According to a report published by the National Center for Cancer Monitoring/ Health Information Systems Unit in the Gaza Strip, the number of new cancer cases that were recorded in the years 2009-2014 was 7069. The incidence of cancer in 2014 was 83.9 per 100000. Breast cancer is the most common cancer, accounting for around 17.8% of all cancer cases, while colon cancer is the second, accounting for about 12% of all cases (Ministry of health, 2016).

Chemotherapy treatment has several groups that can be divided based on their relationship to other drugs, how they work, and their chemical structure. There are many medicines that work in more than one way and can belong to more than one group (Hosoya & Miyagawa, 2014). The chemotherapy drugs have dramatic adverse effects on normal cells, leading to disorders in their function. On the other hand,

many side effects, such as general fatigue, anemia, loss of appetite and bleeding problems result after each chemotherapy dose, and many problems in the heart or damage to lung tissue that appear later after completion of treatment (De et al., 2012). In some cases, resistance to chemotherapy drugs develops and alternative drugs should be used to treat the cancer (Legrand, Gray, Cooke, & Rotello, 2003)

The side effects of chemotherapy treatments and other synthetic chemicals have highlighted the importance of searching for other agents that are effective on cancer cells while having mild effects on normal cells. Various natural products can be used as alternative agents in treatment of diseases, especially cancer. Plants are a natural repository of medical compounds that are almost free of side effects in contrast to manufactured chemicals (Fennell et al., 2004).

Human used medicinal plants for their therapeutic values since the beginning of human civilization. Nature has been a rich source of medicines for thousands of years and an impressive number of modern drugs have been isolated from natural sources. Many of these isolations were based on the uses of the agents in traditional medicine (Pal & Shukla, 2003). In many parts of the world, there is a tradition for using herbal medicine for treatment of many diseases (Brantner & Grein, 1994). This plant-based traditional system of medicine continues to play an essential role in health care, with about 80% of the world's inhabitants relying mainly on traditional medicines for their primary health care and safety (Rasool Hassan, 2012; Singh, Singh, Krishna, & Pandey, 2014).

Palestine is one of the countries that is famous for the existence of many medicinal and useful plants that have been used for long periods of time (Crowfoot & Baldensperger, 1932). In Palestine, the areas rich in plants are the Palestinian mountains. About 2953 plant species were found in this small area of the Mediterranean, of which over 700 appear in the published ethnobotanical data (Dafni, Yaniv, & Palevitch, 1984; Ali-Shtayeh, Salameh, Abu-Ghdeib, Jamous, & Khraim, 2002).

The World Health Organization (WHO) estimates that herbal medicine is still the mainstay of about 75-80% of the world population, mainly in the developing countries, for primary health care because of better cultural acceptability, better

compatibility with the human body, and lesser side effects (Kamboj, 2000; Yadav & Dixit, 2008). More than 50% of the modern drugs used clinically are of natural products and many of them have the ability to control cancer cells. Many natural plants are used in the treatment of different diseases in humans and also in animals since time immemorial (Rosangkima & Prasad, 2004).

Medicinal plants possess antioxidant and immunomodulatory properties, leading to anticancer activities. They are known to have versatile immunomodulatory activity by stimulating both non-specific and specific immunity (Pandey & Madhuri, 2006). Many plant-derived products have been reported to exhibit potent antitumor activity against several rodent and human cancer cell lines. The antioxidants may prevent and cure cancer and other diseases by protecting the cells from the damages caused by free radicals (Rao, Govindaraj, Gundiah, & Vivekchand, 2004).

Cancer cell lines have been widely used for research purposes and have proved, by several researchers, to be a useful tool for the study biological mechanisms and genetic defects involved in cancer. The use of cancer cell lines allowed an increment of the information about the deregulated genes and signaling pathways in this disease (Louzada, Adega, & Chaves, 2012). They were in the origin of the development and testing of anticancer drugs presently used and in the development of new therapies (Vargo-Gogola & Rosen, 2007; Gazdar, Girard, Lockwood, Lam, & Minna, 2010). They are also used as an alternative to transplantable animal tumors in chemotherapeutics testing (Shoemaker, 2006).

Most cancer types occur due to mutations and dysfunction that arise in genes coding for proteins such as growth factors, growth factor receptors, antiapoptotic proteins, transcription factors, and tumor suppressors. All these mutations constitute potential targets for cancer treatment (Millimouno, Dong, Yang, Li, & Li, 2014). Many anticancer drugs, including plant-derived ones act on the proliferating cycle of cell against microtubule function, protein function, or DNA synthesis, and mostly exert cancer cell death effects by inducing apoptosis (Tait, Ichim, & Green, 2014). Apoptosis represents a universal and exquisitely efficient cellular suicide pathway and commonly used in medical oncology (Parrish, Freel, & Kornbluth, 2013). But, in fact, 90 % of cancer patients die because of metastasis due to the resistance of

their cancers to apoptosis inducing drugs (Wilson, Johnston, & Longley, 2009; Zahreddine & Borden, 2013). Anticancer drugs have been classified according to their mode of action into drugs destroying DNA or inhibiting its duplication like alkylating agents, synthesis inhibition of nucleic acid (DNA and RNA) like 5-FU and methotrexate, and inhibition of protein synthesis. Nowadays, there is a great interest to find drugs that play critical roles in the execution of one or more cell death pathways.

The traditional mono-target therapy protocols that is used in the treatment of many cancers is becoming ineffective and may lead to the development of cancer-acquired drug resistance. This resistance is mainly related to the complex signaling pathways involved in cancer and due to genetic redundancy (Faivre, Djelloul, & Raymond, 2006). Nowadays, there is an increasing trend in cancer research to use combination therapy for several solid tumors (Yang et al., 2009). The combination of anticancer agents which have similar or different modes of action may result in synergistic, additive or antagonistic outcomes (Johnson & Walker, 1999).

1.2 Objectives

1.2.1 General Objective

The general objective of the study is the assessment of cytotoxic and antiproliferative effects of natural crude extracts of four medicinal plants on human colorectal cancer cell line (Caco-2).

1.2.2 Specific objectives

1. To study the antiproliferative effect of different natural crude extracts (*Rosmarinus officinalis*, *Avena sativa*, *Punica granatum* & *Portulaca oleracea*) on Caco-2 cancer cells.
2. To study the effect of the crude extract(s) which gives the best antiproliferative effect on the morphology of Caco-2 cells and on apoptosis using DAPI stain.

3. To study the antiproliferative effect of the selected crude extract(s) in combination with the chemotherapeutic drug 5-FU on Caco-2 cells.

1.3 Study Significance

Because cancer is a global disease and a major cause of death, it is a necessary and important objective for scientists to find a cure for this disease. Conventional chemotherapy for treatment of cancers, although quite effective, has been associated with toxicities to normal tissue and organs, which is still a major dose limiting factor. Furthermore, resistance to chemotherapeutic agents is another major obstacle for successful treatment of cancer. Extracts of medicinal plants, on the other hand, have been shown to give effective results in preventing or curing cancer. Many epidemiological studies and human clinical trials revealed that natural compounds, such as flavonoids, polyphenolic compounds and many other phytochemicals play important roles in cancer chemoprevention and chemotherapy. Therefore, assessment of anticancer effects of different plants on cancer cells will help in the process of finding new and safer therapy for cancer.

Chapter II
Literature Review

Chapter II
Literature Review

2.1 Cancer

2.1.1 Overview

Nowadays, the whole world stands face to face with many deadly diseases which kill different organisms. In spite of the tremendous progress in science and the huge sums of money that are spent in various research areas, we still lack an effective treatment for many diseases. Such diseases are still widespread and lives of patients become difficult day after day. Cancer is one of those diseases that constitutes a challenge to the world to find an effective medical drug to eliminate it.

Animals and human beings have had an exposure to cancer throughout the recorded history. People have written about cancer from the dawn of history and there is no surprise in this, some of the earliest evidence of cancer was found among fossilized bone tumors, human mummies in ancient Egypt and ancient manuscripts. Anywise, the word cancer was not used in that time but the oldest description of cancer discovered refers to 3000 BC in Egypt. It is called the Edwin Smith Papyrus and is a copy of part of an ancient Egyptian textbook on trauma surgery. This papyrus was used to describe 8 cases of ulcers or tumors located in breast that were removed by cauterization using a tool called fire drill. The document refers to the disease as an incurable one (American Society of Clinical Oncology, 2016).

2.1.2 Definition

Cancer belongs to a group of malignant diseases, which are characterized by uncontrolled proliferation of abnormal cells due to the inability of the body to make a balance between the cells division and their differentiation, and thus lead to a progressive increase in the number of dividing cells. All proliferative cells unite together to form the tumor or spread to other places in the body and invade adjacent tissue through blood and lymphatic system.

Cancer cells have their own ability to feed themselves through a process known as angiogenesis process in which the growth of a network of blood vessels will increase production of angiogenesis activators and reduce the production of inhibitors of this process (Sakarkar & Deshmukh, 2011).

2.1.3 Cancer statistics

In 2015, an estimated 17.5 million new cases of cancer occurred worldwide and the five most common cancers reported are: breast, lung, bowel, prostate, and stomach cancers. cancer statistics in 2017 as shown in (Table 2.1) (Siegel, Miller, & Jemal, 2015). It is speculated that the number of new cancer cases will rise to nearly 23.6 million by 2030, and global mortality is expected to increase by up to 80%, according to the WHO reports, so cancer now is one of the most pressing health challenges (Dizon et al., 2016). Asia, Africa, Central and South America account for seven of every 10 cancer deaths worldwide. Today global statistics indicate that survival for most cancer types is improving (Quaresma, Coleman, & Rachet, 2015), and the rapid diagnosis and effective treatment can improve patients life (Henley et al., 2015).

2.1.4 Cancer statistics in Palestine

During the past few years like other Arab countries Palestine began to record increasing rates of cancer. However these rates remain low compared to neighboring countries, the crude incidence rate in (2009) was 65.5 per 100,000 While in 2014 it reached to 83.9 per 100,000. The most common type of cancer among Palestinian men is lung cancer followed by prostate and colorectal cancers (CRC), while the most common among women is breast cancer followed by CRC (Ministry of health, 2016).

Table (2.1): Global incidence and deaths for all cancers and 32 cancer groups in (2015) (Fitzmaurice et al., 2017).

	Incident Cases, Thousands[c]			Deaths, Thousands[c]		
Cancer[b]	Total	Male	Female	Total	Male	Female
All cancers	17 481 (16 847-18 177)	9269 (8768-9947)	8212 (7904-8570)	8713 (8539-8894)	5046 (4907-5196)	3667 (3576-3756)
Lip and oral cavity cancer	410 (388-435)	263 (244-283)	148 (136-160)	146 (142-151)	98 (94-101)	48 (46-51)
Nasopharynx cancer	123 (99-144)	88 (65-108)	34 (26-45)	63 (51-67)	46 (34-49)	17 (16-18)
Other pharynx cancer	161 (152-172)	118 (111-128)	43 (39-48)	64 (62-67)	47 (45-49)	17 (16-19)
Esophageal cancer	483 (437-549)	352 (312-416)	130 (116-150)	439 (423-457)	318 (302-335)	121 (115-128)
Stomach cancer	1313 (1238-1404)	872 (806-957)	440 (413-471)	819 (795-844)	535 (516-556)	284 (274-294)
Colon and rectum cancer	1653 (1601-1714)	920 (878-965)	733 (702-767)	832 (812-855)	456 (442-468)	376 (363-391)
Liver cancer	854 (768-961)	591 (517-691)	264 (227-314)	810 (750-863)	577 (524-622)	234 (204-255)
Gallbladder and biliary tract cancer	188 (175-199)	81 (76-87)	107 (96-117)	140 (131-147)	60 (56-62)	81 (73-87)
Pancreatic cancer	426 (412-439)	220 (210-230)	206 (198-216)	412 (404-421)	215 (210-220)	197 (191-203)
Larynx cancer	238 (226-253)	190 (178-205)	48 (45-52)	106 (103-109)	86 (83-90)	19 (19-20)
Tracheal, bronchus, and lung cancer	2019 (1906-2149)	1379 (1281-1499)	640 (602-690)	1722 (1674-1773)	1206 (1165-1252)	517 (497-538)
Malignant skin melanoma	352 (282-445)	190 (124-273)	162 (142-175)	60 (48-73)	32 (21-45)	27 (24-29)
Breast cancer	2422 (2280-2541)	44 (40-49)	2378 (2236-2497)	534 (502-553)	10 (9-11)	523 (492-543)
Cervical cancer	526 (483-571)	NA	526 (483-571)	239 (225-252)	NA	239 (225-252)
Uterine cancer	455 (409-507)	NA	455 (409-507)	90 (86-94)	NA	90 (86-94)
Ovarian cancer	251 (239-266)	NA	251 (239-266)	161 (157-167)	NA	161 (157-167)
Prostate cancer	1618 (1321-2222)	1618 (1321-2222)	NA	366 (303-460)	366 (303-460)	NA
Testicular cancer	72 (67-77)	72 (67-77)	NA	9 (9-10)	9 (9-10)	NA
Kidney cancer	425 (406-447)	268 (253-286)	157 (146-172)	137 (133-141)	89 (86-93)	48 (46-49)
Bladder cancer	541 (517-567)	412 (390-437)	129 (121-137)	188 (183-193)	137 (133-141)	51 (49-53)
Brain and nervous system cancer	321 (293-348)	175 (150-198)	146 (134-160)	229 (210-245)	127 (108-141)	102 (96-106)
Thyroid cancer	334 (310-353)	141 (123-153)	194 (181-210)	32 (29-33)	13 (11-14)	18 (17-20)
Mesothelioma	37 (35-39)	27 (25-29)	10 (9-11)	32 (31-33)	23 (22-24)	9 (9-10)
Hodgkin lymphoma	78 (70-91)	49 (43-61)	28 (24-36)	24 (22-29)	15 (13-19)	9 (7-12)
Non-Hodgkin lymphoma	666 (584-710)	379 (319-415)	287 (249-313)	231 (196-244)	133 (109-143)	98 (82-104)
Multiple myeloma	154 (145-162)	82 (77-87)	72 (66-78)	101 (98-104)	52 (51-54)	49 (46-51)
Leukemia	606 (573-643)	352 (325-385)	254 (235-275)	353 (345-363)	204 (197-212)	149 (144-154)
Acute lymphoid leukemia	161 (141-184)	95 (79-114)	66 (57-78)	110 (101-118)	65 (57-72)	45 (43-49)
Chronic lymphoid leukemia	191 (179-204)	106 (97-116)	85 (78-93)	61 (58-65)	34 (32-38)	27 (25-28)
Acute myeloid leukemia	190 (175-209)	113 (98-131)	78 (71-85)	147 (137-157)	85 (76-95)	62 (59-64)
Chronic myeloid leukemia	64 (60-68)	39 (35-43)	25 (23-27)	35 (33-38)	20 (19-23)	15 (14-16)
Other neoplasms	756 (680-809)	386 (329-429)	370 (335-399)	372 (336-392)	191 (160-206)	181 (162-192)

NA: not applicable

Table (2.2) shows the number of new cases and incidence of cancer in the years (2009-2014) in Gaza strip according to the Palestinian Health Information Center report for the year 2017.

Table (2.2): The number of new cases and incidence of cancer in Gaza strip in the years (2009-2014) (Ministry of health, 2016).

Year	Nu.	Incd. /100,000
2009	945	65.6
2010	940	60.1
2011	1037	64.2
2012	1231	73.6
2013	1414	81.7
2014	1502	83.9
Total	7069	--

2.1.5 Classification of Cancer

Cancer is classified into two types, benign and malignant. The first type, benign tumors are not considered as cancerous cells because they grow in specific places, are limited and restricted to an area, surrounded by a capsule and slow growing.

While the other type is malignant tumors which can invade other tissues by metastasis if left untreated, characterized by its fast growing and not capsulated, so this type is more deadly and represent the most lethal aspect of carcinogenesis (Gurcan et al., 2009). Figure (2.1) represents the encapsulated benign tumors (unable to metastasize) and malignant tumors which are able to metastasize away from the original tumor site.

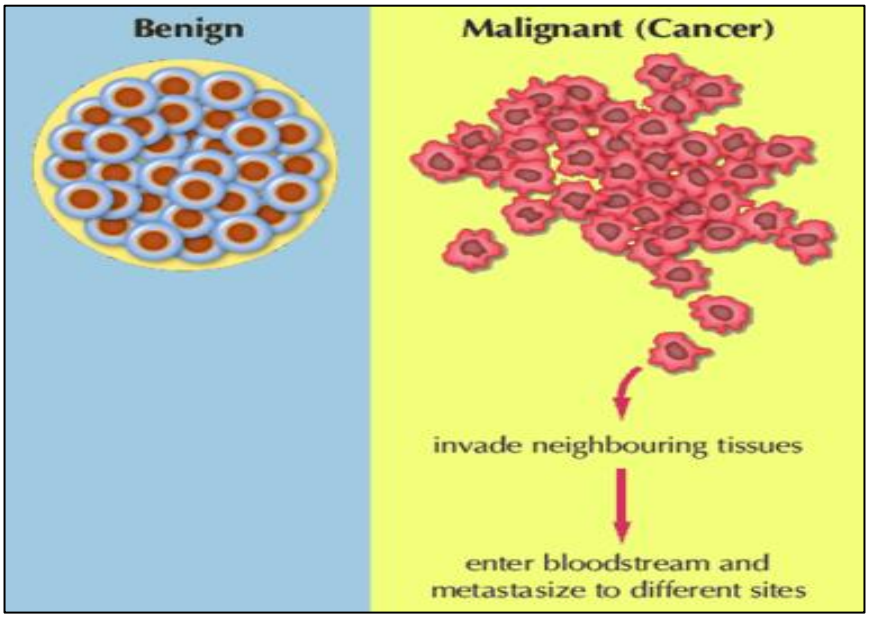

Figure (2.1): Comparison between malignant tumor and benign tumor (https://www.siyavula.com, 2017).

2.1.6 Types of Cancer

Cancer is often described based on the area in which it arises or originated. However, there are some parts in the body that contain multiple types of tissues, so for greater precision, cancers are additionally classified by adding the tissue type that the tumor cells originated from. Table (2.3) lists the common cancers that affect human which exceed 100 types according to organs.

Table (2.3): Most common types of cancer according to organs (American Cancer Society, 2017).

Bladder Cancer	Liver Cancer
Breast Cancer	Melanoma
Colon and Rectal Cancer	Non-Hodgkin Lymphoma
Endometrial Cancer	Pancreatic Cancer
Kidney Cancer	Prostate Cancer
Leukemia	Thyroid Cancer
Lung Cancer	

2.1.7 Cell cycle

The progress and dividing process in the cells is known as the cell cycle, which lies at the heart of cancer. The normal cells control the cell cycle by a complex series of signaling pathways by which a cell grows and divides, to give two daughter cells. In mammals, the cell cycle consists of four distinct phases: two gap phases, G1 and G2, during which RNA synthesis and protein synthesis occur; S-phase during which DNA is replicated; and M-phase, in which cells undergo mitosis and cytokinesis (Figure 2.2) (Vermeulen, Van Bockstaele, & Berneman, 2003).

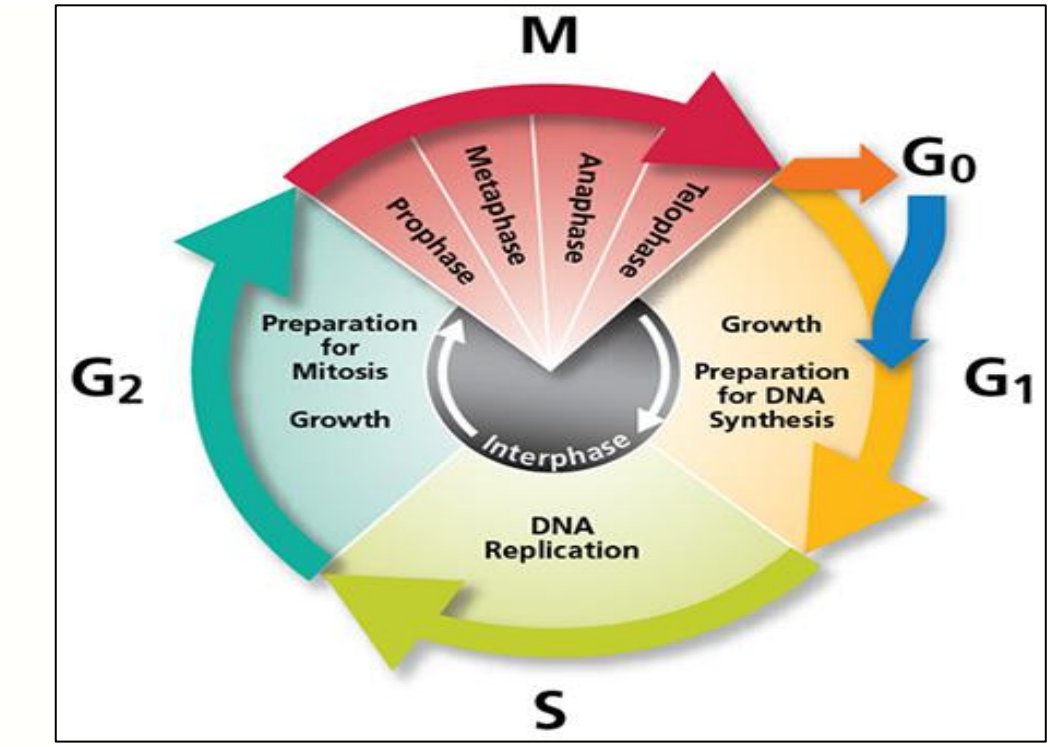

Figure (2.2): The cell cycle (http://www.bdbiosciences.com, 2017).

If the cells are not dividing, they remain in a quiescent or resting state called G0 phase. G1, S, and G2 phases are referred to collectively as interphase (Garrett, 2001). This process also includes mechanisms to ensure errors are corrected, and if not, the cells commit suicide (apoptosis). In cancer, as a result of genetic mutations, this regulatory process malfunctions, resulting in uncontrolled cell proliferation.

2.1.8 Risk factors for cancer

In developing countries, the burden of cancer is increasing rapidly, due to a variety of reasons, including increasing people's modifiable and susceptibility to risk factors in general such as: lack of exercise, ingestion of western diets, smoking, and environmental pressures. In addition, infectious diseases also have a role in increasing cancers of infectious diseases origin. Lack of access to prevention and control cancer services, late stage diagnoses and inadequate treatment facilities also lead to higher cancer mortality in different countries (Kanavos, 2006).

2.2 Colon and rectum

The colon is located in the last part of the digestive system. Its function is to extract water and salts from the solid wastes before it is disposed of from the body. The length of the adult male colon is 166 cm on average, while the female has a length of about 155 cm (Hounnou, Destrieux, Desme, Bertrand, & Velut, 2002).

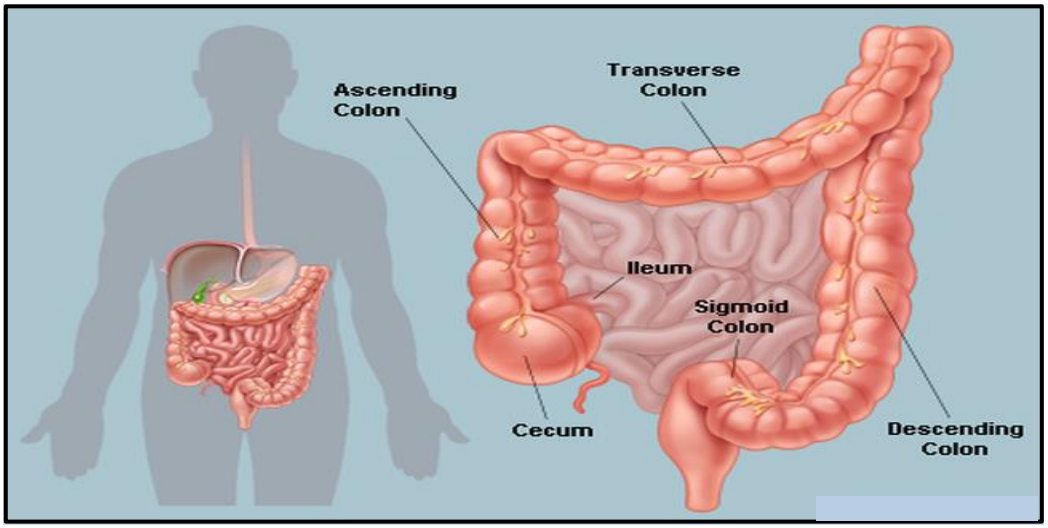

Figure (2.3): The five main sections of colon (http://www.newhealthguide.org, 2017).

In mammals the colon is divided into five main sections: the cecum plus the ascending colon, the transverse colon, the descending colon, the sigmoid

colon and the rectum (Kumar, Majumdar, Panda, & Pathak, 2014), as shown in Figure (2.3).

The fermentation process of unabsorbed material occurs in the colon, carried out by the microbiota (largely bacteria) present there. Unlike the small intestine, the colon does not play a major role in absorption of food and nutrients, about 1.5 liters of water arrives daily into colon (Krogh, 2011). The rectum is the final straight portion of the large intestine in humans and some other mammals, and the gut in others (Rex et al., 2009). The human rectum is about 12 centimeters, and begins at the recto sigmoid junction (the end of the sigmoid colon). The rectum acts as a temporary storage site for feces (Bruce, James, & David, 2007). As the rectal walls expand due to the materials filling it from within, stretch receptors from the nervous system located in the rectal walls stimulate the desire to defecate (Talley & O'Connor, 2013). One of the diseases that affect the rectum is rectal cancer, a subgroup of colorectal cancer specific to the rectum.

2.3 Colorectal cancer

2.3.1 Definition

Colorectal cancer (CRC) is a cancer of the large bowel and includes all cancer originating from the cecum to the anus. Colorectal cancer can be subdivided into colon cancer, which ranges from cecum to the sigmoid (approximately 15 cm above the anal verge), and rectal cancer, that ranges from the recto-sigmoid to the anus. Rectal cancer constitutes approximately 25% of all colorectal cancers (Siesling, Van Dijck, Visser, Coebergh, & Registry, 2003).

One of the most common malignancies in many regions of the world is colon cancer, and is thought that the colorectal cancer arises from a mutations which accumulates in a single epithelial cell of the colon and rectum. Colon cancer and rectal cancer have many features in common, and because of that they are often grouped together. Most colorectal cancers begin as a growth on the inner lining of the colon or rectum called a polyp. Some types of polyps can change into cancer over

the course of several years, but not all polyps become cancer. The nature of the polyp determines the chance of its transformation into cancer (American Cancer Society, 2014).

2.3.2 Colon cancer types

When doctors talk about colorectal cancer, they are almost always talking about Adenomatous type, which is the first type of colorectal cancer and makes up more than 95% of colorectal cancers. This type of cancer begins in cells that form the glands that make mucus to lubricate the inside of the colon and rectum. The second type of CRC is Gastrointestinal Stromal Tumors (GISTs) which start from specialized cells in the wall of the colon called the interstitial cells of Cajal, where some are non-cancerous (benign). It is unusual to find them in the colon but these tumors can be found anywhere in the digestive tract. The third type is known as Lymphomas, called also the cancers of immune system, cells that typically start in lymph nodes, but they can also start in the colon, rectum, or other organs. The last type is Sarcomas that can start in blood vessels, muscle layers, or other connective tissues in the wall of the colon and rectum. Sarcomas of the colon or rectum are rare (American Cancer Society, 2014).

2.3.3 Colorectal cancer stages

One of the main reasons, considered as a major obstacle, in the effective treatment of colorectal cancer is the reduced sensitivity of advanced stages of disease to chemotherapy. Therefore, the survival rate of the patient is highly dependent on the stage of the disease at the time of diagnosis (Gonzalez-Pons & Cruz-Correa, 2015). Most colorectal cancer cases are asymptomatic until it progresses to advanced stages, so implementation of programs that can be used for early detection of cases is essential to reduce incidence and mortality rates (Zavoral et al., 2014).

The extent of cancer in the body is described according to the stage of cancer which is also considered as one of the most important factors for successful cancer

treatment. The staging system of American Joint Committee on Cancer (AJCC) is the most often used staging system for colorectal cancer (Greene et al., 2002).

The TNM system is based on 3 key pieces of information: Primary tumor (T), Regional lymph nodes (N) and Metastasized (M). Numbers or letters after T, N, and M provide more details about each of these factors, stage of 0, I, II, III, or IV (Hari et al., 2013).

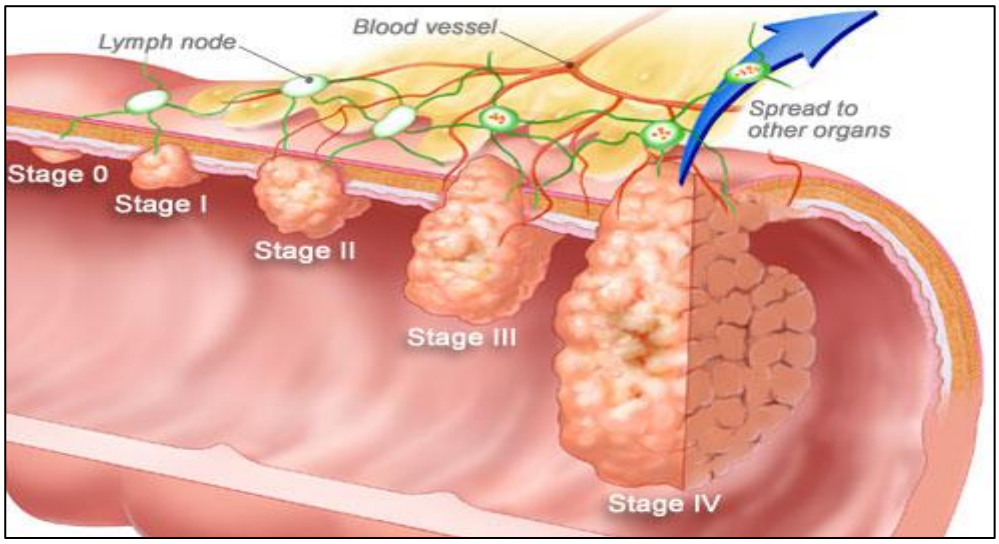

Figure (2.4): Colorectal cancer stages (http://img.webmd.com, 2017).

2.3.4 Risk factors of colon cancer

2.3.4.1 Diet

Fiber, especially in the form of fruits and vegetables, has been considered beneficial in helping to lower one's risk for CRC (Martinez & Willett, 1998). Many studies pointed to the importance of high fiber content and to the benefits of dietary fibers. Proposed mechanisms regarding the benefit from fiber include increased folic acid consumption, lower colonic pH, decreased colonic transit time, and increased production of short chain fatty acids as well as micronutrients found in vegetables including anti-oxidants (Kritchevsky, 1995). On the other hand, consumption of red meat in abundance, especially in the form of beef or lamb has been linked with an increased risk for CRC (Norat et al., 2005).

2.3.4.2 Gender

Although, researchers in many studies have observed that the risk of advanced colon and rectal cancer is increasing in men than in women, the overall lifetime risk for CRC for men and women is almost similar (Roy & Bianchi, 2009).

2.3.4.3 Alcohol

Rectal and colon cancer risk is thought to increase due to Ethanol-based beverages, through a variety of mechanisms including abnormal DNA methylation and repair, induce cytochrome p450 enzymes to increase carcinogen production and alter bile acid composition (Choi et al, 1999).

2.3.4.4 Tobacco

Tobacco exposure, most commonly in the form of cigarette smoking, has only been recognized as an important risk for CRC in both men and women (Giovannucci, 2004).

2.3.4.5 Obesity

Several studies have demonstrated that obesity increases the risk of CRC in men and women, although this association appears to be stronger in males (Dai, Xu, & Niu, 2007; Larsson & Wolk, 2007). The men with the highest body mass index (BMI) had a twofold increased risk for CRC as compared to the thinnest men (Giovannucci et al., 1995).

2.3.4.6 Race

Colorectal cancer rates are the highest for African Americans for both incidence (Murphy et al., 2011), as well as overall mortality when compared to white patients of both genders (Kelly et al., 2010).

2.4 Colorectal cancer treatment

Colorectal cancer treatment is based largely on the stage of the cancer, but also other important factors can be included. For people who have colorectal cancer that is not spread to distant areas, surgical intervention is usually the primary treatment, and adjuvant chemotherapy may also be used. Most adjuvant treatment is given for about 6 months. Other forms of treatment include radiation therapy and targeted therapy (American Cancer Society, 2014).

2.4.1 Drugs used for treatment of CRC

The Food and Drug Administration (FDA) approved many drugs for the treatment of colon and rectal cancer. Some of these drugs are listed in Table (2.4). A combination drugs in pill form, often 2 or more of these drugs, are combined to try to make them more effective (see Table 2.5 for examples). Sometimes, chemo drugs are given along with a targeted therapy drug. Most of the chemotherapy drugs listed in Tables (2.4) and (2.5) are used in Palestine to treat colorectal cancer.

2.5 Drug combination in cancer treatment

Combination therapy has been the standard of care, especially in cancer treatment, since it is a rationale strategy to increase response and tolerability and to decrease resistance. Today, there is growing interest in combination anti-cancer drugs, whose primary objective is to achieve maximum effectiveness as well as reduce toxicity through the patient's dose reduction (Zoli, Ricotti, Tesei, Barzanti, & Amadori, 2001; Ramsay, Santos, Dragowska, Laskin, & Bally, 2005; Mayer & Janoff, 2007).

Table (2.4): Drugs used to treat colorectal cancer according to FDA (http/www.Cancer.gov, 2017).

Drugs Approved for Colon and Rectal Cancer	
5-FU (Fluorouracil Injection)	Capecitabine (Xeloda)
Leucovorin Calcium	Eloxatin (Oxaliplatin)
Oxaliplatin	Trifluridine and Tipiracil
Cyramza (Ramucirumab)	Irinotecan Hydrochloride

Many studies have presented the enhanced effects of drugs combinations in treatment of cancer cells using western drugs, isolated pure herbal extracts or a combination of both.

A number of good chemotherapeutic agent combinations have been developed to treat cancers and they showed positive cytokinetic and biological interaction with reduced toxicity (Fitzgerald, Schoeberl, Nielsen, & Sorger, 2006).

Evaluation and calculation of the effect of drug combination is very important in all areas of medicine, especially in chemotherapy treatments where combination therapy is commonly used (Alzaharna, Alqouqa, & Cheung, 2017). The combination index (CI) method is the most commonly used method in the analysis of drug combination effects. Table (2.6) gives an illustration of the description and symbols used in drug combination studies using the CI method to describe synergism or antagonism.

Table (2.5): Drug combination used to treat colorectal cancer according to FDA (https://www.cancer.gov, 2017).

Drug Name	Chemotherapeutic Agents
CAPOX	CAP: Capecitabine, OX: Oxaliplatin
FOLFIRI	FOL: Leucovorin Calcium, F: Fluorouracil, IRI: Irinotecan Hydrochloride
XELOX	XEL: Capecitabine (Xeloda), OX: Oxaliplatin
FOLFOX	FOL: Leucovorin Calcium, F:Fluorouracil, OX: Oxaliplatin
XELIRI	XEL: Capecitabine (Xeloda), IRI: Irinotecan Hydrochloride
FOLFIRI-BEVACIZUMAB	FOL: Leucovorin Calcium (Folinic Acid), F:Fluorouracil, IRI: Irinotecan Hydrochloride & Bevacizumab

Table (2.6): Description and symbols of synergism or antagonism in drug combination studies analyzed with the Combination Indix method. Modified from (Chou, 2006).

Range of CI	Description	Graded Symbols
< 0.1	Very strong synergism	+++++
0.1–0.3	Strong synergism	++++
0.3–0.7	Synergism	+++
0.7–0.85	Moderate synergism	++
0.85–0.90	Slight synergism	+
0.90–1.10	Nearly additive	±
1.10–1.20	Slight antagonism	–
1.20–1.45	Moderate antagonism	– –
1.45–3.3	Antagonism	– – –
3.3–10	Strong antagonism	– – – –
> 10	Very strong antagonism	– – – – –

2.6 Natural products

Natural products are obtained mainly from plants, fungi and microorganisms that are present in soil. They are chemical compounds or substances produced by a living organism that is found in nature (Samuelson, 1999). In the broadest sense, natural products include any substance produced by life. The history of natural products is long and full of beneficial use by humans to treat many diseases. These materials may be useful in their structurally original form or may be *derived* by chemical synthesis to enhance potency or pharmacologic properties such as water solubility or thermostability (Foye, Lemke, & Williams, 2008).

The term "natural product" is generally taken to mean a compound that has no known primary biochemical role in an organism. Such compounds are also called "secondary metabolites", and apparently are produced by the organism for ecological or defensive purposes, thus promoting its survival (Messina & Barnes, 1991). The National Cancer Institute collected about 35,000 plant samples from 20 countries and has screened around 114,000 extracts for anticancer activity (Shoeb, 2006).

2.6.1 Medicinal plants

Medicinal plants can be defined as plants which at least one of its parts contains substances that can be used for therapeutic purposes. Traditional medicine (TM) refers to health practices, approaches, knowledge and beliefs that include medicines of animal and plant, minerals, spiritual therapies, manual techniques and exercises, which are applied individually or in combination to treat, diagnose, cure/prevent illnesses and maintain well-being (Sofowora, 1982).

2.6.1.1 History of medicinal plants

The ancient Egyptians are considered the first nation that practiced medicine with proper rules. Ancient manuscripts showed that ancient Egyptians (Ebers papyrus; 1550 B.C.) and Greeks used plants for medicinal purposes and pointed out their importance. Hippocrates (377-460 B.C) was famous for his medical recipes and writings in the field. In addition, Theophrastus (285- 372 B.C) wrote a very

important book entitled: Etiology of plants (be Historia Plantarum). This famous book includes 500 medicinal plants (Edwin Smith, 2017).

Many factors contributed to the exponential growth in the use of medicinal plants to treat different diseases and gaining popularity both in developing and developed countries. First of all, and the most important is the spreading of new diseases that are not eliminated until now, and the belief of the societies' members that the natural materials have no harmful effects. Additionally, the appearance of ecological movements around the world, to pay attention to the medicinal use of plants as a safer and more successful treatment than manufactured synthetic drugs that are costly and have many adverse side effects (Schulz, Hansel, & Tyler, 1998).

Medicinal plants have a promising future because there are about half million plants around the world, and most of their medical activities have not been investigated yet, and their medical activities could be decisive in the treatment of present or future studies (Rasool Hassan, 2012).

2.6.1.2 Characteristics of Medicinal Plants

Medicinal plants have many benefits when used in treatment, they include:

- Synergistic effects which indicate that the plant’s ingredients can interact together, so can neutralize their possible negative effects or complement or damage others.
- Support of medicine: the components of the plants proved to be very effective treatment in complex cases such as cancer diseases.
- Preventive medicine: the plants components has been proven that the components characterized by its ability to prevent the appearance of some diseases. This will help to reduce the use of the chemical remedies which will be used when the disease is already present i.e., reduce the side effect of synthetic treatment (Van Wyk, B. E., & Wink, M, 2017).

2.6.1.3 Medicinal plants in Palestine

Palestine is distinguished by its unique geographical location at the meeting point of three continents; Asia, Africa, and Europe. It has a large desert and a lot of mountains; it is at the coast of the Mediterranean. This geographical diversity leads to weather and climate changes and this, in turn, leads to biodiversity (Mendelssohn & Yom-Tov, 1999). More than 700 species of medicinal plants are known to exist, and approximately 63 of these are actively used for the preparation of traditional medicines (Ali-Shtayeh, Yaniv & Mahajna, 2000; Sawalha, 2008; Ali-Shtayeh, Jamous, & Jamous, 2012).

In Palestine, like other developing countries, using medicinal plants play an important role in primary health care. They are used as complementary and alternative medicine (CAM) in the Traditional Arabic Palestinian Herbal Medicine (TAPHM), for health maintenance and to treat various illnesses including chronic diseases (Husein et al., 2014).

Many plants are used as anticancer agents in Palestinian traditional medicine (Table 2.7). At the beginning of the eighties of the last century, an ethnobotanic study was made about the medicinal plants which were in use in north of "Occupied Palestine". The study involved 66 informants, and produced information on about 150 species of the plants which were used in folkloric medicine (Dafni, Yaniv, & Palevitch, 1984).

Table (2.7): Plants used as anticancer agents in Palestine modified (Ali-Shtayeh, Jamous, & Jamous, 2011).

Scientific name (Family)	Common name	Arabic name	Plant part used
Zingiber officinale Rose. (Zingiberaceae)	Ginger	Zangabel	AP, LE, FL, RT, FR
Allium sativum L. (Liliaceae)	Garlic	Thoum	RT, FR
Curcuma longa L. (Zingiberaceae)	Turmeric	Korkom	AP, FL, RT, SD
Rosmarinus officinalis L. (Lamiaceae)	Rosemary	Ekleel El-jabl	AP, LE, FL
Allium cepa L. (Liliaceae)	Onions	Basal	AP, LE, RT
Camellia thea Link. (Theaceae)	Tea	Shai Akhdar	LE
Triticum aestivum L. (Poaceae)	Wheat	Qamh	AP, FR, SD
Punica granatum L. (Punicaceae)	Pomegranate	Rumman	FR
Vitis vinifera L. (Vitacea)	Grape	Inab	FR
Citrus limon (L.) Burm. Fil (Rutaceae)	Limon Tree	Laimoon	RT, FR
Cinnamomum zeylanicum Blume. (Lauraceae)	Cinnamon Tree	Qerfeh	LE, BA
Ficus carica L. (Moraceae)	Fig Tree	Teen	FR
Portulaca oleracea L. (Portulaceae)	Purslane	Baqleh	LE

AP, aerial parts; BA, bark; LE, leaves; FL, flowers; FR, fruits; SD, seeds; ST, stem; RT, roots.

2.7 Plants used in the present study

2.7.1 *Portulaca oleracea*

P. oleracea belongs to the *Portulacaceae* family (Table 2.8), a small family with 21 genera and 580 species. It is an important plant naturally found as a weed in field crops and lawns, also called purslane or Portulaca (Danin & Reyes-

Betancort, 2006). Purslane is widely distributed around the globe, they are succulent, annual herbaceous, and erect up to 30-40 cm high (Figure 2.5) (Uddin et al., 2014).

Table (2.8): Classification of *Portulaca oleracea* (USDA, 2017).

Rank	Scientific Name
Kingdom	Plantae
Subkingdom	Tracheobionta
Division	Magnoliophyta
Class	Magnoliopsida
Order	Caryophyllales
Family	*Portulacaceae*
Genus	*Portulaca* L. – purslane
Species	*Portulaca oleracea* L.

P. oleracea is used as a pickle or for cooking. Its medicinal value is evident from its use for treatment of headache, and diseases related to the intestine, liver, stomach, cough, burns, shortness of breath, and arthritis. It is used as a purgative, cardiac tonic, muscle relaxant, anti-inflammatory and diuretic treatment (Oliveira et al., 2009).

P. oleracea has been described as a power food because of its antioxidant, high nutritive properties (Liu et al., 2000). *P .oleracea* is rich in vitamin A which is a natural antioxidant and has the highest content of this vitamin among green leafy vegetables. It also contains omega-3 fatty acid and alpha linolenic acid. Polysaccharides from *P. oleracea* display several biological activities, such as anticancer, antioxidant, anti-inflammation, and immunity enhancing properties (Yang, Zhao, Yang, & Ruan, 2008).

Figure (2.5): The plant *Portulaca oleracea* (USDA, 2017).

2.7.2 *Rosmarinus officinalis*

R. officinalis, is a shrubby herb that grows wild in the Mediterranean basin, today this plant is cultivated worldwide due to its diverse uses as a common household culinary spice for flavoring. *R. officinalis* commonly known as rosemary, belongs to the *Lamiaceae* family (Table 2.9). *R. officinalis* has pharmacological and decorative values, it is a sustainable plant, aromatic and has wooden stalks with 50 cm to up to 2 m height (Frankel, Huang, Aeschbach, & Prior, 1996; Nascimento, Locatelli, Freitas, & Silva, 2000) (Figure 2.6).

R. officinalis has been used as a medicinal herb for centuries due to significant activities such as hepatoprotective (Sotelo-Felix et al., 2002), antimicrobial (Campo, Amiot, & Nguyen-The, 2000; Bozin, Mimica-Dukic, Samojlik, & Jovin, 2007), antithrombotic (Yamamoto, Yamada, Naemura, Yamashita, & Arai, 2005), diuretic (Haloui, Louedec, Michel, & Lyoussi, 2000), antidiabetic (Bakırel, Bakırel, Keleş, Ülgen, & Yardibi, 2008), anti-inflammatory (Altinier et al., 2007), antioxidant (Pérez-Fons, GarzÓn, & Micol, 2009), and anticancer (Lo, Liang, Lin-Shiau, Ho, & Lin, 2002; Visanji, Thompson, & Padfield, 2006).

Table (2.9): Classification of *Rosmarinus officinalis* (USDA, 2017).

Rank	Scientific Name
Kingdom	Plantae
Subkingdom	Tracheobionta
Division	Magnoliophyta
Class	Magnoliopsida
Order	Lamiales
Family	*Lamiaceae*
Genus	*Rosmarinus*
Species	*Rosmarinus officinalis*

The main active constituents of *R. officinalis* are flavonoids, phenolic acids, diterpenes, steroids, and triterpene (Frankel et al., 1996; Nascimento et al., 2000). Rosemary essential oil is also used as an antibacterial, antifungal (Oluwatuyi, Kaatz, & Gibbons, 2004; Kabouche et al., 2005; Fernandez-Lopez, Zhi, Aleson-Carbonell, Perez-Alvarez, & Kuri, 2005) and anticancer agent (Leal et al., 2003).

Figure (2.6): The plant *Rosmarinus officinalis* (USDA, 2017).

2.7.3 *Avena sativa*

Classification of *Avena sativa* is listed in Table (2.10). *A. sativa L*. is an annual grass about 1.5 meters high; culms tufted or solitary, erect or bent at the base, and smooth (Figure 2.7). The leaves are non-articulate, green, and the sheaths rounded on the back; the ligules are blunt and membranous (Gibbs et al., 1955). *Avena sativa L. (Gramineae)*, commonly known as Oat, Groats, Haber, Hafer, Avena, Straw, Oatmeal, is a species of cereal grain grown for its seed. In folk medicine, oats are used to treat nervous exhaustion, insomnia, and weakness of the nerves. They are considered as antispasmodic, antitumor, cyanogenetic, demulcent, diuretic, neurotonic, stimulant, tonic, and vulnerary. A tea made from oats was considered to be useful in rheumatic conditions and to treat water retention (Singh. R, Singh, De, & Belkheir, 2013).

Table (2.10): Classification of *Avena sativa* (USDA, 2017).

Rank	Scientific Name
Kingdom	Plantae
Subkingdom	Tracheobionta
Division	Magnoliophyta
Class	Liliopsida
Order	Cyperales
Family	*Poaceae / Gramineae*
Genus	*Avena L.*
Species	*Avena sativa L.*

A tincture of the green tops of oats has been used as a nerve stimulant and also to help in withdrawal from tobacco and opium addiction. Oats were often used in baths to treat insomnia and anxiety as well as in a variety of skin conditions,

including burns and eczema. Its use in the bath water helps to keep the skin soft due to its emollient action. The seeds are folk remedies for tumors while straws are said to be useful in rheumatism (Duke & Wain, 1981).

β-glucans from oats have been used in immune-adjuvant therapy for cancers and tumors since 1980. The ability of β-glucan to inhibit tumor growth in a variety of experimental tumor models is well established. There is a large collection of research data that demonstrates β-glucans as having antitumor and anticancer activity (Hong et al., 2004).

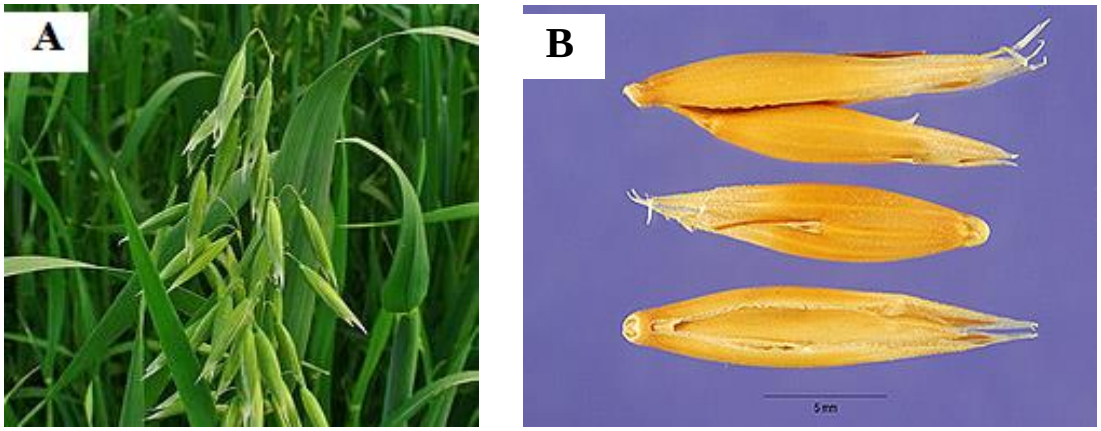

Figure (2.7): (A) The plant *Avena sativa L.* (B) The *Avena sativa L.* seeds (USDA, 2017).

2.7.4 *Punica granatum*

The pomegranate (*Punica granatum L.),* which belongs to the *Punicaceae* family (Table 2.11), is a nutrient dense food source rich in phytochemical compounds (Miguel, Neves, & Antunes, 2010). It is a large shrub up to 10m in height with smooth, dark grey bark (Figure 2.8). Flowers are scarlet red or sometime yellow, fruits are globose (Yogeeta, Ragavender, & Devaki, 2007).

Table (2.11): Classification of *Punica granatum* (USDA, 2017).

Rank	Scientific Name
Kingdom	Plantae
Subkingdom	Tracheobionta
Division	Magnoliophyta
Class	Magnoliopsida
Order	Myrtales
Family	*Lythraceae*
Genus	*Punica* L.
Species	*Punica granatum* L.

Figure (2.8):The plant *Punica granatum* (USDA, 2017).

Previous studies reported that phytochemicals have been identified from various parts of the pomegranate tree and from pomegranate fruit: peel, juice and seeds (Singh, Chidambara Murthy, & Jayaprakasha, 2002; Elfalleh et al., 2011). They have many benefits (Leja, Mareczek, & Ben, 2003), having free radical

scavenging capacity and antioxidant ability (Kähkönen, Hopia, & Heinonen, 2001). Pomegranate Peel exhibited high antioxidant activity in various in vitro models (Li et al., 2006). The peel of the pomegranate has been extensively used in folk medicine (Ahmad & Beg, 2001).

Chapter III
Materials and Methods

Chapter III

Materials and Methods

3.1 Materials

3.1.1 Equipment

The equipment used in the present study are listed in Table (3.1).

Table (3.1): List of the equipment used in the study.

No.	Apparatus	Manufacture/model	Country
1	Automated ELISA Reader	Multiskan FC	Republic or Korea
2	Heat drying oven	Boxun	China
3	Inverted microscope	EVOS XL -AMG	USA
4	Centrifuge	Hettich	USA
5	Soxhlet extractor	Biology department	IUG
6	Incubator	HH.CP.01	China
7	Safety cabinet	Boxun	China
8	IR Concentrator	N- Biotek	Korea
9	Autoclave	Tuttnauer	Germany
10	Refrigerator (4°C) and freezer (-20°C)	Selecta	China
11	Liquid nitrogen tank (-196°C)	International Cryogenics	USA
12	Pipettes 10, 50, 200, and 1000 µL, Multichannel Pipette, and Motorized Pipetting Device	Dragon Med	China

3.1.2 Chemicals and culture media used

Table (3.2) represents the reagents which were used in the present study.

Table (3.2): List of reagents used in the present study.

No.	Items	Cat. No.	Company
1	Trypsin-EDTA solution C	03-051-5c	Biological industries
2	Thiazolyl- blue tetrazolium bromide (MTT)	194592	MP Biomedical, LLC
3	Phosphate buffer saline (PBS)	1452627	Biological industries
4	Ethanol	21050802	Advancid tich
5	DMSO	BCBD5259V	Sigma
6	Trypan blue solution (0.5%)	1434285	Biological industries
7	Copper sulphate	50972	Biomark
8	Chloroform	HO210	Homed
9	DMEM medium	1643856	Biological industries
10	L-Glutamine solution	0L003860	Biochemica
11	Penicillin-Streptomycin solution	03-031-1b	Biological industries
12	Fetal Bovine serum (FBS)	04-007-1a	Biological industries

3.1.3 Disposables

The disposables used in the present study are shown in Table (3.3).

Table (3.3): List of disposables used in the present study.

No.	Items	Company
1	Cell culture flask with filter cap (75, 25 cm^2)	Greiner Bio- One
2	Millex-GP syringe filter units, pore size 0.22 μm, filter diam. 33 mm, sterile; γ-irradiate	Sigma-Aldrich
3	96 and 6 wells suspension culture plate with lid, clear, sterile (flat bottom)	Greiner Bio- One
4	Falcon Conical Graduated tubes (15 ml, 50 ml)	Greiner Bio- One
5	Sterile serological pipettes, pipette tips, Petri dishes	Labcon
6	Eppendorf tubes	Greiner Bio- One

3.1.4 Plant collection

The plants used in the present study were collected from different agricultural areas from Gaza. *Portulaca oleracea* was collected from agricultural areas in the north of Gaza Strip in August-2016. *Rosmarinus officinalis* was collected from agricultural land in central Gaza Strip in January-2017. *Punica granatum* was purchased from Gaza`s local markets in Gaza Strip in November-2016 and *Avena sativa* was collected from agricultural land in eastern Gaza Strip in May - 2016. Plants were identified at the Biology Department - Islamic University of Gaza. The tested plants are listed in Table (3.4).

Table (3.4): List of medicinal plants tested for their anti-cancer activity.

No.	Binomial name	Vernacular	Arabic Name	Part/s Tested
1	*Rosmarinus officinalis*	Rosemary	إكليل الجبل	Arial parts
2	*Punica granatum*	Pomegranate	رمان	Peel
3	*Portulaca oleracea*	Purslane	بقله	Arial parts
4	*Avena sativa*	Oats	شوفان	Seed

3.2 Methodology

3.2.1 Cell line and cell culture

The cell line used in the present study, the colorectal cancer cell line (Caco-2), was kindly provided by Prof. Rana Abu-Dahab (University of Jordan) from American Type Culture Collection (ATCC) and normal lymphocytes where obtained from the Genetic Diagnosis unit (The Islamic University of Gaza). Caco-2 cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin at 37^{o}C in a humidified 5% CO_2 incubator. The medium was changed every 2-3 days for the stock culture.

3.2.2 Preparation of plants extracts

The aerial parts of the two plants (*Rosmarinus officinalis* & *Portulaca oleracea*), the seeds of *Avena sativa* and the peels of *Punica granatum* were washed thoroughly with tap water (in order to remove soil, dust and any insects that may be present) and dried on clean tissue papers. Then, all plants were air-dried for one day. Plants were then completely dried by using hot oven at 37°C for one week. Dried plants were grinded using blender mixer and converted to powder and stored in well-sealed sterile cups.

3.2.3 Extraction of plants material

The ground plants were extracted by using Soxhlet extractor apparatus with different solvents (distilled water, ethanol and chloroform).

For all plants, the ethanolic, chloroformic and distilled water extracts were prepared as follows: in a Soxhlet extractor apparatus, 20g of the ground plants/ seeds were weighed and then added to filter paper and mixed with 200-300 ml ethanol 70% (v/v). The weight/ solvent volume ratio was 1/10, the system was left at 60-100 °C according to solvent type for 6-8 hours until the extraction process completed. The extracts were then allowed to evaporate in an infrared (IR) concentrator at 37-40 °C (depending on the type of plant and solvent used). Table (3.5) illustrates the temperature and time needed for drying the extract and heat mode for each solvent. The dried extract was stored in a refrigerator at 4°C in sterile well-sealed tube until used (Harbone, 1998; Rangari, 2009).

Table (3.5): Temperature and time needed for drying the extract by IR concentrator as recommended by manufacturer (Labconco, 2017).

Solvent	Temp (°C)	Time to dry (min.)	Heat mode
Ethanol	40	270	IR/Heat
	50	60	IR/Heat
	60	39	IR/Heat
	65	39	IR/Heat
Chloroform	50	49	IR/Heat
	60	34	IR/Heat
	65	31	IR/Heat
Water	50	330	IR/Heat
	60	180	IR/Heat
	65	129	IR

IR: infrared

3.2.4 Preparation of stock solutions

The first stock solutions of ethanolic and chloroformic crude extracts were prepared by dissolving 400 mg of crude extracts in 1ml of dimethyl sulfoxide (DMSO). The stock was vortexed until completely dissolved, filter-sterilized (0.22µm) and then stored at 4^{o}C. The distilled water stock solutions of the different extracts were prepared by adding 10 mg from different dried crude extract to 1 ml of distilled water. The working solution was prepared by adding 1µl from the stock solution to 1ml of DMEM and then the different concentrations were prepared by serial dilution (Rios & Recio, 2005).

3.2.5 Recovery of cells

The required tube was taken out from liquid nitrogen, rapidly thawed in a 37^{o}C water bath and then the contents of the vial were transferred to a 15 mL sterile falcon centrifuge tube containing 10 mL of pre-warmed complete medium. The tube was spun for 5 minutes at 1000 rpm. Then, the supernatant was discarded and the cell pellet was re-suspended in fresh medium. The cell suspension was transferred to a culture flask, the suitable amount of medium was added and the flask was incubated at 37°C with 5% CO_2.

3.2.6 Cells harvesting and counting

After Caco-2 culture flask reached complete confluence, cells were trypsinized with 3 ml of trypsin-EDTA solution (75 cm^2 flask). The flask was incubated at 37°C for 3.5 minutes in 5% CO_2 incubator, then 5 ml of complete media were added to stop trypsin action. The cells were harvested and transferred to a 15 ml sterile falcon. The tube was centrifuged for 5 minutes at 1000 rpm. Then, the supernatant was removed and the pellet was re-suspended in 3 ml media. 100 µl of the cells suspension were transferred to a 1.5 ml Eppendorf tube and 100 µl of trypan blue were added, then they were mixed well and left for 5 minutes. Twenty µl of the mixture were transferred to a clean hemocytometer chamber as shown in (Figure 3.1). The number of viable (seen as bright cells) and non-viable cells (stained blue)

were counted in the four large corner squares and the following formula was used to calculate viable number of cells.

$$\text{Viable cells number/ml} = \frac{\text{Number of viable cells}}{4} \times 10^4 \times \text{dilution factor}$$

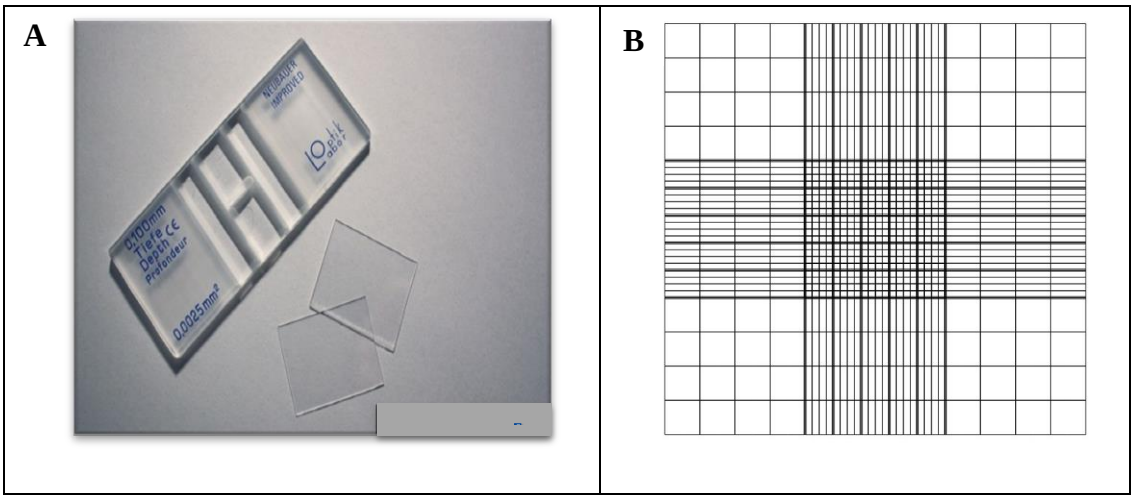

Figure (3.1): A. Haemocytometer for cell counting. B. Gridlines seen under the microscope.

3.2.7 Seeding cells for experiment

After counting cells by using a hemocytometer (Figure 3.1), the cells were then seeded in a 96-wells plate (Figure 3.2) at a density of 10,000 cells/well for Caco-2 cell line and 40,000 cells/well for normal lymphocyte in 100 µl of full DMEM, then incubated at 37°C in 5% CO_2 incubator. The cells were left for 24 h to attach and recover.

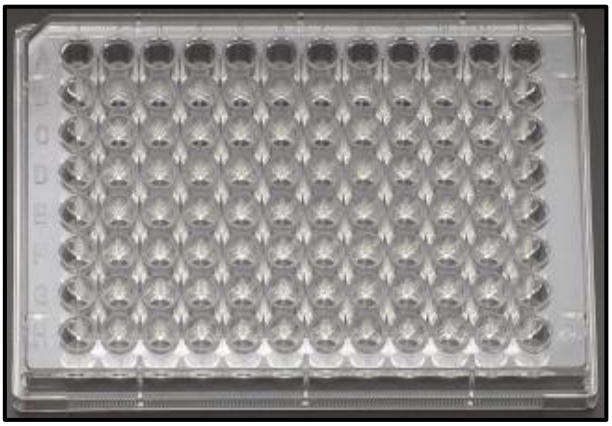

Figure (3.2): A 96-wells plate used for viability experiments.

After 24 h of cells seeding, the cells have attached well to the surface of the wells and become confluent 60-80%, the medium was removed from the wells and replaced with media containing different concentrations of the test plant crude extracts. The concentrations of ethanolic and chloroformic plant extracts were 6.25, 12.5, 25, 50, 100 and 200 µg/ml. While the concentrations used for distilled water extracts were 31.25, 62.5, 125, 250, 500 & 1000 µg/ml.

3.2.8 Combination studies

Cells were seeded in 96 wells plate at a density of 10,000 cells/well in 100µl of full DMEM. Then chloroformic crude extracts of *R. officinalis* and ethanolic crude extract of *A. sativa* were added at different concentrations (6.25, 12.5, 25, 50, 100, and 200 µg/ml) combined with different concentrations of the 5-FU (1.625, 3.25, 6.5, 13, 26 & 52). The crude extracts and 5-FU were combined at a constant ratio (3.85:1). The combination of 5-FU with different plant crude extracts was always set at a fixed ratio.

All treated plates were incubated for 24 h to 48 h at 37°C in 5% CO_2 incubator. All experiments were independently repeated at least three times for each plant extract (Jumarie & Malo, 1991).

3.2.9 Determination of IC_{50} and analysis of combinations effect using CompuSyn software

The computer software CompuSyn was used for the determination of half maximal inhibitory concentration (IC_{50}), Combination Index (CI) and the dose reduction index (DRI) for the single or combined compounds. This software is based on the median-effect principle of Chou and Talalay (Chou, 2006). The cytotoxic effects of the single compounds or combination of different crude extracts with 5-FU were first determined and then input in the software which calculated the CI of different combinations. The CI was calculated by using fixed ratio combinations of crude extracts with 5-FU which will give better CI – Fa simulation. A CI value of more than 1 means antagonism, CI value equals to 1 means additivity and CI value of less than 1 means synergism (Table 2.5). It should be borne in mind that if the combined effect is more than of each drug alone does not necessarily indicate synergism (Chou, 2010). The DRI represents the decrease in dosage when compounds are combined together compared to single compounds treatments DRI > 1 indicates that there is a reduction in the dosage (Ohnstad et al., 2011).

3.2.10 MTT Assay

After the treatment periods, medium containing drugs was removed, wells washed carefully with PBS for one time and then 100 µl of medium containing MTT at 0.5 mg/ml was added to all the microtiter plate wells. The plate was incubated for 3 hours in a CO_2 incubator at 37^oC, then the MTT was removed and 100 µl of DMSO were added to dissolve the formazan crystals that were formed. The plate was covered with a tinfoil for 15 minute and agitated on orbital shaker for 30 seconds. Then, the Optical Density (OD) of the MTT formazan was determined at 550 nm in a micro- plate reader in figure (3.3).

Figure (3.3): Micro-plate reader.

Finally, cell viability was defined as the ratio in (%) of absorbance of treated cells to untreated cells (control) after subtraction of the blank reading (Mosmann, 1983). Percentage viability at each extract concentration was calculated as following:

$$\text{Viability (\%)} = \frac{\text{Mean OD of sample} - \text{OD of Blank}}{\text{Mean OD of control} - \text{OD of Blank}} \text{ X } 100$$

3.2.11 Determination of morphological changes of the cells in culture using DAPI stain assay

The Caco-2 cells were grown on clean and sterile coverslips. The coverslips were put into 6 wells cell culture plates and then the Caco-2 cells were seeded into the wells at a density of $4x10^4$- $8x10^4$ cells per well. The cells were left overnight to attach, then treated with the crude extracts for the specified time. After treatment, the medium was removed and cells were washed with PBS briefly. The cells were then stained for 10 min. Using DAPI dye solution (5 µg/ml) to stain DNA at room temperature and then mounted on a slide. Finally, morphological changes were observed under a fluorescent microscope at excitation/emission 357/447 nm (Kubota, Nara, & Yoshino, 1987).

Chapter IV
Results

Chapter IV

Results

4.1 Antiproliferative effects of different crude plant extracts on Caco-2 Cells

The effect of the different plant extracts on the viability of the colon cancer cell line (Caco-2) was examined. The different crude extracts used in this study showed variable antiproliferative effects against Caco-2 cancer cells. Cells in the exponential growth phase were treated with different concentrations of ethanolic, chloroformic (0, 6.25, 12.5, 25, 50, 100 and 200 µg/ml) or distilled water crude extracts (0, 31.25, 62.5, 125, 250, 500 and 1000 µg/ml) at 24 and 48 h. Viability of cells was determined using MTT assay. At least three independent experiments were done.

4.1.1 Antiproliferative effects of *R. officinalis* ethanolic crude extract on Caco-2 cells

The inhibitory effect of different concentrations of *R. officinalis* ethanolic crude extract at different time points on Caco-2 cells was studied. *R. officinalis* ethanolic crude extract increased the inhibitory effect in dose and time dependent manner (Figure 4.1). The inhibitory effect increased from 1.6% to 85.7% as the concentration of *R. officinalis* increased from 12.5 – 200 µg/ml for 24 h (Table 4.1). When the treatment time was increased to 48 h, the inhibitory effect increased from 16.4% to 97.8% as the concentration of *R. officinalis* increased from 6.25 – 200 µg/ml (Table 4.2). The calculated IC_{50} is 98 µg/ml and 24.7 µg/ml at 24 h and 48 h, respectively.

Table (4.1): The inhibitory effect of *Rosmarinus officinalis* ethanolic extract at 24 & 48 h on Caco-2 cells.

Conc. µg/ml	*R. officinalis* ethanolic crude extract					
	24 h			48 h		
	Viability (%)		Inhibitory effect %	Viability (%)		Inhibitory effect %
	Mean	±SD		Mean	SD	
6.25	100.4	15.2	-0.4	83.7	0.6	16.4
12.5	98.4	3.2	1.6	71.7	0.1	28.4
25	93.2	6.5	6.8	68.3	6.9	31.8
50	90.6	10.3	9.4	41.5	4.4	58.5
100	35.1	14.2	64.9	6.7	0.9	93.4
200	14.3	8.1	85.7	2.2	2.2	97.8

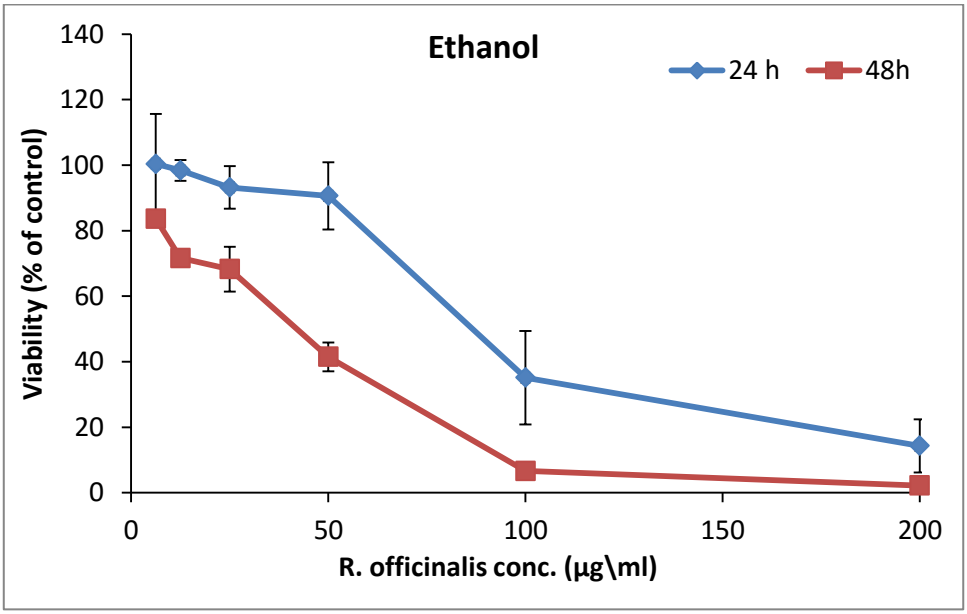

Figure (4.1): Effect of *Rosmarinus officinalis* ethanolic crude extract on Caco-2 cells for 24 and 48 h. Values are expressed as mean ± SD of at least 3 independent experiments.

4.1.2 Antiproliferative effects of *R. officinalis* chloroformic crude extract on Caco-2 cells

The inhibitory effect of different concentrations of *R. officinalis* chloroformic crude extract at different time points on Caco-2 cells was studied. The crude extract increased the inhibitory effect in a dose and time dependent manner (Figure 4.2).

The inhibitory effect increased from 11% to 90% as the concentration of the crude extract increased from 6.25 – 200 µg/ml for 24 h (Table 4.2). On the other hand, the increase in the treatment time to 48 h increased the inhibitory effect from 16% to 99% as the concentration of *R. officinalis* increased from 6.25 – 200 µg/ml (Table 4.2). The calculated IC_{50} is 77 µg/ml and 21 µg/ml at 24 h and 48 h, respectively.

Table (4.2): The inhibitory effect of *Rosmarinus officinalis* chloroformic extract at 24 & 48 h on Caco-2 cells.

Conc. µg\ml	***R. officinalis* chloroformic crude extract**					
	24h			**48h**		
	Viability (%)		**Inhibitory effect %**	**Viability (%)**		**Inhibitory effect %**
	Mean	**±SD**		**Mean**	**SD**	
6.25	88.6	7.8	11.4	83.6	6.0	16.4
12.5	91.1	3.3	8.9	74.8	6.6	25.2
25	88.3	6.4	11.7	59.2	7.3	40.8
50	83.5	15.7	16.5	26.1	0.8	73.9
100	49.9	10.5	50.2	4.5	2.5	95.5
200	9.1	5.0	90.9	1.0	0.3	99.0

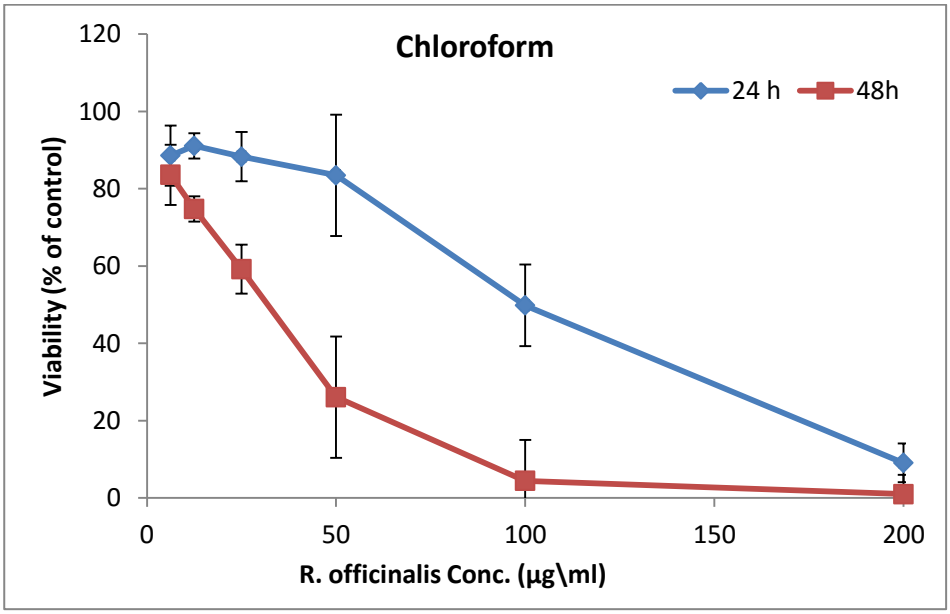

Figure (4.2): Effect of *Rosmarinus officinalis* chloroformic crude extract on Caco-2 cells for 24 and 48 h. Values are expressed as mean ± SD of at least 3 independent experiments.

4.1.3 Antiproliferative effects of *R. officinalis* distilled water crude extract on Caco-2 cells

The inhibitory effect of different concentrations of *R. officinalis* distilled water crude extract at different time points on Caco-2 cells was investigated (Figure 4.3). *R. officinalis* distilled water crude extract enhanced the proliferation of Caco-2 cells differently at the different used concentrations for 24 h (Table 4.3). In contrast, there was a slight change in the inhibitory effect of *R. officinalis* distilled water extract when the incubation time was increased to 48 h. The inhibitory effect of *R. officinalis* distilled water extract increased to 9.2% when the incubation time was increased to 48 h. Additionally, at 48 h incubation, the inhibitory effect increased from 9.2% to 19.4% as the concentration of the crude extract was increased from 250 to 1000 µg/ml (Table 4.3).

Table (4.3): **The inhibitory effect of *Rosmarinus officinalis* distilled water extract at 24 h and 48 h on Caco-2 cells.**

Conc. µg\ml	*R. officinalis* distilled water crude extract					
	24h			48h		
	Viability (%)		Inhibitory effect (%)	Viability (%)		Inhibitory effect (%)
	Mean	±SD		Mean	SD	
31.25	106.0	6.1	-6.0	94.5	28.3	5.5
62.5	114.9	10.6	-14.9	107.5	31.0	-7.5
125	120.4	12.4	-20.4	100.7	26.2	-0.7
250	115.3	15.0	-15.3	90.8	11.8	9.2
500	110.7	18.5	-10.7	88.7	9.4	11.3
1000	109.1	17.0	-9.1	80.6	7.3	19.4

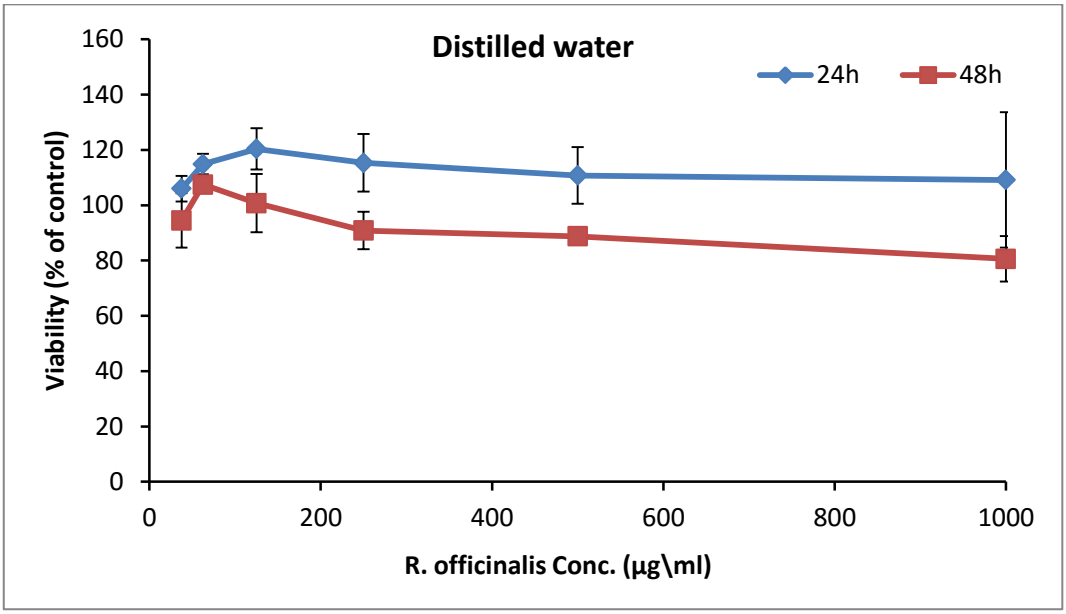

Figure (4.3): Effect of *Rosmarinus officinalis* distilled water crude extract on Caco-2 at 24 and 48 h. Values are expressed as mean ± SD of at least 3 independent experiments.

4.1.4 Antiproliferative effect of ethanolic crude extract of *A. sativa* on Caco-2 cells

The inhibitory effects of *A. sativa* on Caco-2 cells increased in a dose and time dependent manner (Figure 4.4). As shown in Table 4.4, the inhibitory effect of *A. sativa* ethanolic crude extract on Caco-2 cells increased from 18% to 63% as the concentration increased from 6.25 – 200 µg/mL at 24 h. Moreover, the inhibition of cell growth was increased from 33% to 82% as the concentration of the crude extract increased from 6.25 – 200 µg/ml at 48 h (Table 4.4). The IC_{50} for *A. sativa* was 67 µg/ml at 24 h and 11 µg/ml at 48 h.

Table (4.4): The inhibitory effect of *Avena sativa* ethanolic crude extract on Caco-2 cells at 24 and 48 h.

Conc. µg\ml	***A. sativa* ethanolic crude extract**					
	24 h			**48 h**		
	Viability (%)		**Inhibitory effect %**	**Viability (%)**		**Inhibitory effect %**
	Mean	**±SD**		**Mean**	**SD**	
6.25	81.8	6.1	18.2	66.6	4.2	33.4
12.5	75.4	0.2	24.7	43.4	7.7	56.6
25	61.0	5.0	39.0	32.3	5.3	67.7
50	51.8	9.1	48.2	26.0	4.6	74.0
100	42.5	3.0	57.5	18.0	1.7	82.0
200	36.9	6.5	63.1	17.6	4.7	82.4

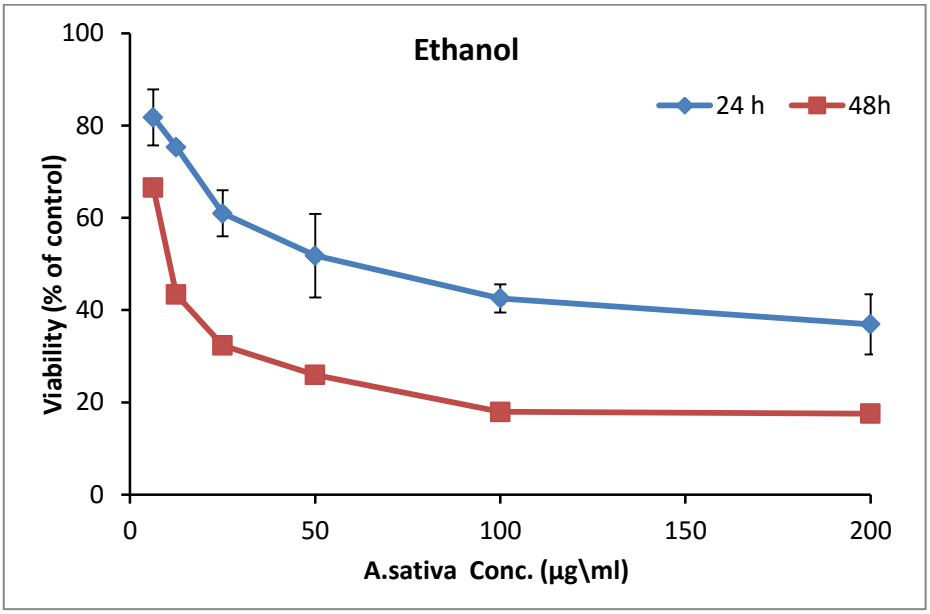

Figure (4.4): Effect of *Avena sativa* ethanolic crude extract on Caco-2 cells at 24 and 48h. Values are expressed as mean ± SD of at least 3 independent experiments.

4.1.5 Antiproliferative effect of chloroformic crude extract of *A. sativa* on Caco-2 cells

The inhibitory effect of *A. sativa* chloroformic crude extract on Caco-2 cells increased in a dose and time dependent manner (Figure 4.5). As shown in Table 4.5, the inhibitory effect of *A. sativa* increased from 9% - 61% as the concentration of *A. sativa* chloroformic crude extract increased from 6.25-200 µg/mL at 24 h. Meanwhile, the inhibitory effect increased from 26% - 81% as the concentration of the crude extract increased from 6.25-200 µg/mL at 48 h. The IC_{50} for *A. sativa* chloroform extract is 98 and 22.8 µg/mL at 24 and 48 hours, respectively.

Table (4.5): The inhibitory effect of *Avena sativa* chloroformic crude extract on Caco-2 cells at 24 and 48 h.

Conc. µg\ml	*A. sativa* chloroform crude extract					
	24 h			48 h		
	Viability (%)		Inhibitory effect %	Viability (%)		Inhibitory effect %
	Mean	±SD		Mean	SD	
6.25	91.4	6.4	8.6	74.0	10.0	26.1
12.5	88.6	11.2	11.5	60.4	6.3	39.6
25	81.1	12.3	18.9	45.4	3.7	54.6
50	55.7	11.7	44.3	33.4	3.9	66.6
100	45.8	9.5	54.3	27.9	2.7	72.1
200	38.9	7.1	61.2	18.6	5.5	81.4

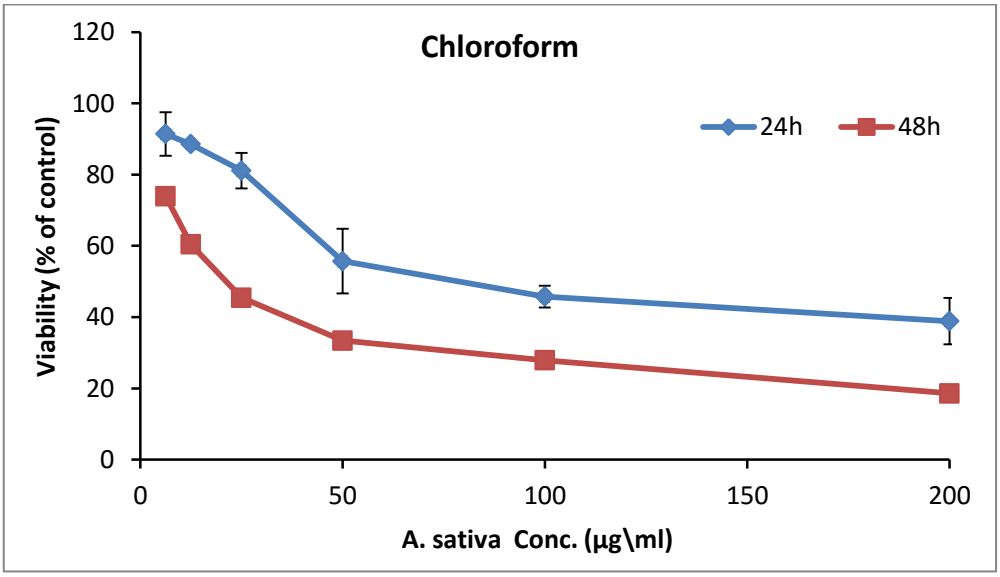

Figure (4.5): Effect of *Avena sativa* Chloroform crude extract at 24 and 48 h on Caco-2. Values are expressed as mean ± SD of at least 3 independent experiments.

4.1.6 Antiproliferative effect of distilled water crude extract of *A. sativa* on Caco-2 cells

The inhibitory effect of different concentrations of *A. sativa* distilled water crude extract at different time points on Caco-2 cells was studied (Figure 4.6). Generally, *A. sativa* distilled water crude extract increased the proliferation of Caco-2 cells differently at different concentrations for 24 h and 48 h (Table 4.6).

Table (4.6): The inhibitory effect of *Avena sativa* distilled water extract at 24 and 48 h on Caco-2 cells.

Conc. µg\ml	***A. sativa* distilled water crude extract**					
	24 h			**48 h**		
	Viability (%)		**Inhibitory effect %**	**Viability (%)**		**Inhibitory effect %**
	Mean	**±SD**		**Mean**	**SD**	
31.25	96.7	6.0	3.3	97.1	4.6	2.9
62.5	99.3	3.4	0.7	103.6	1.8	-3.6
125	105.5	4.8	-5.5	106.5	3.3	-6.5
250	117.6	11.3	-17.6	103.2	2.5	-3.2
500	124.5	7.9	-24.5	105.8	5.6	-5.8
1000	132.2	6.0	-32.2	112.7	15.0	-12.7

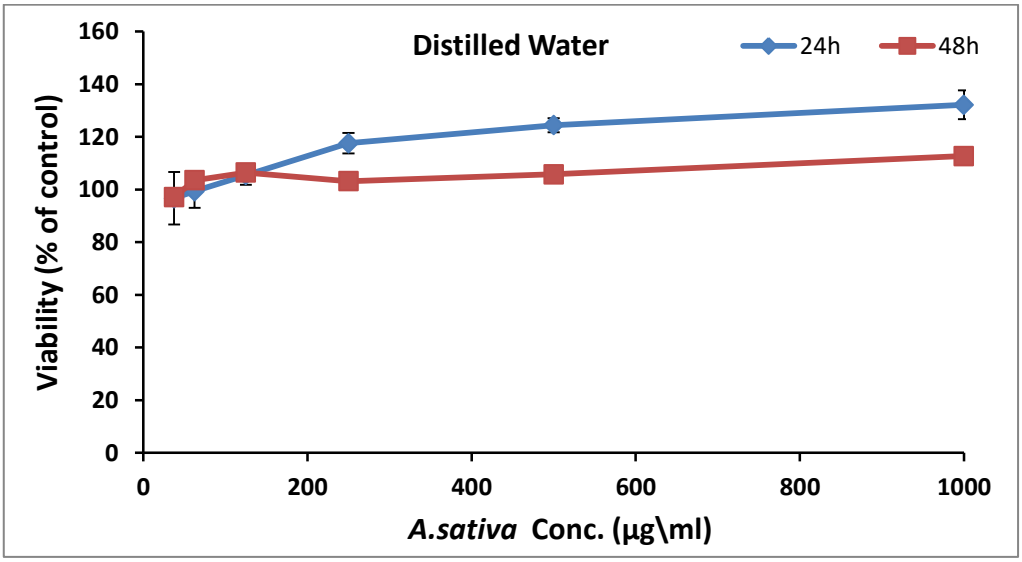

Figure (4.6): Effect of *Avena sativa* distilled water extract at 24 and 48 h on Caco-2. Values are expressed as mean ± SD of at least 3 independent experiments.

4.1.7 Antiproliferative effect of ethanolic crude extract of *P. oleracea* on Caco-2 cells

Figure (4.7) shows the effect of the *Portulaca oleracea* ethanolic extract on colon cancer cells (Caco-2). The results showed that the use of ethanolic crude extract inhibits the growth of Caco-2 in the range of 4.8% to 12.1% at different used concentrations (6.25 – 400 µg/mL) at 24 h. The rate of inhibition of cell growth did not differ significantly when time was increased to 48 h (Table 4.7).

Table (4.7): The inhibitory effect of *Portulaca oleracea* ethanolic crude extract at 24 and 48h on Caco-2 cells.

Conc. µg\ml	***P. oleracea* ethanolic crude extract**					
	24 h			**48 h**		
	Viability (%)		**Inhibitory effect %**	**Viability (%)**		**Inhibitory effect %**
	Mean	**±SD**		**Mean**	**SD**	
6.25	95.2	8.8	4.8	88.5	0.3	11.5
12.5	87.1	2.3	12.9	91.8	10.5	8.3
25	86.9	2.9	13.1	83.4	4.1	16.6
50	93.0	5.3	7.0	87.5	5.7	12.5
100	90.3	3.3	9.7	88.8	12.9	11.2
200	87.5	3.7	12.5	80.4	12.0	19.6
400	88.4	2.4	11.6	93.1	0.3	6.9

4.1.8 Antiproliferative effect of chloroformic crude extract of *P. oleracea* on Caco-2 cells

Figure (4.8) shows the effect of the *Portulaca oleracea* chloroformic extract on Caco-2 cells. The results show that the use of chloroformic crude extract inhibits the growth of Caco-2 in the range of 1% to 23% at different used concentrations (6.25 – 400 µg/mL) at 24 h. While the percentage of inhibition ranged from 19.3% to 28.3% at different used concentrations (6.25 – 400 µg/mL) at 48 h (Table 4.8).

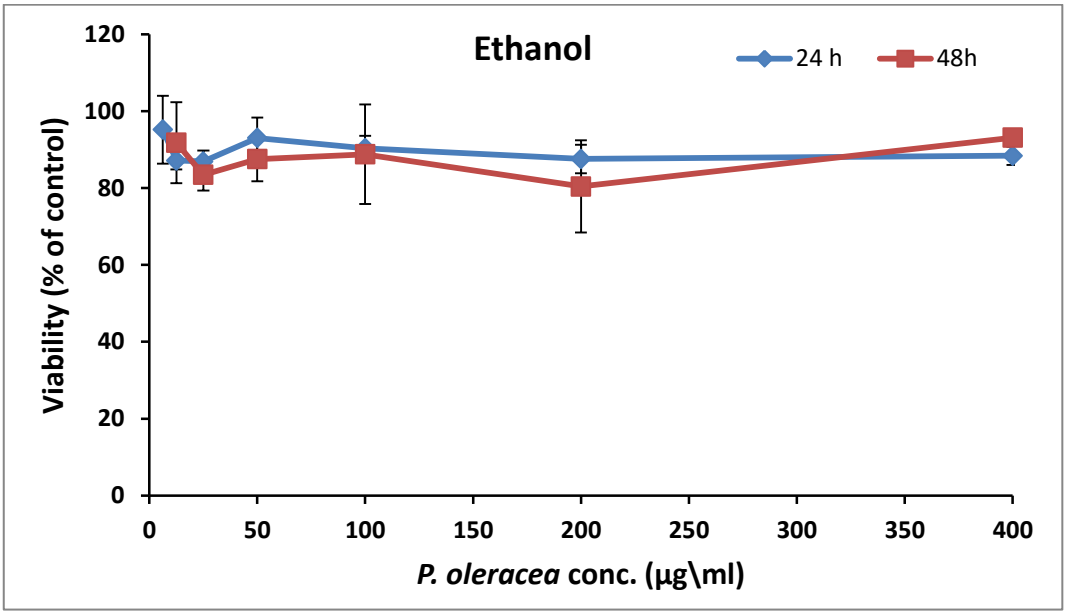

Figure (4.7): Effect of *Portulaca oleracea* **ethanol extract at 24 and 48 h on Caco-2 cells. Values are expressed as mean ± SD of at least 3 independent experiments**.

Table (4.8): The inhibitory effect *Portulaca oleracea* chloroformic crude extract at 24 and 48 h on Caco-2 cells.

Conc. µg\ml	***P. oleracea* chloroformic crude extract**					
	24 h			**48 h**		
	Viability (%)		**Inhibitory effect %**	**Viability (%)**		**Inhibitory effect %**
	Mean	**±SD**		**Mean**	**SD**	
6.25	99.0	5.7	1.0	77.7	4.4	22.3
12.5	89.7	15.0	10.3	74.9	11.7	25.1
25	92.2	15.1	7.8	71.7	6.3	28.3
50	92.0	12.3	8.0	80.0	3.9	20.0
100	85.6	10.9	14.4	80.7	7.9	19.3
200	85.9	14.0	14.1	76.2	9.4	23.8
400	77.0	0.7	23.1	74.6	14.9	25.5

4.1.9 Antiproliferative effect of distilled water crude extract of *P. oleracea* on Caco-2 cells

The inhibitory effect of different concentrations of *P. oleracea* distilled water crude extract at different time points on Caco-2 cells was studied (Figure 4.9). Generally, *P. oleracea* distilled water crude extract inhibited the proliferation of Caco-2 cells differently at different used concentrations (31.25 – 1000 µg/mL) for 24 h and 48 h (Table 4.9). The maximum inhibitory effect was 15.5% at 250 µg/mL at 24 h and 11.1% at 125 µg/mL at 48 h.

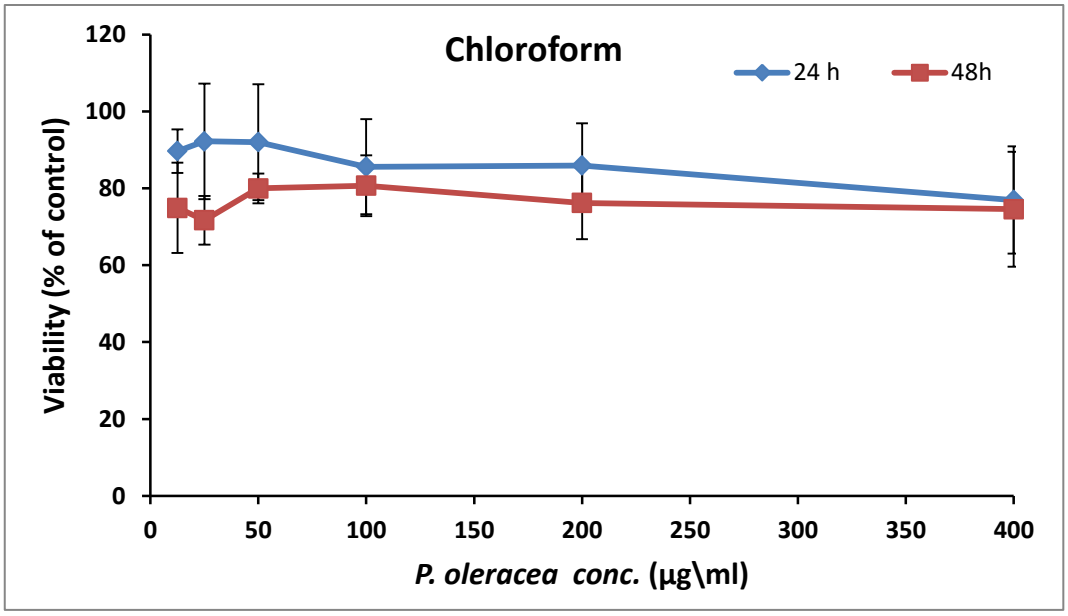

Figure (4.8): Effect of *Portulaca oleracea* chloroformic crude extract at 24 and 48h on Caco-2 cells. Values are expressed as mean ± SD of at least 3 independent experiments.

Table (4.9): The inhibitory effect of *Portulaca oleracea* distilled water crude extract at 24 & 48 h on Caco-2 cells.

Conc. µg\ml	***P. oleracea* distilled water crude extract**					
	24 h			**48 h**		
	Viability (%)		**Inhibitory effect %**	**Viability (%)**		**Inhibitory effect %**
	Mean	**±SD**		**Mean**	**SD**	
31.25	92.5	10.6	7.5	94.1	8.2	5.9
62.5	85.4	10.3	14.7	92.2	1.1	7.8
125	91.2	10.4	8.8	88.9	6.8	11.1
250	84.6	7.4	15.5	98.1	10.5	2.0
500	92.4	3.7	7.7	92.7	1.8	7.3
1000	96.6	4.7	3.4	98.5	9.8	1.6

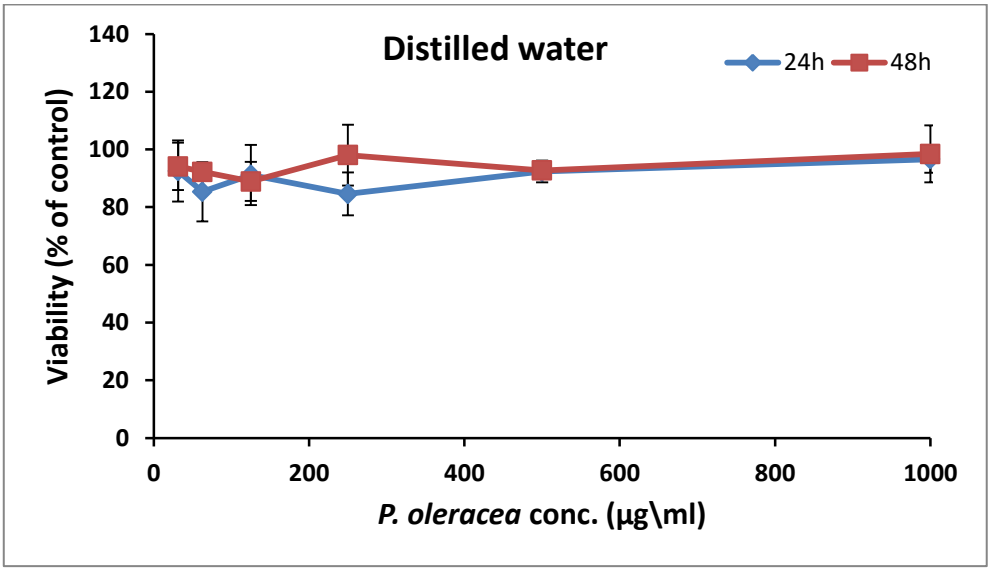

Figure (4.9): Effect of *Portulaca oleracea* distilled water extract at 24 & 48 h on Caco-2 cells. Values are expressed as mean ± SD of at least 3 independent experiments.

4.1.10 Antiproliferative effect of *P. granatum* ethanolic crude extract on Caco-2 cells

The inhibitory effect of different concentrations of *Punica granatum* ethanolic crude extract at different time points on Caco-2 cells was studied. *Punica granatum* ethanolic crude extract increased the inhibitory effect in a dose and time dependent manner (Figure 4.10).

Table (4.10): The inhibitory effect of *Punica granatum* ethanolic crude extract at 24 & 48 h on Caco-2 cells.

Conc. µg\ml	***P. granatum*** **ethanolic extract**					
	24 h			**48 h**		
	Viability (%)		**Inhibitory effect %**	**Viability (%)**		**Inhibitory effect %**
	Mean	**±SD**		**Mean**	**SD**	
6.25	100.5	4.0	-0.5	128.6	0.6	-28.6
12.5	98.9	3.2	1.1	108.8	10.3	-8.8
25	92.3	5.1	7.7	93.6	1.5	6.5
50	87.0	7.5	13.1	81.6	1.9	18.5
100	69.5	7.3	30.5	63.2	3.3	36.9
200	55.0	6.3	45.0	46.8	2.7	53.2

The inhibitory effect increased from 1.1% to 45% as the concentration of *Punica granatum* increased from 12.5 – 200 µg/ml for 24 h (Table 4.10). When the treatment time was increased to 48 h, at lower concentrations the crude extract increased the proliferations of cells while increasing the concentration to 25 µg/ml inhibited the proliferation of cells by 6.5%. The maximum inhibitory effect was at concentration of 200 µg/ml, which inhibited the growth of cells by 53.2%. The calculated IC_{50} is 125.5 µg/ml and 120.6 µg/ml at 24 h and 48 h respectively.

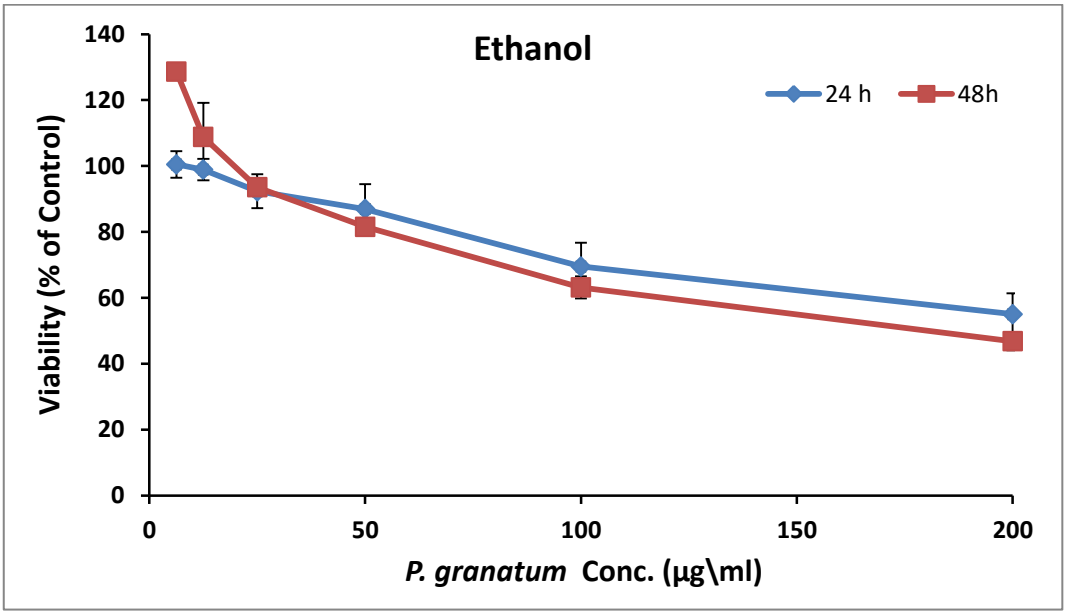

Figure (4.10): Effect of *Punica granatum* ethanolic crude extract at 24 and 48h on Caco-2 cells. Values are expressed as mean ± SD of at least 3 independent experiments.

4.1.11 Antiproliferative effect of *P. granatum* chloroformic crude extract on Caco-2 cells

The inhibitory effect of different concentrations of *Punica granatum* chloroformic crude extract at different time points on Caco-2 cells was studied (Figure 4.10). The inhibitory effect of the extract increased from 5.6% to 12.2% as the concentration increased from 6.25 – 200 µg/mL at 24 h (Table 4.11). On the other hand, increasing the time to 48 h increased the inhibitory effect from 2.8% – 24.8% as the concentration was increased from 12.5 – 200 µg/mL. The IC_{50} of *P. granatum* at 48 h is 191.7 µg/mL.

Table (4.11): The inhibitory effect of *Punica granatum* chloroformic extract at 24 and 48h on Caco-2 cells.

Conc. µg\ml	*P. granatum* chloroformic extract					
	24 h			48 h		
	Viability (%)		Inhibitory effect %	Viability (%)		Inhibitory effect %
	Mean	±SD		Mean	SD	
6.25	84.7	5.6	15.3	100.0	10.2	0.0
12.5	93.0	6.9	7.0	97.2	8.4	2.8
25	85.6	9.2	14.4	85.5	5.3	14.5
50	92.5	9.2	7.5	82.7	8.1	17.3
100	88.8	10.2	11.2	87.2	5.3	12.8
200	96.6	12.2	3.4	75.2	4.6	24.8

4.1.12 Antiproliferative effect of *P. granatum* distilled water crude extract on Caco-2 cells

The inhibitory effect of different concentrations of *Punica granatum* distilled water crude extract at different time points on Caco-2 cells was studied (Figure 4.12). As shown in Table 4.12. The crude extract increased the proliferation of cells at concentrations 31.25 – 250 µg/ml at 24 h. As the concentration was increased from 500 – 1000 µg/ml the inhibitory effect increased from 41.7% – 56.5%. On the other hand, the inhibitory effect was increased from 25.5% - 88.1% as the concentration of the extract increased from 125 – 1000 µg/ml at 48 h. The IC_{50} of the *P. granatum* distilled water extract at 24 and 48 h is 786.2 µg/ml and 232.9 µg/ml respectively.

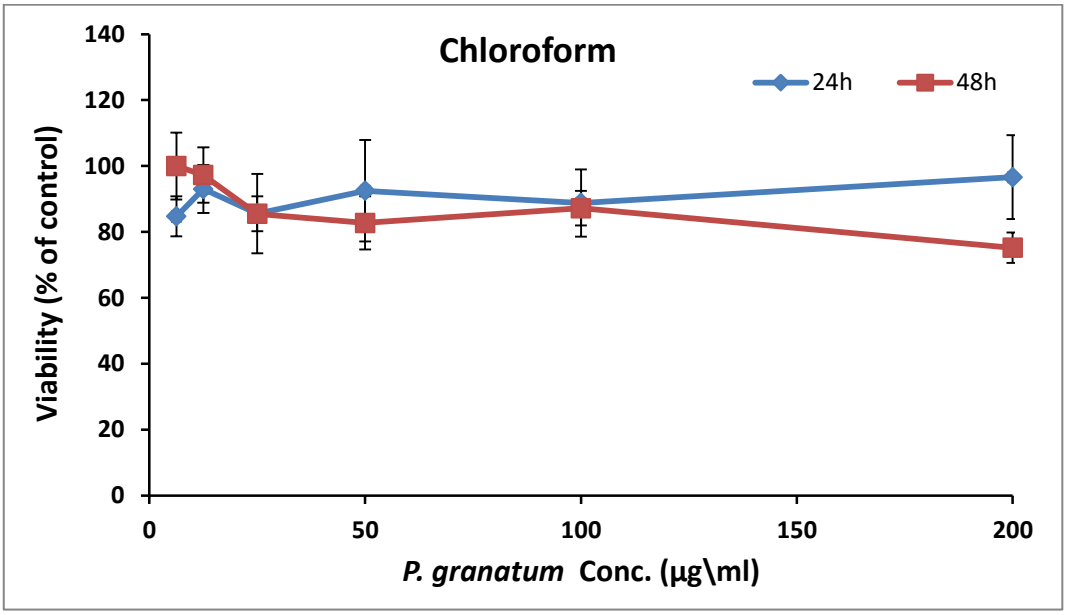

Figure (4.11): Effect of *Punica granatum* chloroformic extract at 24 and 48h on Caco-2 cells. Values are expressed as mean ± SD of at least 3 independent experiments.

Table (4.12): The inhibitory effect *Punica granatum* distilled water extract at 24 and 48 h on Caco-2 cells.

Conc. µg\ml	*P. granatum* distilled water extract					
	24 h			48 h		
	Viability (%)		Inhibitory effect %	Viability (%)		Inhibitory effect %
	Mean	±SD		Mean	SD	
31.25	121.0	17.8	-21.0	116.4	14.0	-16.4
62.5	131.1	10.1	-31.1	105.4	15.7	-5.4
125	126.9	6.9	-26.9	74.5	12.9	25.5
250	107.5	3.3	-7.5	47.4	9.6	52.6
500	58.3	7.1	41.7	18.9	2.2	81.1
1000	43.5	4.8	56.5	11.9	1.0	88.1

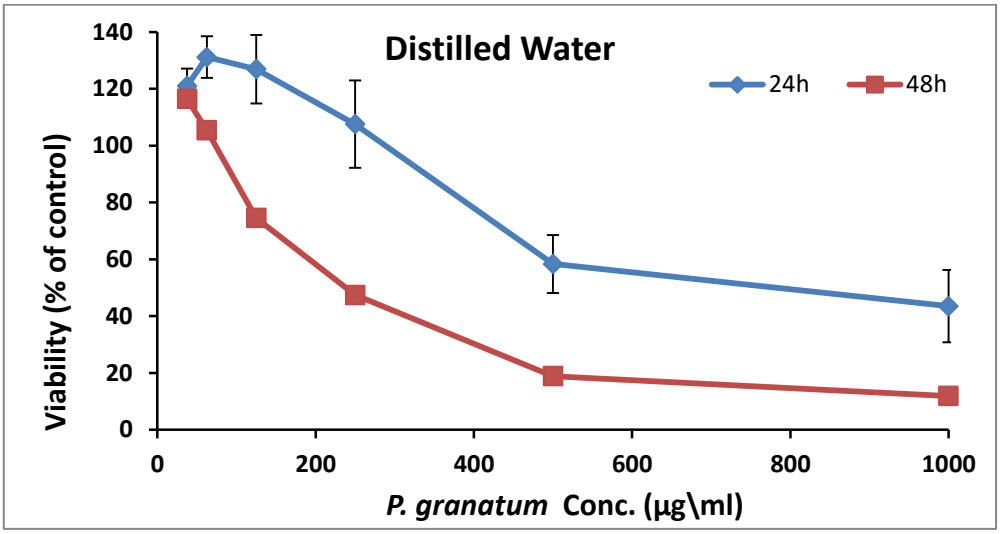

Figure (4.12): Effect of *Punica granatum* distilled water extract at 24 and 48h on Caco-2 cells. Values are expressed as mean ± SD of at least 3 independent experiments.

4.1.13 The effect of *R. officinalis* chloroformic extract and *A. sativa* ethanolic extract on normal cells (human lymphocytes)

The crude extracts with the best inhibitory effect was used to treat normal cell (lymphocytes). Table (4.13) shows that the effect of the two extracts was much lower on normal cells as compared to cancer cells (Caco-2 cells) as shown in Figure (4.13).

Table (4.13): The effect of *R. officinalis* chloroformic extract and *A. sativa* ethanolic extract on normal cells (human lymphocytes).

Conc. µg\ml	24 h					
	R. officinalis chloroformic extract			*A. sativa* ethanolic extract		
	Viability (%)		Inhibitory effect %	Viability (%)		Inhibitory effect %
	Mean	SD		Mean	SD	
6.25	90.6	0.2	9.4	81.3	0.3	18.7
12.5	97.9	0.4	2.1	83.9	0.2	16.1
25	95.8	0.5	4.2	81.6	0.1	18.4
50	99	0.1	1	82.9	0.4	17.1
100	100.4	2.6	-0.4	79.4	0.1	20.6
200	115.9	0.7	-15.9	80.5	0.3	19.5

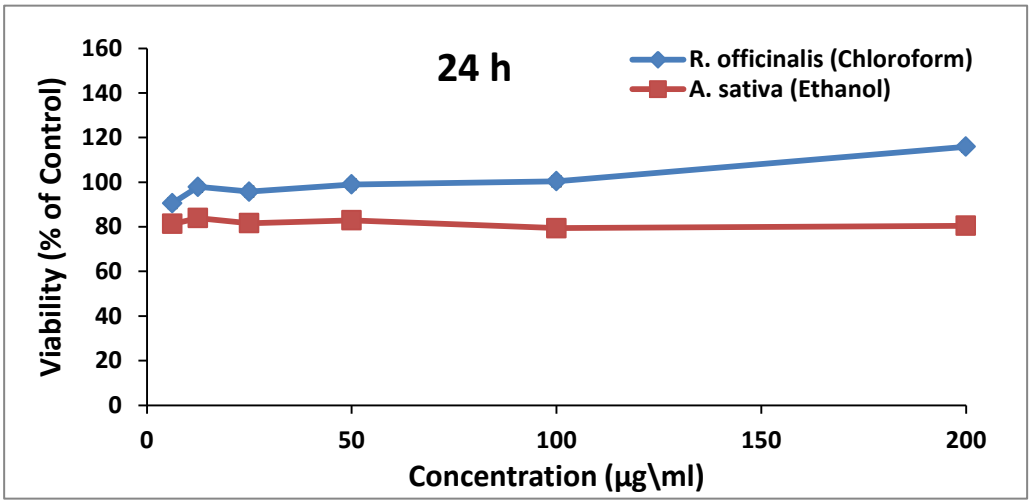

Figure (4.13): The effect of *Rosmarinus officinalis* chloroformic extract and *Avena sativa* ethanolic extract on normal cells (lymphocytes) for 24 h.

4.2 Effect of plant crude extracts on cell morphology

The Caco-2 cell line is originally derived from human epithelial colorectal adenocarcinoma cells. However, one of its most advantageous properties is its ability to spontaneously differentiate into a monolayer of cells with many properties typical of absorptive enterocytes as found in the small intestine. Caco-2 cell line is the most common and extensively characterized cell-based model for the assessment of absorption of drugs (Artursson, 1990; Rubas et al., 1996). Figure (4.14) shows the morphology of Caco-2 cells in culture as seen under the inverted microscope using 40x power.

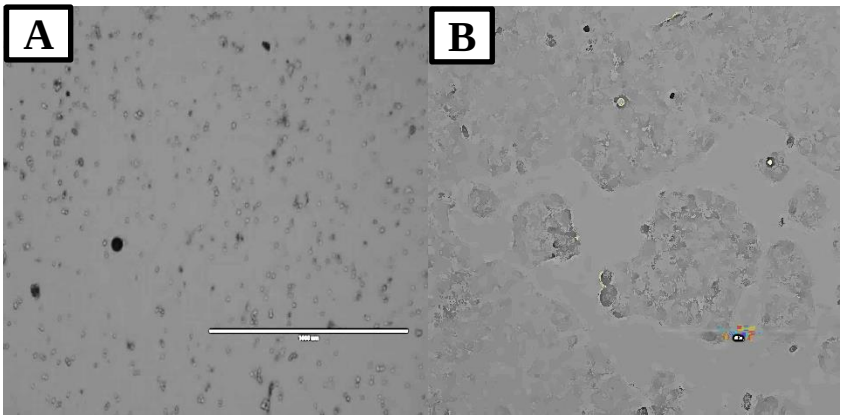

Figure (4.14): Caco-2 cells under the inverted microscope, A: Cells after subculture; B: Cells at 90% confluence.

4.2.1 Effect of treatment of different crude extracts on Caco-2 cells

As shown in Figure (4.15 A) untreated (control) Caco-2 cells, as observed under the inverted microscope, are healthy and abundant with sharp morphology, whereas, the treated cells (Figure 4.15 B to E) underwent morphological changes and intense reduction in number (which could be due to arrested growth or death by apoptosis) upon treatment with the plants extracts.

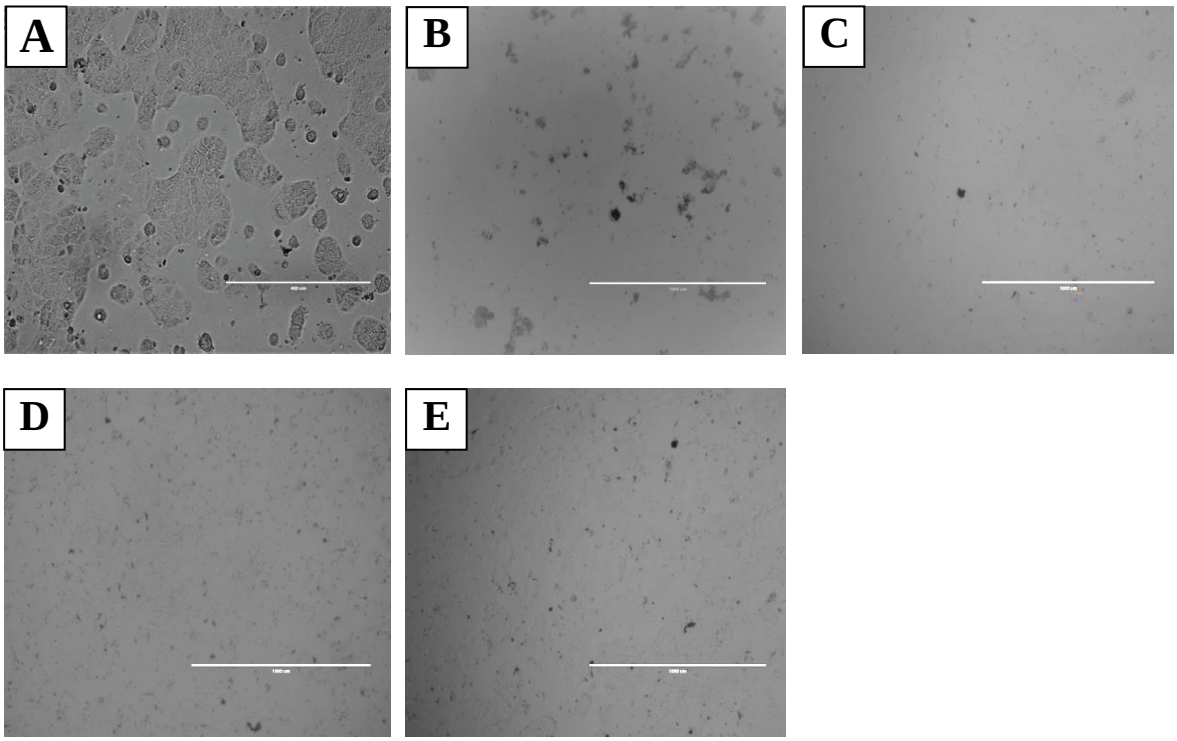

Figure (4.15): Morphological changes of Caco-2 cells after treatment with different crude extracts. A: Caco-2 control cells without treatment after 48 h of seeding; B: Caco-2 cells treated with *Rosmarinus officinalis* ethanolic extract 200 µg/ml after 24 h of treatment; C: Caco-2 cells treated with *Rosmarinus officinalis* chloroformic extract 200 µg/ml after 24 h of treatment; D: Caco-2 cells treated with *Avena sativa* ethanolic extract 200 µg/ml after 24 h of treatment; E: Caco-2 cells treated with *Avena sativa* chloroformic extract 200 µg/ml after 24 h of treatment.

4.2.2 Effect of *R. officinalis* chloroformic extract and *A. sativa* ethanolic extract on chromosomal condensation in Caco-2 cells

Changes in nuclear morphology and cell distribution were examined by using DAPI stain observing the cells under the inverted microscope. Figure (4.16 A) illustrates the untreated control cells whereas (Figure 4.16 B and C) indicate the cells after 24 h treatment with *R. officinalis* chloroformic extract and *A. sativa* ethanolic extract, respectively.

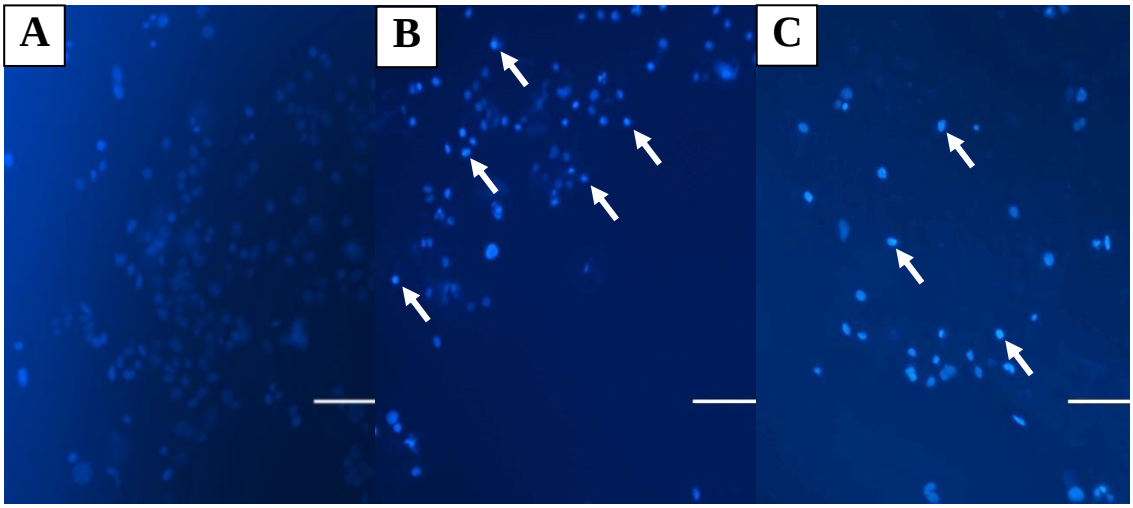

Figure (4.16): Effect of different crude plant extract on nuclear condensation of Caco-2 after 24 h. Cells were seeded on coverslips as described in materials and methods and after 24 treated with different crude plant and left for 24 h. Cells were then stained using DAPI stain, mounted on a slide using mounting medium and observed under the fluorescence microscope. A: control Caco-2 without treatment; B: Caco-2 treated with 200 µg/ml of *Rosmarinus officinalis* chloroformic extract; C: Caco-2 treated with *Avena sativa* ethanolic extract 200 µg/ml concentration, magnified 400 times. White arrows indicate cells with nuclear condensation.

4.3 Combination studies

4.3.1 The antiproliferative effect of *R. officinalis* chloroformic extract and *A. sativa* ethanolic extract combined with the chemotherapeutic drug 5-FU

The interaction effect of the two crude extracts with 5-FU on the proliferation of Caco-2 cells was assessed. Crude extracts at different concentrations were combined with different concentrations of the 5-FU. The concentrations used for crude plant extracts were (0, 6.25, 12.5, 25, 50, 100 & 200 µg/ml) and those for 5-FU were (0, 1.625, 3.25, 6.5, 13, 26 & 52 µg/ml). The inhibitory effects data were analyzed by using the CompuSyn software to determine the type of interaction which occurred between *R. officinalis* chloroformic extract and *A. sativa* ethanolic extract combined with 5-FU chemotherapeutic agent individually.

The combination index (CI) value, fraction affected (Fa), dose response index (DRI) and single dose concentrations were calculated by using CompuSyn software. A CI value > 1 means antagonism; CI = 1 means additive effect and CI < 1 means synergism (Table 4.13). Fraction affected (Fa) indicates the fraction of cells affected after treatment.

4.3.1.1 The inhibitory effect of *R. officinalis* chloroformic extract combined with 5-FU chemotherapy

Different concentrations of *R. officinalis* chloroformic extract were combined with different concentrations of 5-FU at a fixed ratio of 3.85:1 (Table 4.14). The concentration that killed 50% of the cells of each drug alone is 98 and 249 µg/mL for *R. officinalis* and 5-FU, respectively.

The concentration was reduced to a combination dose of 52.2 µg/mL of both drugs at combination ratio 3.85:1 of both *R. officinalis* and 5-FU respectively. The dose reduction index is 2.4 and 23 times for *R. officinalis* and 5-FU, respectively (Figure 4.17). The combination index (CI < 1) was synergistic at all combinations (Figure 4.18).

Table (4.14): The inhibitory effect of *Rosmarinus officinalis* combined with 5-FU on Caco-2 cells after 24 h treatment.

	Single Dose Conc. (µg/ml)		***R. officinalis* combined 5-FU (3.85:1)**				
Fa	***R. officinalis***	**5-FU**	**CI**	**Comb. Dose (µg/ml)**	***R. officinalis* + 5-FU**	**DRI**	
						R. officinalis	**5-FU**
0.25	46	46	**0.42**	19.15	15.17 + 3.83	3	12
0.5	98	249	**0.46**	52.2	41.76 + 10.44	2.4	23
0.75	212	1345	**0.55**	142.2	113.76 + 28.44	2	46
0.9	459	7270	**0.68**	387.6	310.08 + 77.52	1.5	91

Combination Index (CI) < 1, = 1, > 1 indicates synergism, additive effect, and antagonism, respectively; DRI: The dose-reduction index; Fa: fraction affected.

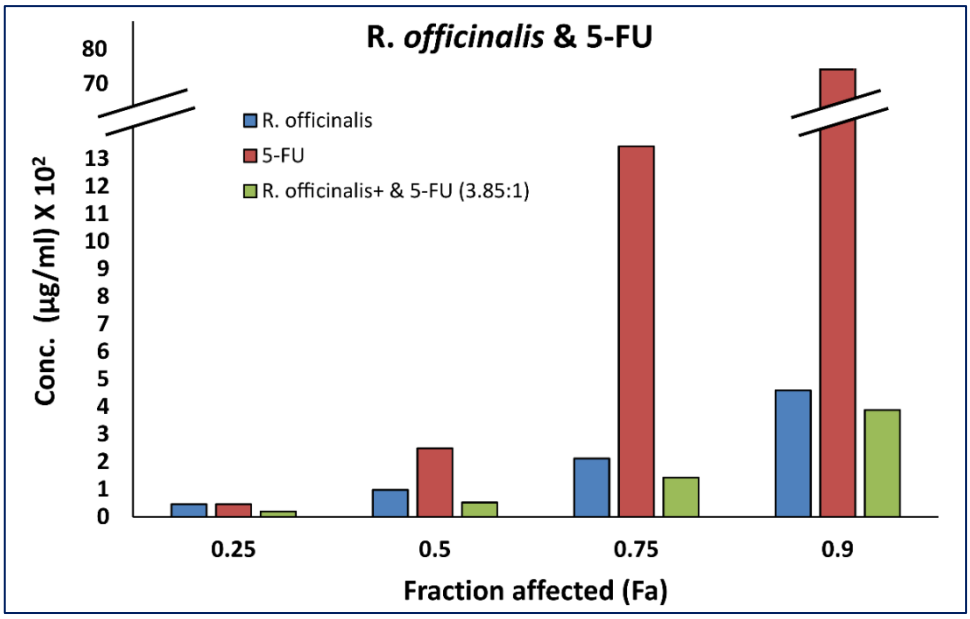

Figure (4.17): A diagram representing the decrease in the total concentration of *Rosmarinus officinalis* and 5-FU when used in combination as compared to single doses of *Rosmarinus officinalis* and 5-FU at different Fa ratios.

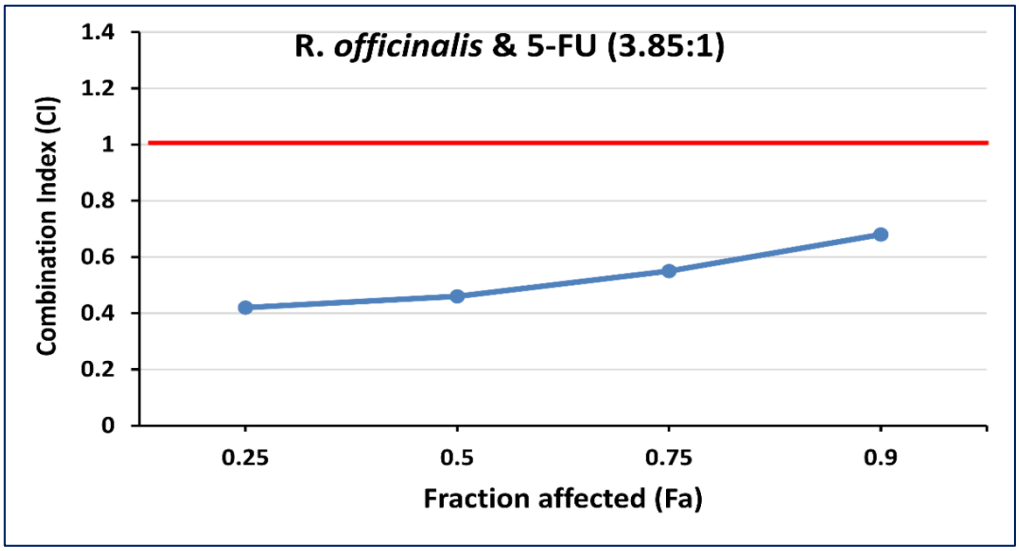

Figure (4.18): The CI values of different combination ratios of *Rosmarinus officinalis* and 5-FU plotted against different Fa values. CI < 1, = 1, > 1 indicates synergism, additive effect, and antagonism, respectively.

4.3.1.2 The inhibitory effect of *A. sativa* ethanolic extract combined with 5-FU

Different concentrations of *A. sativa* ethanolic extract was combined with different concentrations of 5-FU at fixed ratio which is 3.85:1 (Table 4.15). The concentration that killed 50% of the cells of each drug alone is ~ 122 and 249 µg/mL for *A. sativa* and 5-FU, respectively. The combination dose was reduced to ~ 86 µg/mL. The dose reduction index is 1.8 and 14 times for *A. sativa* and 5-FU, respectively (Figure 4.19). The combination index (CI < 1) was synergistic at all combinations (Figure 4.20).

Table (4.15): The inhibitory effect of *Avena sativa* ethanolic extract combined with 5-FU on Caco-2 cells upon 24 h treatment.

	Single Dose Conc. (µg/ml)		*A. sativa* combined 5-FU (3.85:1)				
Fa	***A. sativa***	**5-FU**	**CI**	**Comb. Dose (µg/ml)**	***A. sativa* + 5-FU**	**DRI**	
						A. sativa	**5-FU**
0.25	16.3	46	**0.57**	10.8	8.64 + 2.16	2	21
0.5	121.9	248.6	**0.63**	85.8	68.64 + 17.16	1.8	14
0.75	912	1345	**0.7**	682	545.6 + 136.4	1.7	9.6
0.9	6820	7270	**0.78**	5422	4337.6 + 1084.4	1.6	6.5

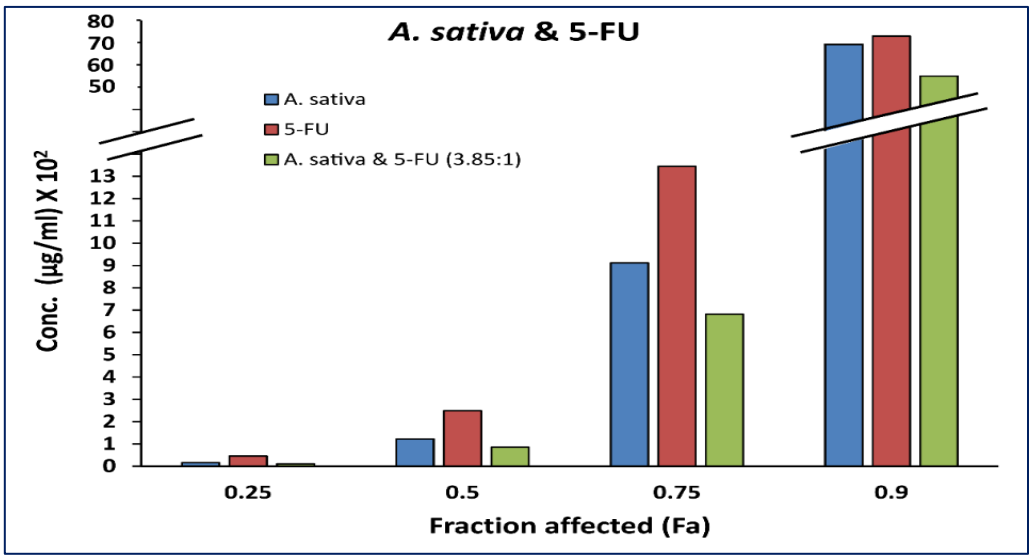

Figure (4.19): A graph representing the decrease in the total concentration of *Avena sativa* and 5-FU when used in combination as compared to single doses of *Avena sativa* and 5-FU at different Fa ratios.

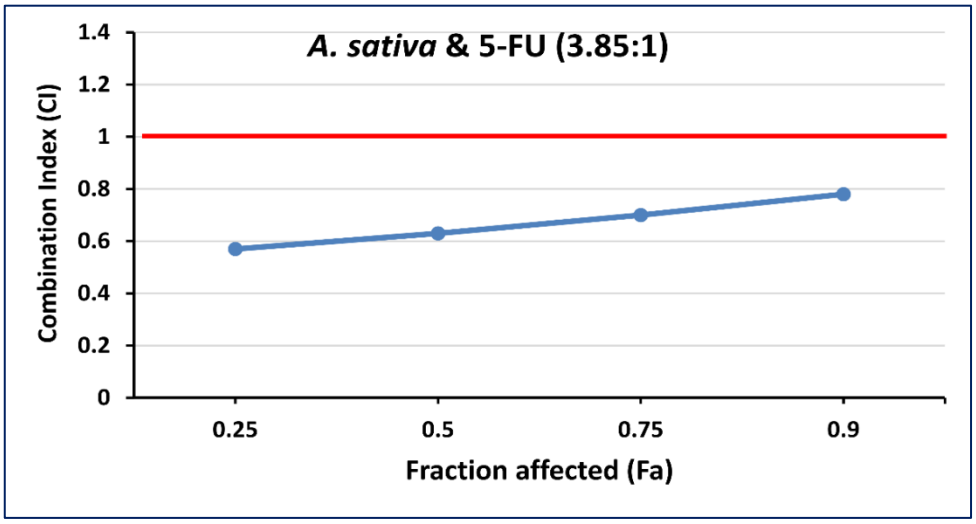

Figure (4.20): The CI values of different combination ratios of *Avena sativa* and 5-FU plotted against different Fa values. CI < 1, = 1, > 1 indicates synergism, additive effect, and antagonism respectively.

Chapter V
Discussion

Chapter V
Discussion

Cancer is among the leading causes of death worldwide. Research is conducted every day globally to find an effective and safe treatment for cancer. There is a remarkable widespread interest in recent years for verification and investigation of the properties and the biologically active substances of plants products that can be used as anti-cancer agents. There is a great need to examine reliable and inexhaustible sources of natural substances. This interest is due to the fact that conventional cancer treatments (chemotherapy, surgery and radiotherapy) are ineffective in some cases due to their resistance mechanisms and cause very serious side effects due to its high toxicity. There is considerable confidence and clarity in the safety of natural products, which contributed to the popularity of traditional medicine.

One reason for choosing plants is that they are readily available (Tandon & Yadav, 2017), and play important role in human life and animals (Samie, Obi, Bessong, & Namrita, 2005). Although, there are around half million plants around the world, medical activities of most of these plants have not been investigated yet (Patwardhan, Warude, Pushpangadan, & Bhatt, 2005). Only 15% of those plants that have been effectively investigated were found to be useful. Consequently, 85% of higher plants should be investigated for their anticancer effects. Some of the plants which have been investigated were found to be useful and are being used for curative purposes. More than one thousand plants have been found to possess significant anticancer properties (Neelima, Sudhakar, Patil, & Lakshmi, 2017).

During this study, four plants were collected from different agricultural areas from Gaza strip. The extracts of these plants were obtained by Soxhlet apparatus by using three solvents for extraction namely ethanol solution (70%), chloroform and distilled water. All extracts were obtained after filtration and evaporation. Crude extracts of the plants may have mixtures of active compounds which act synergistically and their overall bioactivity is usually greater than individual compounds (Céspedes, 2013).

Extraction of active compounds from plants depends on many factors including: the geographical origin, the plant variety, the ecological characteristics (Jamshidi, Afzali, & Afzali, 2009; Zaouali, Bouzaine, & Boussaid, 2010; Ojeda-Sana, van Baren, Elechosa, Juárez, & Moreno, 2013). Additionally, the method of extraction and nature of the solvents used in the extraction (Bousbia et al., 2009). Other factors include the harvest time (McGimpsey, Douglas, Van Klink, Beauregard, & Perry, 1994), drying method, soil conditions and storage conditions (Bernard et al., 2014). All these factors may contribute to the differences in the anticancer effects which can be obtained during the investigation of different plant extracts.

The present study objectives were to assess the antiproliferative effects of the *R. officinalis*, *A. sativa*, *P. granatum* and *P. oleracea* extracts on Caco-2 cells. In addition, investigating the combination effect of 5-FU with the plant extracts: *R. officinalis* (chloroform extract) or *A. sativa* (ethanol extract) on the proliferation of Caco-2 cells.

MTT assay was used to evaluate the antiproliferative effect of the plants extracts on both Caco-2 cells and normal lymphocyte cells (Mosmann, 1983). The assay measures the activity of living cells via mitochondrial dehydrogenases. The cleavage of the tetrazolium salt MTT (3- (4,5dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) into a blue colored product (formazan) by mitochondrial dehydrogenases is potentially very useful for assaying cell survival and proliferation. The conversion takes place only in living cells and the amount of intracellular formazan produced, which can be measured spectrophotometrically after dissolving it, is proportional to the number of viable cells present.

5.1 Antiproliferative activity of ethanolic crude plants extract on Caco-2 cells

The results of the present study showed that the ethanolic crude extracts of the different used plants have different antiproliferative effects. The highest antiproliferative effect at 24 h was that of *A. sativa* followed by *R. officinalis* and then *P. granatum*. While, *P. oleracea* showed low antiproliferative effects on Caco-2 cells. The calculated IC_{50} at 24 h for *A. sativa*, *R. officinalis* and *P. granatum* are 67, 98 and 125.5 µg/ml respectively.

Different studies have investigated the effect of ethanolic extracts of *A. sativa*, *R. officinalis*, *P. granatum* and *P. oleracea* on different cell lines. *R. officinalis* crude extract has been studied for its antitumor activities both in-vivo and in-vitro against different cancer cell lines and a normal cell line (mouse embryonic fibroblasts) (Horváthová, & Robichová, 2002; Bai et al., 2010; Rashid et al., 2011; Petiwala & Johnson, 2015; Varoni, Faro, Sharifi-Rad, & Iriti, 2016).

The results of our study are in agreement with a study of Cheung et al (2007) which showed that *R. officinalis* ethanolic crude extract has antiproliferative effect on human leukemia and breast cancer cell lines (Cheung & Tai, 2007; Amar et al., 2017). In addition, the results in the present study are in agreement with those of Moore et al (2016) which showed that the *R. officinalis* ethanolic extract drastically decreased colony formation of Caco-2 (Moore, Yousef, & Tsiani, 2016). Yi & Wetztein (2011) achieved that the rosemary ethanolic crude extract decreased cell growth of different colorectal cancer cell line such as SW620 and DLD-1 at a concentration of 31.25 µg/mL and IC_{50} around 25 µg/mL (48 h) (Yi & Wetzstein, 2011). In the present study, the IC_{50} of the *R. officinalis* ethanolic crude extract was 98 µg/ml at 24h and 24.7 µg/ml at 48 h which agrees with those of Yi & Wetztein (2011).

This study provides evidence that extract of *R. officinalis* possesses significant antitumor activities against the colorectal cancer cell line (Caco-2). This anticarcinogenic activity of *R. officinalis* may be due to the major bioactive compounds such as phenolic compounds, flavonoids, carnosic acid, rosmarinic acid,

and carnosol (Fernández-Ochoa et al., 2017). Flavonoids, a subclass of polyphenols, are phytochemicals which have been reported in many research for their significant contribution in the reduction of cancer risk and also in cancer therapy (Singh, Dhanalakshmi, & Agarwal, 2002; Scalbert, Johnson, & Saltmarsh, 2005).

Our results are in consistence with the results of a study by Sato et al (2016). They investigated the anti-proliferative activities of *A. sativa* against different cancer cell lines like colon (HCT116), lung (NCI-H460) and breast (MCF7). Ethanol extracts indicated higher anti-proliferative activities against HCT116 with inhibition (69.5%), NCI-H460 (75.2%), and MCF7 (84.8%) cells compared with other extracts like methanol and acetone, and ethyl acetate extracts (Sato et al., 2016). In another study which determined the phenolic contents and investigated the activities of *A. sativa* on growth of HepG2 cell line, the results indicated that the *A. sativa* ethanolic extract have antiproliferative and antioxidant capacity (Chen et al., 2017).

The results of the present study showed that *P. oleracea* has low antiproliferative effects on Caco-2 cells. Several studies have been conducted using the ethanolic crude extract of *P. oleracea*. The studies showed that the *P. oleracea* exerts cytotoxic, apoptotic, antiproliferative and cell cycle arrest effects against several cancer cell lines including murine mammary adenocarcinoma (AMN3), human breast adenocarcinoma cells (MCF-7) and RD, HepG2 (Zakaria & Hazha, 2013; Al-Sheddi et al., 2014; Farshori et al., 2014).

Khatibi et al (2016) reported that the *P. oleracea* ethanolic extract has antitumor activity and significantly reduced the survival and the growth rate of HeLa cell line in a dose and time dependent manner after 24 and 48 hours (Khatibi, Taban, & Roushandeh, 2016). In our study the inhibitory effect of ethanolic crude extract of *P. oleracea* on Caco-2 cells was low (5% to 12%) at different used concentrations (6.25 to 400 µg/mL). This may be due to the higher concentrations (700, 1000, 1200, and 1500 µg/mL) of ethanolic extract of *P. oleracea* , and the percentage of the solvent that was used (80% ethanol) by khatibi et al (2016). In addition, different crude extracts may exert different effects on different cell lines. As we mentioned

before many other factors are also involved in the inhibitory effect of different plant extracts of the same plant. Therefore, the difference in the results may be due to one or more of these factors.

Our results showed that ethanolic crude extract of *P. granatum* peel has antiproliferative effects on Caco-2 cells. A study performed by Malik and Mukhtar (2006) showed that *P. granatum* peel inhibit the growth of human prostate cancer cells (PC3) and MCF-7 in a dose dependent manner (Malik & Mukhtar, 2006). In another study by Khan et al (2007), they found that 50, 100, and 200 µg/mL of the *P. granatum* peel ethanolic crude extract decrease the human lung carcinoma (A549) cell line viability at rates of 33%, 44%, and 47% (Khan, Afaq, & Mukhtar, 2007). This result agrees to a certain degree with our results which showed that 200 µg/ml of the extract decreased the viability of Caco-2 cells to 45%.

5.2 Antiproliferative activity of plant chloroformic crude extract against Caco-2 cell line

The results of the present study showed that the chloroformic crude extracts of the different used plants have different antiproliferative effects. The highest antiproliferative effect at 24 h was that of *R. officinalis* followed by *A. sativa* and then P. granatum. While, *P. oleracea* showed low antiproliferative effects on Caco-2 cells. The calculated IC_{50} at 24 h for *R. officinalis* and *A. sativa* are 77 and 98 µg/ml respectively. While the calculated IC_{50} of *P. granatum* at 48 h is 191.7 µg/ml.

Concurred with these results, one previous study by Choi et al (2009) which investigated the effect of *R. officinalis* on the inhibition of growth of Hela, HepG2, non-small cell lung cancer such as A549, AGS cells and HT-29 cells. The results showed that the hexane and chloroform fractions of rosemary have cytotoxic effect which are related to the activity of the essential oil in the rosemary (Choi, Kim, & Lee, 2009).

Despite intensive literature search we couldn't find any published reports regarding to chloroformic extract of *A. sativa*, therefore to the best of our knowledge

this is the first study that investigated the anticancer activity against Caco-2 cell line, it is noticeable from the results we obtained that *A. sativa* chloroformic extract significantly reduced the proliferation of Caco-2.

The results of the present study showed that the rate of inhibition of the chloroformic crude extract of *P. oleracea* ranged from 19.3% to 28.3% at different used concentrations (6.25 – 400 µg/mL) at 48 h. The results of Mali (2015) are in agreement with our results which showed that the chloroformic crude extract of *P. oleracea* was less efficient or does not have cytotoxic activity against human colon adenocarcinoma cell line (Mali, 2015).

In another study, Rather et al (2010) studied the effect of *P. granatum* peel chloroformic extracts as antioxidants and anticancer agents against Hep-2 and MCF-7. The results showed that the anticancer activity of the extract is low at the used concentrations (10, 50, 100, 150 µg/ml) (Rather, Swetha, & Rajagopal, 2010). These results are also in full agreement with our result on Caco-2 cells.

5.3 Antiproliferative activity of plant distilled water crude extract against Caco-2 cell line

The results of the present study showed that the antiproliferative effects of distilled water crude extract was low in all used plants extracts except for *P. granatum.*

Some Chinese herbs and plants have been found to have deleterious effects on certain cancer cell lines, and has been long known to induce inflammation and carcinogenesis in different types of cells. As reported in study by Vito (2012) the pure ingredients of some herbs extracts were found to have significant proliferative activity when human embryonic kidney cells were used (HEK-293) (Vito, 2012). It has also been reported that some herbal extracts can stimulate cancer cell proliferation such as MCF7 cells (Niu, Zhan, Wang, Chen, & Ma, 2007).

Distilled water plant extract contains different components including anthocyanins, starches, tannins, saponins, lectins and others. Some of these components e.g. lectins can induce the proliferation of normal and cancer cells in cell

culture. Lectins can bind to cell receptors acting as a growth factor, modulating the receptor leading to increase in cell division. The results of some studies showed that some dietary lectins has a mitogenic effect on the cells of normal colon and colorectal cancer cells (Valentineri, Fabiani, Schumacher & Leathem, 2003). Therefore, the components of the plant extract and how they also interact with each other give the final effect of the crude plant extract which is either stimulatory or inhibitory proliferation effect.

R. officinalis distilled water crude extract increased the proliferation of Caco-2 cells differently at different used concentrations for 24 h instead of inhibiting its proliferation. On the other hand, *A. sativa* distilled water crude extract increased the proliferation of Caco-2 cells differently at different used concentrations for 24 h and 48 h. While, the maximum inhibitory effect of *P. oleracea* was 15.5% at 250 µg/mL at 24 h and 11.1% at 125 µg/mL at 48 h. In contrast, the IC_{50} of the *P. granatum* distilled water extract at 24 and 48 h is 786.2 µg/ml and 232.9 µg/ml respectively.

In one study performed by Salih et al (2015) on the effect of *R. officinalis* on RD cells. They demonstrated that the aqueous extracts of *R. officinalis* exerted cytotoxic and anti-tumor effects in a dose and time dependent manner. The inhibitory effect was 10-15% at different used concentrations (50 -100 -250 -500 -1000 µg/mL) after 48 h. However, our study showed slightly higher antiproliferative effect against Caco-2 which was from 6-19% after 48 h (Salih et al., 2015).

As reported in the study performed by Chen et al (2010) the water-soluble polysaccharides that were isolated from *P. oleracea* aqueous crude extract have a suppressive effect on the growth of HeLa and HepG2 cells in vitro (Chen et al., 2010). These results are different to the results of our study which showed that *P. oleracea* extract has low antiproliferative activity against Caco-2. This difference may be due to extraction method and other factors which were mentioned earlier in the discussion.

A study done by El Awady et al (2015) showed that the *P. granatum* peel aqueous crude extract has antitumor activity in dose dependent manner against two cancer cell lines, which was Colon cancer (Caco-2) and Hepatocellular carcinoma

(HepG2) cell line. The results of this study in Caco-2 cell line were fairly close to our results and agreement with it. When El Awady et al (2015) using low concentration 10 μg/mL the viability of cells was 96% and after increasing the concentration to 1000 μg/mL the viability decrease to reach 3% (El-Awady, Awad, & El-Tarras, 2015). Also other different studies showed the same results and conducted to evaluate the anticancer effect of pomegranate extracts against different cancerous cell lines (Motaal & Shaker, 2011; Aqil et al., 2012).

5.4 Effect of *R. officinalis* chloroformic extract and *A. sativa* ethanolic extract on normal cells

The successful treatment returns to drugs and material which exhibits its therapeutic effects without causing any serious side effects, or toxicity to organs or to any part of the body cells (Tripathi et al., 2017). Therefore, it is not sufficient to judge on treatment activity without assessing its toxicity against normal cells. If we cannot rule out cellular toxicity or at least reduce it, we cannot consider this drug to be a successful therapeutic drug.

In our study the extract of *R. officinalis* chloroformic and *A. sativa* ethanolic crude extract has showed a dose dependent inhibitory effect on Caco-2 cell line with an IC_{50} value of 77 μg/ml, and 67 μg/ml respectively. Its inhibitory effect on normal lymphocyte cells was much lower compared to cancer cells, this finding means that *R. officinalis* chloroformic and *A. sativa* ethanolic crude extracts at certain concentrations was significantly more potent to cancer cells than normal cells.

5.5 Effect of different crude plants extracts on nuclear condensation

The morphological results of the present study show that the different used ethanolic and chloroformic extracts show various degrees of inhibitory effect on Caco-2 cells. To further study the effect of these extracts on the induction of apoptosis in Caco-2 cells we used the DAPI stain. This will also discriminates between cytotoxic and cytostatic effects of the extracts. DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) is a nucleic acid stain that binds to A-T rich regions

of DNA along the minor groove. DAPI is predominantly impairment to live cells, allowing it to be used as a viability dye in unfixed cells to discriminate intact from membrane-compromised cells.

In our study, the nuclei of the control cells were stained uniformly blue; and the size of nuclei was similar, with some cells undergoing mitosis. After the treatment of Caco-2 cells with 200 µg/ml of *R. officinalis* chloroformic extract and *A. sativa* ethanolic extract, the chromatin of some cells was condensed showing a smaller and a brighter nuclei. This chromatin condensation is an important feature of apoptosis. Therefore, the results indicate that apoptosis may be induced by the use of these two extracts but further studies should be performed to confirm this conclusion.

5.6 The antiproliferative effect of *R. officinalis* chloroformic extract and *A. sativa* ethanolic extract combined with the chemotherapy 5-Fluoruracil (5-FU)

The concept of combination chemoprevention seeks to increase the chemo preventive effectiveness of agents, while decreasing toxicity by dose reduction (Thompson, Meeker, & Becci, 1981). In this study, we examined the effects of combining *R. officinalis* chloroformic extract and *A. sativa* ethanolic extract with the chemotherapy 5-FU that is used as colorectal cancer treatment in Palestinian Ministry of health.

We found that the CI indicates a synergistic effect at combination ratio 3.85:1 of both *R. officinalis* chloroformic extract and 5-FU respectively. When the concentration that killed 50% of the cells of each drug alone is 98 and 249 µg/mL for *R. officinalis* and 5-FU, respectively. These results are in agreement with the result obtained by González et al (2013), who investigated the anti-tumor effect of 5-FU which enhanced by rosemary (*R. officinalis*) extract in both drug resistant and sensitive human colon cancer cells SW620 and DLD-1. The results indicate that the *R. officinalis* sensitizes the 5-FU resistant cells to therapeutic activities of this drug and there was a synergistic effect between 5-FU and rosemary extract. These effects were related to the modulation of the gene expression of thymidylate synthetize

(TYMS) and thymidine kinase 1 (TK1), which are enzymes involved in the mechanism of resistance of 5-FU (González-Vallinas et al., 2013).

In addition, *A. sativa* ethanolic extract indicates a synergistic effect at combination ratio 3.85:1 when combined with 5-FU, although there were no previous results for this, but the results we have obtained are promising.

Verma (2014) stated that "cancer is considered as a complicated disease with complex processes and requires a multi-target therapeutic approach to battle it" (Verma, 2014). The traditional use of one drug protocol for treatment of cancer is becoming increasingly ineffective. Currently, many research groups use combination therapy, which contain either two or more drugs for treatment of cancer (Yang et al., 2009). This combination of anticancer agents may cause synergism, additive or antagonistic effects. The pure natural or synthetic drugs used in western medicine usually affects a single target. On the other hand, crude extracts of natural products contain many components, in various combinations and formulations, aimed at multiple targets. This combination therapy contains many bioactive components which can target different cell cycle regulators causing cell cycle arrest and induce cell death mechanisms in cancer cells. Further studies are needed to determine the mechanisms involved in the synergistic effect.

Chapter 6
Conclusions and Recommendations

Chapter VI
Conclusions and Recommendations

6.1 Conclusions

In conclusion, in this study the in vitro antiproliferative and cytotoxic effects of *R. officinalis*, *A. sativa*, *P. granatum* and *P. oleracea* extracts on human colon cancer Caco-2 cell line were assessed. In addition, the inhibitory effects of *R. officinalis* chloroformic, and *A. sativa* ethanolic extract on normal peripheral blood lymphocyte cells were evaluated.

According to the findings of this study, the following conclusions could be drawn:

- The present study found that both extracts chloroformic *R. officinalis* and ethanolic *A. sativa* have concentration dependent antiproliferative effect on Caco-2 cell line. These extracts are selective towards cancer cells than normal cells. *R. officinalis* ethanolic extract has also promising results for its ability to inhibit cell growth of Caco-2.
- *P. granatum* chloroformic extract, *A. sativa* aqueous extract and *P. oleracea* ethanolic, aqueous and chloroformic crude extracts did not exhibit any antiproliferative effects towards Caco-2 cells at the applied concentrations and exposure time. As a result, it is unpredicted that these extracts may possess significant therapeutic characteristics for colorectal cancer.
- *P. granatum* ethanolic and aqueous extracts have antiproliferative effects against Caco-2 cells at the applied concentrations and exposure time. This important finding gives a hope that *P. granatum* ethanolic and aqueous extract active materials can be useful in colorectal cancer treatment development.
- On the other, hand *R. officinalis* chloroformic extract and *A. sativa* ethanolic extract show synergetic effect when combined with 5-FU chemotherapeutic agent at different concentrations and fixed ratio of 3.85:1 and can be considered as new strategy for treatment and development of new anticancer agent.

6.2 Recommendations

In light of the above conclusions and based on the results of this study, the following recommendations are suggested:

1. More research should be conducted using the different tested extracts on other types of cancer using different cancer cell lines.
2. Stability studies for plants extracts will be of value before any further testing.
3. Further investigations in animal models to determine plant extract toxicity (LD50) *in vivo*.
4. It is recommended that plant extracts which have high antiproliferative effects to be further studied to check their efficiency *in vivo* and in normal colorectal cells.
5. Molecular investigations of the combination studies should be further studied to determine the mode of death of the cells.
6. The combination effect of the extracts with other chemotherapeutic agents should be tested.
7. Palestine has a unique flora which were not fully explored for their biologically active components, which may represent a cheap resource for future medicine. Therefore, more research should be conducted to determine the Flora with medicinal effects.
8. More research should be conducted to determine the different bioactive components in the most effective extracts.

Chapter VII

References

Chapter VII

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