An-Najah National University
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In vitro evaluation of the apoptotic and antimitotic (cytostatic) effects of *Arum palaestinum* and *Peganum harmala*

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Dedication

To My parents …

To my patient wife…

To my brothers and sisters …

To my sweet kids (Fatima, Shaima and Mahdi) …

I present this work.

Acknowledgments

First of all, I would like to express my sincere appreciation to my supervisors, Dr. Ashraf Sawafta (An-Najah National University) and Dr. Hilal Zaid (Arab American University) for their guidance, help and encouragement throughout research work and writing.

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الاقرار

انا الموقع ادناه مقدم الرسالة التي تحمل العنوان:

In vitro evaluation of the apoptotic and antimitotic (cytostatic) effects of *Arum palaestinum* and *Peganum harmala*

التقييم المخبري لنباتي الحرمل واللوف الفلسطيني على تحفيز الموت المبرمج وتثبيط انقسام الخلايا السرطانية

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List of Abbreviations

A549	Lung cancer cell line
AO	Acridin Orange
A. palaestinim	*Arum palaestinum*
AV	Annexin V-CY3
CAM	Complementary and alternative medicine
DMEM	Dulbecco's modified Eagle's medium
FDA	US food and drug administration
HCl	Hydrochloric acid
HCT	Colon cancer cell line
IC50	Lethal dose which kill 50% of cells
L6	Muscle normal cell line
LDH	Lactate dehydrogenaze
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
NCEs	New chemical entities
OD	Optical density
PC3	Prostate cancercell line
P. harmala	*Peganum harmala*
PS	Phosphotydilseren
WHO	World health organization
µg	Microgram
µl	Microliter

In vitro evaluation of the apoptotic and antimitotic (cytostatic) effects of *Arum palaestinum* and *Peganum harmala*

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Abstract

The incidence of cancer is increasing in the developed countries and even more so in developing countries parallel to the increase in life expectancy. Cancer is a result of an accelerated and uncontrolled cellular proliferation and low rate of apoptosis (programmed cell death) leading to an increasing mass of cells termed as tumor. Mitochondria play a crucial role in the induction of this apoptosis. It is involved in the release of apoptogenic intermediates such as cytochrome c from the intermembrane space. These apoptogenic intermediates appear to play a central role in initiation of a cascade that leads to programmed cell death . Advanced tumors are treated usually by chemotherapy and although these drugs are effective, they are associated with severe adverse events and drug resistance. Several studies have revealed that natural products exhibit an extensive spectrum of biological activities such as stimulation of the immune system, antibacterial, antiviral, anti-hepatotoxic, anti-ulcer, anti-inflammatory, antioxidant, anti-mutagenic, anti-cancer effects, as well as apoptosis induction. The traditional Arab-Islamic herbal-based medicines might be promising candidates for new cancer therapeutics, especially natural herbal products with low toxicity and minimal side effects. Two

medicinal plants were selected to investigate their anti-cancer effect: *(Arum palaestinum* and *Peganum harmala* (AP and PH). Three cancer cell lines: Colon, Prostate and Lung (HCT-116, PC3, A549) and one normal (control) cell line (skeletal muscle, L6) were selected to test the efficacy of AP and PH in apoptosis induction. The cells were treated with an increasing concentration of 50%water/50% ethanol plant extracts (0 , 8, 16, 32, 62, 125, 250, 500 and 1000 µg/ml) for 24h. Then we used MTT assay to test cytotoxicity of the extracts and Annexin V-Cy3 to test apoptosis. Results shows that *Peganum harmala* has non-toxic effect on all treated cell lines at concentrations less than 250 µg/ml. However, it had induced apoptosis in prostate cancer cell line (PC3), muscle cell line (L6), and lung cancer cell line (A549). Surprisingly, *Arum palaestinum* had no cytotoxic or apoptotic effect in all selected cell lines, even at 1000 µg/ml.

Chapter One

Introduction

Chapter One

1. Introduction

1.1 General background

Cancer is a major health problem in the world. In many countries, Cancer is one of the common and considered as the second leading cause of death after heart diseases in the world. More than 20% of all deaths among the world's population is due to cancer (1, 2, 3). Cancer affects both developing and developed countries. In 2004, half of the 10 millions of cancerous people were in the developed countries (4). For example, among the cancer patients in the USA, the use of complementary and alternative medicine, represented mainly by plants, ranges between 30-75% (5). This in turn justifies the interest in search of possible anticancer agents from the flora of different countries.

The body maintains a system of checks and balances on cell growth so that cells divide to produce new cells only when new cells are needed or when normal cells grow old or get damaged. Disruption of this system results in an uncontrolled division and proliferation of cells that are build up an extra cells often forms a mass of tissue known as a tumor, which grow or proliferate throughout the tissues of the body and it may progress and cause death (6).

Tumors can be benign or malignant, about the term cancer refers usually to malignant tumors. Benign tumors usually can be removed and do not spread to other parts of the body. Malignant tumors, on the other hand, grow aggressively and invade other tissues of the body, allowing entry of tumor cells into the bloodstream or lymphatic system and then to other sites and organs in the body such as bone, brain and liver, and they overwhelm these sites by consuming their oxygen, nutrients, and space. This process of spread is termed metastasis, the areas of tumor growth at these distant sites are called metastases.

Benign tumors don't invade the tissues around them, don't spread to other parts of the body and usually don't need to be removed. Malignant tumors can invade nearby organs and tissues, can spread to other parts of the body and often can be removed but may grow back.

There are different causes of cancer, among these causes are chemicals, radiation, smoking, viral infection, dietary factors, and environmental factors. Physicians and researchers need to find a comprehensive cancer treatment that is based on the increased awareness of the role of traditional and complementary medicine (7). According to number of global deaths, the most frequent types of cancer worldwide are lung, stomach, liver, colon (colorectal), prostate among men, while among women, they are breast, lung, stomach, colorectal and cervical (8).

1.2 Lung cancer

Lung cancer is the leading cause of cancer death worldwide, and it is the most common cause of cancer-related death in men and women, and is responsible for 1.38 million deaths annually, as of 2008 (9). For instance, death in the United States, with approximately 222,520 new cases diagnosed and 157,300 deaths in 2010 (10).

Lung cancers can arise in any part of the lung, but 90%-95% of cancers of the lung are thought to arise from the epithelial cells, the cells lining the larger and smaller airways (bronchi and bronchioles), for this reason, lung cancers are sometimes called bronchogenic cancers or bronchogenic carcinomas.

Lung cancer is a disease characterized by uncontrolled cell growth in tissues of the lung. If left untreated, this growth can spread beyond the lung in a process called metastasis into nearby tissue and, eventually, into other parts of the body like adrenal glands, liver, brain, and bone which are the most common sites for lung cancer metastasis. Moreover, lung is a common place for metastasis of tumors from other parts of the body. Secondary cancers are classified by the site of origin, e.g., breast cancer that has spread to the lung is called metastatic breast cancer (11). Once it is formed, it tends to spread or metastasize very early stages, so it is a very life-threatening cancer and one of the most difficult cancers to treat.

The most common cause of lung cancer is long-term exposure tobacco smoke (12) which causes 80–90% of lung cancers (13) Nonsmokers account for 10–15% of lung cancer cases, and these cases are often attributed to a combination of genetic factors, asbestos and air pollution (14).

The literature shows that many fruits and vegetables, including leafy green and yellow/orange vegetables, are associated with a lower risk of lung cancer (15) since they contain b-carotene that has a relation to the risk of lung cancer.

1.3 Prostate cancer

Prostate cancer is considered to have an impact on society as well, as far as biologic, economic, and personal (16). It is the most common cancer among males in developed countries. Surgical removal of the prostate effectively cures the primary disease but the metastatic disease is refractory to most forms of chemotherapy. External beam radiation therapy is one of the standard treatment modalities for treating patients with prostate cancer, about 30% of all prostate cancer patients are treated by radiotherapy (17). Yet, novel treatment strategies that exploit the mode of action of both conventional which is the chemotherapy, and alternative drugs which is the medicinal plants (18).

1.4 Colon cancer

These tumors are referred to as colorectal cancer, because the end portion of the colon may be affected. Most colon cancers are adenocarcinomas-tumors that develop from the glands lining the colon's inner wall.

Cancers of the colorectum are common in economically developed areas (19). Colorectal cancer is the second most common malignancy in western societies and the second leading cause of death related to cancer (20). Deaths from colorectal cancer rank third after lung and prostate cancer for men and third after lung and breast cancer for women (20).

Hence, colon cancer is a preventable disease (21). Diet-based strategies hold promise for both prevention and treatment of colon cancer (21, 22).

1.5 Cancer treatment

1.5.1 Chemotherapy

Cancer is treated with chemical compounds in a process called chemotherapy. Chemoprevention is defined as the use of non-toxic chemical substances or their mixtures to inhibit, retard or delay the overall process of multi-stage carcinogenesis. A wide array of compounds, of both synthetic and natural origin, have been reported to exert anti-mutagenic and anti-carcinogenic effects in numerous animal and cell culture systems.

Chemotherapy may be used alone, with radiation therapy, or after surgery. Chemotherapy uses drugs to kill cancer cells. When radiation therapy and chemotherapy are given at the same time, the side effects may be worse.

Cancer chemotherapy has faced dramatic problems. Poor selectivity of anticancer agents, kills both malignant and normal cells (23). Contentious treatment with chemotherapy may lead to drug-resistant (24). This in turn justifies the interest in search of possible anticancer agents from the flora of different countries (25). Some products of plant origin have strong biological activity and can be used as an effective sources of chemotherapeutic agents without side effects. This attracted the attention of many scientists to screen plants and to study their chemical, pharmacological and biological activity (26).

In studies conducted in the Middle East, during chemotherapy treatment (27), about half of the cancer patients uses the complementary and alternative medicine (CAM) in Turkey (28), 35% in Jordan (29) and in Iran, 75% of cancer patients uses CAM (30). CAM is also used by patients with hematological, malignancies, gynecological (31) and pediatric (32). People who use a high level of natural herbal product have a low incidence of gastric cancer (33, 34), as example, a high consumption of soybean products in Asian countries reduce the incidence of colon cancer (35), a high consumption of vegetables reduces the risk of colon cancer mortality (36), and recently, medicinal plant extracts have the ability to control the proliferation of prostate cancer cells (37, 38).

The countries , where cancer and infectious diseases are spread, have a high consumption level of medicinal plants as a source of drug discovery. Anticancer and anti-infectious preparations drugs from medicinal plants that approved by US Food and Drug Administration (FDA) share about 60% and 75% respectively (39). It is worthy to focus on the vivid current interest in discovery of natural drugs for cancer treatment and chemoprevention (40). Huge number of plant species is screened and bioassayed for this purpose worldwide (5, 41).

1.6 Medicinal plants

Epidemiological studies provide robust evidence for a protection effect of the Mediterranean diet against cardiovascular disease and cancer. Since a long time, plants have been the source of medicines for the treatment of many diseases. Plants remains an important part of the health care in many countries, mainly the developing ones (42). In 2000 the WHO reported that a big percentage of the world's population depends on plants as the main source for the treatment of many disease in the primary health care (43). Nowadays, plants are being used as complementary and alternative therapies in the drug market in the developed countries.

In the last years, herbal medicine has been gaining interest in the scientific research, specifically, regarding cancer prevention or treatment (44). Herbal medicine is still used in Arab and Islamic societies, especially the Mediterranean region. Throughout Muslim history, Greco-Arab and

Islamic herbal medicine were the first choice of treatment for many disease involving epilepsy, infertility, depression and cancer (7).

In the Mediterranean region a high percentage of individuals collect and consume wild edible plants as part of their traditional source of food with low health risks (7).

1.6.1 Herbal-Based Prevention and Therapy

There are about 260,000 higher plants, 120,000 plant species can be used to create biologically active products, which are used for the treatment of different diseases (45), such as olive, black seeds, onion, grapes, Peganum harmala and Arum Palaestinum(46, 47).

Various wild plants contain high amount of nutritional minerals with relatively low energy and antioxidant property, mainly from phytochemicals (48). Herbal-based molecules, which are called phytochemicals, that are isolated from medicinal plants have a significant importance to reduce or prevent some types of cancer and inhibit the development and spread of tumors in the tested animals (7). Phytochemicals have relatively low or nontoxic nature (49), so we can use these compounds for cancer treatment. Flavonoids, which is the main important part of plant extract, have long been recognized to have antiviral, anti-inflammatory, antiallergenic, anti-proliferative and anti-oxidative activities (50), and lower the risk of lung cancer (51), stomach cancer (52), coronary heart disease (53) and stroke (54).

1.6.2 Medicinal plants and drugs

Medicinal plants are the most preventive source of drugs for most of the world's population, and possess an important position in the drug discovery and development. Medicinal plants remain an important source of new drugs (55), despite the advantages of the synthetic chemicals and molecular modeling (39), and many modern drugs have their origin in traditional medicine of different cultures.

Herbs and plant products were used in medicine in treating many diseases since thousands of years. The interest in studying the effects of traditional medicinal plants for treatment of illness has been increasing all over the world (56). Moreover, The combination between traditional medicine and other new biotechnological tools have to be established in order to make new drug development (6).

People use medicinal plants due to their nutritional components, In addition, they are used for treating a wide spectrum of diseases, and they have been tested for their potential uses as alternative remedies and to reduce the toxic and oxidants of foods (57). Many plants have been found to have components that have anticancer activities, antifungal and antibacterial,. Other plants are used in traditional medicine due to their antioxidant properties (58).

The studies reported that of the 877 small molecule new chemical entities (NCEs) introduced between 1981 and 2002 nearly the half (49%)

were natural products, semi-synthetic natural products, semi-synthetic natural products analogues or synthetic compounds based on natural products (46). Among FDA reported anticancer and anti-infectious drugs which have natural origin are 60% and 75% respectively (39).

1.6.3 Anticancer activity of medicinal plants

Several studies have revealed that natural products exhibit an extensive spectrum of biological activities such as stimulation of the immune system, antibacterial, antiviral, anti-hepatotoxic, anti-ulcer, anti-inflammatory, antioxidant, anti-mutagenic, anti-cancer effects, and induction of apoptosis (59, 60) (table 1.1) , and medicinal plants are still used despite the advantages of modern synthetic drugs. Moreover, medicinal plants are more natural and more accessible than manufactured drugs, so people believe that the use of the medicinal plants for the treatment of the diseases is more save (61) . Modern synthetic drugs can't be used to cure diseases for many reasons including side-effects and toxicity, multi-drug resistance microorganisms and the inability of modern medicine to find effective cures for a number of diseases.

More than 70% of the developing world's population now depends on traditional medicinal system, otherwise known as complementary or alternative systems of medicine (62, 63). Between 30-75% of cancer patients in the USA uses the medicinal plants as complementary and alternative medicine (5). This encourage the researchers to search for

possible anticancer agents from plants in different countries. Indeed, the use of medicinal plants by people in their food systems will open the window for many important new pharmaceuticals. In the last 20 years more than 25% of drugs are directly derived from plants, while the other 25% are chemically altered natural products (64). Pharmacologically active chemicals present in medicinal plants are now proving their potential for use in cancer therapy (63).

Table 1.1 Medicinal plants used to treat cancer based on Traditional Arab Medicine.

Plant species	Preparation	Uses
Allium cepa	Bulb juice	Diabetes, liver diseases, and coughing
Arum palaestinum	Foliage decoction	bacterial infection, poisoning, and circulatory system
Peganum harmala	Roots and seeds	anti-bacterial activity, cytotoxicity antitumoral activity, and anti-oxidant activity.
Crataegus azarolus	Fruit and flower decoction	Cardiovascular diseases, diabeties, and sexual diseases
Quercus calliprinos	Fruit and bark decoction	Ulcer, diabetes, and skin diseases
Zea mays	Kernel and fiber decoction	Blood pressure, joint inflammation, and weight loss
Triticum aestivum	Shoot decoction	Anemia, and skin diseases

Many nutritional agents are believed to be critical in carcinogenesis (65) Evidences from epidemiologic studies indicates that diets that contain a high fruits and vegetables such as cabbage, broccoli, tomatoes, apples and grapes (66), are associated with a lower risk of different cancer (67), such as prostate, oral cavity, lung, breast and colon (68, 49). Moreover, several

organizations, such as National research council of the national academy of sciences (65), the national cancer institute (69), and the American cancer society (70), encourage the increase intake of citrus fruits, green and yellow vegetables. Fruits and vegetables are rich in vitamins A and C that lower cancer risk (71).

In Palestine, the screening of flora for pharmacological active compounds started in the late sixties (72). There are more than 2900 plant species found on a very small geographical area, this large number is due to the diversity of the soil and climatic conditions (73) and it is considered as a major advantage of studying the Palestinian flora. Two of these plants that are widely used to treat cancer patients (*Arum Palaestinum* and *Peganum Harmala*) were selected to test their efficacy in the treatment of cancer (72, 44), in vitro (Apoptosis induction).

1.7 Apoptosis

Apoptosis is a mode of cell death which is responsible for deletion of unwanted cells in normal tissues, and occur in specific pathologic way. Morphologically, apoptosis involves sequential events that lead to the cell death such as rapid condensation and budding of the cell, formation of membrane-enclosed apoptotic bodies containing well-preserved organelles, which lastly are phagocytosed and digested by nearby resident cells. There is no associated inflammation with the outpouring of specialized phagocytes into the tissue, such as occurs with necrosis (74).

The healthy animal cells have an important structural which is the asymmetric distribution of phospholipids between the both sides of the cell plasma membrane. Spatially phosphatidylserine (PS) which is found exclusively in the inner part of the membrane and usually constitutes less than 10% of the total phospholipid in the membrane. During the early stages of programmed cell death (apoptosis), the phospholipid distribution will be affected and this will lead loss of cell membrane phospholipid asymmetry and to the translocation of phosphatidylserine which will appear on the cell surface (outer part of the membrane). So, the detection of phosphatidylserine is a straightforward and widely used assay for apoptosis. The assay usually employs a 36 kDa protein with a calcium-dependent affinity for membranes that are enriched in anionic phospholipids, which is called annexin V and the binding is observed as red fluorescence (figure 1.1). A wide range of annexin V–dye conjugates is now commercially available. It is apparent that a small-molecule substitute for annexin V that binds PS rich membranes in a Ca2+-independent manner would be a very useful reagent for detecting apoptosis (75).

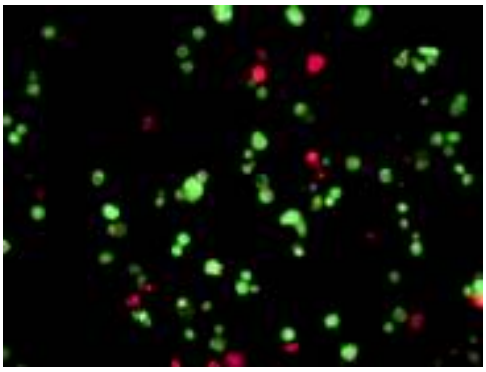


Figure 1.1: Apoptotic (red) and alive (green) cells stained with Annexin V-CY3,under fluorescent microscope .

1.8 *Arum palaestinum* (Palestinian Arum)

Figure 1.2: *Arum palaestinum* from different sites in the West bank.

Arum is edible plant and is widely used in Palestine, Arum palaestinum i**s** one of many delicious arums from the mountains of the Middle East, and it is one of our favorite arums that has thrived in our garden for more than two decades.

It is considered as an anticancerous plant in Palestine (especially for colon cancer), according to a survey conducted in 2008 (53). Also, *A. palaestinum* is effective against internal bacterial poisoning, infections and disturbances of the circulatory system (7). However, *A. palaestinum* may cause negative side effects when it is used for treating tumor. For instance, flavonoid iso-orientin isolated from *A. palaestinum* possesses myolytic activity on rat(76).

1.9 *Peganum harmala*

A **B**

Figure 1.3: Peganum harmala: (A) the whole plant, (B) seeds.

Peganum harmala, and so-called ‘‘Harmal’’ is native in the steppe areas of semiarid and predesert regions, such as Palestinian area (77). The most important compounds are so called alkaloids which are found in the seeds (contain about 2 to 6%) and the roots. Alkaloids include β-carbolines such as: harmine, harmaline, harmalol and Harman (78). Harmaline is almost twice as toxic as harmine and in moderate doses causes clonic convulsions (79). Lethal doses bring about convulsions, which are soon followed by motor paralysis. Respiration is paralyzed and a decrease in body temperature occurs. Harmine Pharmacologically resembles harmaline in its actions but is less toxic, and it is highly active against *Myco-bacterium tuberculosis* (80). Alkaloids are used as psychoactive drug to treat Parkinson’s disease (81), and other different bioactivities, such as against human cancer cell lines (82), anti-bacterial activity (83) cytotoxicity antitumoral activity (84), anti-oxidant activity (85), enzyme

inhibition (86), immunomodulator properties (87) and vasodilator activity on rat aorta (88).

1.9.1 Medicinal uses

Peganum harmala is used as an analgesic, anti-inflammatory agent and antibacterial activity and Anticancer (89). *Peganum harmala* seeds have been used to treat skin cancer and subcutaneous cancers traditionally. Seed extracts are powerful against different tumor cell lines both in vitro and in vivo. The fruit and seed stimulates digestive and uterine (90, 91), and they are taken internally to treat urinary and sexual disorders, epilepsy, menstrual problems, mental and nervous illnesses (91). Seeds contain 'harmine' which used in research into mental disease, and inflammation of the brain (90). It has been used in the past as a truth drug (92). The root has been used internally in the treatment of rheumatism and nervous conditions (60).

1.10 The aim of the study

In accordance with this worldwide trend, the current study was undertaken to investigate the anti-cancer effect of ethanolic extracts of two medicinal plants found in the Palestinian flora. These medicinal plants(*Arum palaestinum* and *Peganum harmala*) are recommended by the traditional healers for the treatment of cancer . So we assessed their efficacy in apoptotic induction after examining their cytotoxic activity on three cancer cell lines.

Chapter Two

Material and Methods

Chapter Two

Material and Methods

2.1 Plant material

Plants (*Arum palestinum* and *Peganum harmala*) were collected from different places in Tulkarm and Jenin (Palestine).

2.2 Preparation of Plant Extracts

One-hundred grams of air-dried plant material (leaves) were added to 1 L of distilled water and boiled for 10 min. The extract then were filtered using filter paper and frozen at -70°C until use. *A. Palestinum* yielded (20000 µg/ml) and *P.Harmala* yielded (23720 µg/ml). These crude extracts were used for the whole experiments (93).

2.3 Cell culture

2.3.1 Cell lines

Human prostate cancer cells (PC3, ATCC number: CRL-1435, from human prostate), colon cancer (HCT116, ATCC number: CCL-247, human, from the epithelial tissue of the colon), lung cancer cells (A547, SIGMA-Aldrich, catalog number: 86012804, from the epithelial tissue of the lung) and normal muscle cells (L6, ATCC number: CRL-1458, from the skeletal muscle) were grown in RPMI, RPMI-1640, Dulbecco's modified Eagle's medium (DMEM), and alpha MEM, respectively, supplemented with 10%

fetal calf serum, 1% penicillin streptomycin,1% amphotricine B, 1% nonessential amino acids and 1% L-glutamin, all chemicals were purchased from SIGMA company. Cell lines were maintained in a humidified atmosphere of 95% air, 5% CO2 at 37°C.

2.4 Determining cell viability

2.4.1 Cytotoxicity, using MTT Assay

The tetrazolium dye, MTT, is widely used to assess the viability of cells. The MTT mediated cytotoxicity and cytostatic assay, based upon the ability of living cells to reduce the yellow 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) into purple formazan crystals by mitochondrial succinate dehydrogenase in viable cells (94, 74), which provides a quantitative determination of viable cells. Cells with 70-80% confluence, were detached from the cultured flask by treatment with 0.05% trypsin- EDTA and a suspension of 100 µl (2.0×10^4 cell/well) of viable cells were seeded/well in a 96-well plate and incubated for 24 h. Cells then were incubated with stock solutions of crude extracts from plants serially diluted to reach concentrations of 500.0, 250.0, 124, 62.5, 31.25, 15.625, 7.8 µg/ml. After 24 hour of incubation, 100 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) solution (0.5mg/ml) were added to each well and incubated at 37 °C for 4 hours. MTT solution was then removed, and the formazan product was solubilized with acidified isopropanol (0.1N HCl).The plate were covered with tinfoil and agitated on

orbital shaker for 15 min. The optical density (OD) of the MTT formazan then will determined at 570 nm in an enzyme-linked immunosorbent assay (ELISA) reader.

2.4.2 Cytostatic effect, using MTT Assay

To test the antimitotic activity of the extract, we seeded less number of cells in each well. 1.0×104 cells/well, which were plated in 100 µl of the medium in 96-well plate for 24 hours and were treated with the plant extracts in different increasing concentrations as mentioned in the previous section and were incubated for 24 hours at 37°C. Following the removal of plant extracts from each well, the cells were incubated in DMEM to which MTT (0.5 mg/ml) was added to each well (100µl), and then the cells were incubated for 4h at 37°C. The medium were removed and 100 µl of isopropyl alcohol were added to dissolve the formazan crystals. The plate were covered with tinfoil and agitated on orbital shaker for 15 min. The optical density (OD) of the MTT formazan then were determined at 570 nm in an enzyme-linked immunosorbent assay (ELISA) reader.

2.4.3 Apoptosis: using Annexin V-Cy3 protocol for the staining of cells

Apoptosis was induced by different doses of *Peganum harmala* and *Arum palaestinum* extracts . we used three plant concentrations To determine apoptosis, cells were seeded with growth medium into 6- well plate at 200,000 cell/well and incubated for 24 hours, then cells were treated with three plant concentrations: (A) 0 mg/ml, (B) 0.125 mg/ml, (C)

0.25 mg/ml. after 24 hours we washed the cells with 500 µl of PBS. After trypsenization with 500 µl trypsin , cells were centrifuged at 1500*g for 5 minutes at room temperature and re-suspended in 500 µl of binding buffer. After centrifugation at 1500*g for 5 minutes, cells were subjected to staining with 1 µl Annexin V-Cy3 (AV) and were incubated at room temperature for 20 minutes, then 1 µl of Acridine Orange (AO) were added . The cells were then visualized by fluorescence microscopy and images were recorded by a digital camera (74).

Chapter Three

Results and Discussion

Chapter Three

3. Results and Discussion

As cancer is one of the major world health problems and because of the side effects of the chemotherapy used, we selected two medicinal plants, *Arum palaestinum* and *Peganum harmala*, based on a Ethno botanical studies reported an anti-cancer therapeutic usage of these two medicinal plants. Accordingly, we selected the two medicinal plants to show their anti-tumor on different cancer cell lines, colon cancer cells (HCT116), lung cancer cells (A549), prostate cancer cells (PC3) and a control normal cell line which was muscle cell line (L6). For the cytostatic effect (antimitotic), there was no different between cytotoxic and cytostatic effect, so we take just the cytotoxic effect.

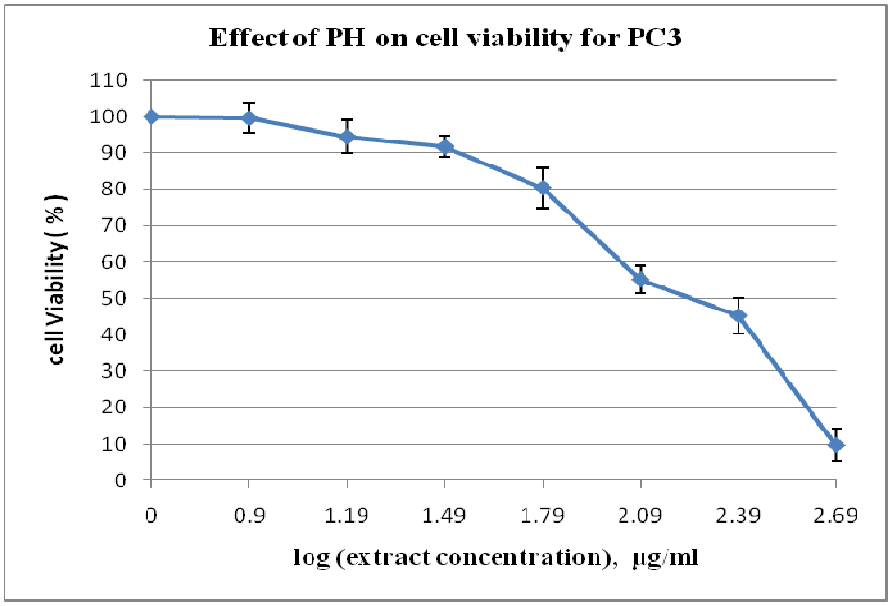
3.1 Cytotoxic and apoptotic effect of *Peganum harmala*

Cells were seeded on 96 well plate, $2*10^4$ cell/well with 40-50% confluence, and left for 24 hours. Then the cells were treated with the plant extract (0, 8 , 16 , 32 , 65 , 125 , 250, 500 and 1000 μg/ml), after 24 hours of incubation, cytotoxicity of plant extract were measured using MTT assay. After four hours of adding MTT, isopropanol was added for 15-20 minutes in dark to dissolve the formazan crystals, then absorbance was measured at 570 nm using ELISA reader.

3.1.1 Prostate cancer cell line (PC3)

Results shows that *Peganum harmala* (*P. harmala*) has a toxic effect on PC3 cells at high concentrations, IC50 was 174 µg/ml (Figure3.1, Table 3.2). However, apoptosis induction was evident (90%) at 250 µg/ml of *P. harmala's* extract (table 3.1). At nontoxic concentrations such as 62 µg/ml, 30% of the cells were apoptotic (Table 3.1) while according to the MTT assay (toxicity), only 20% of the cells were dead (Figure 3.1). The difference is due to the fact that MTT measures the dead cells, while apoptosis measures the dead cells and the cells that start preparing for programmed cell death, so the death in apoptosis is more than MTT. So we can conclude that *P. harmala* extract has an apoptotic effect on prostate cancer cells at non-toxic concentrations.

A



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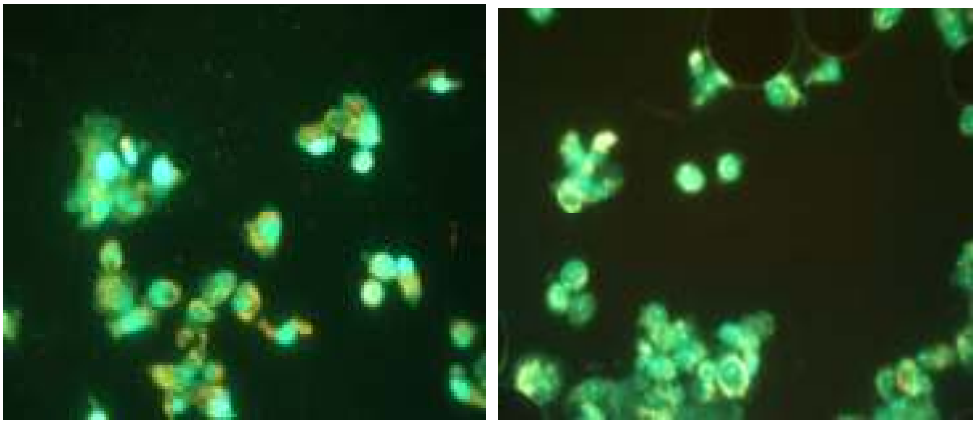
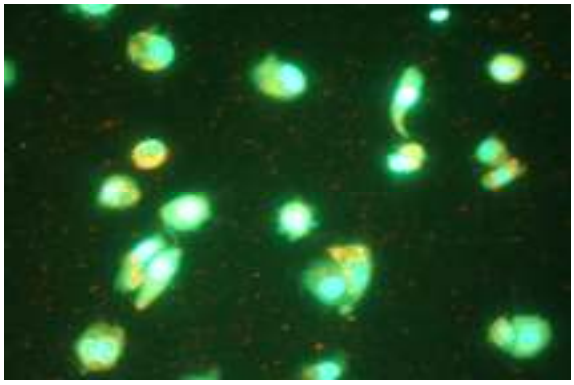


PC3- 250 µg/ml PC3- 62 µg/ml



PC3-250 µg/ml

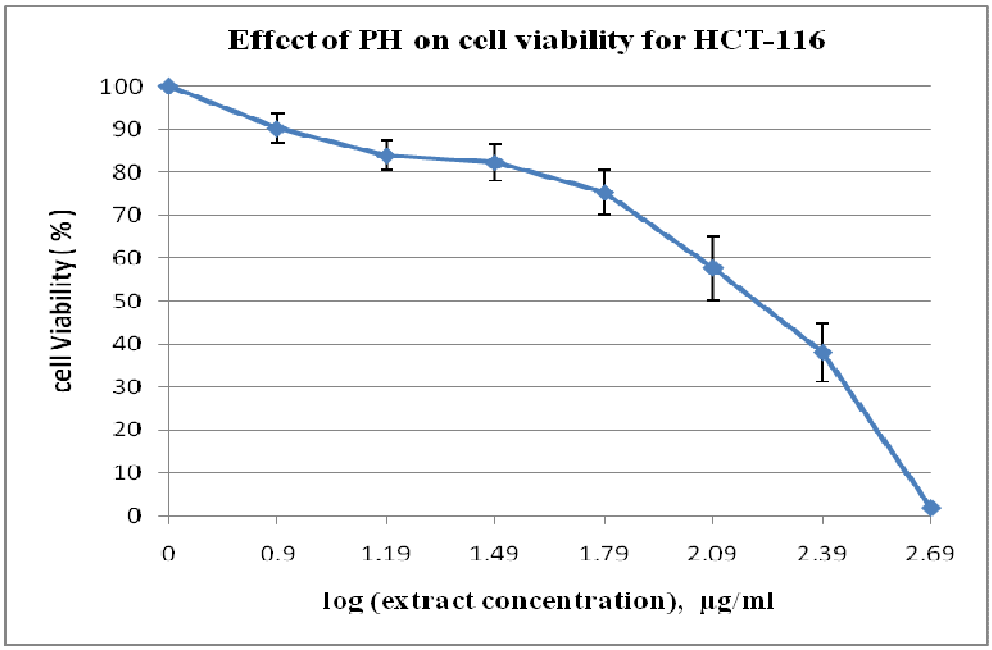
Figure 3.1 : PC3 cell line, (A) effect of different concentrations, in logarithmic scale, (0-500 µg/ml) of *P. haemala* extract on cell survival of human prostate cancer cell line (PC3) obtained by MTT colorimetric assay. Results are expressed as the percentage of surviving cells. (B) Apoptotic effect of *P. haemala* extracts (62 and 250 µg/ml) after using Annexin v-cy3 under the florescent microscope .

3.1.2 Colon cancer cell line (HCT-116)

P. harmmala also has a toxic effect on HCT cancer cells at high concentrations, IC50 was 155µg/ml (Figure 3.2, Table 3.2). , Apoptosis was evident (70%of cells apoptotic, Table 3.1) at a non-toxic concentration (250 µg/ml). According to MTT assay (cytotoxic), only 38% of cells were dead at the same concentration of *P. harmmala* (250 µg/ml).

We conclude that *P. harmmala* extract can induce apoptotis on colon cancer cells at a non cytotoxic concentrations.

A



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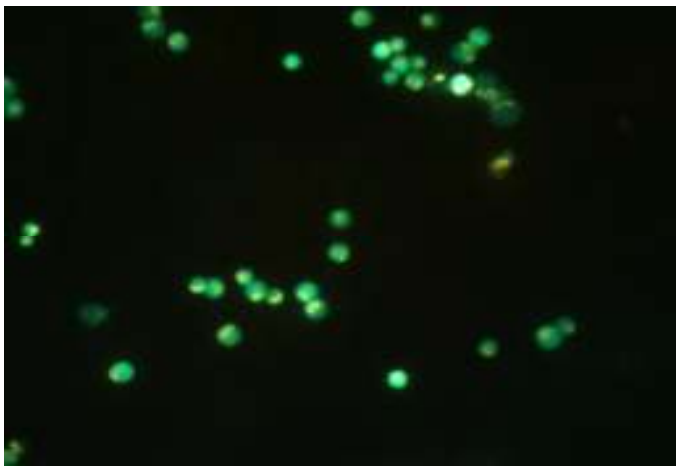


HCT-250 µg/ml

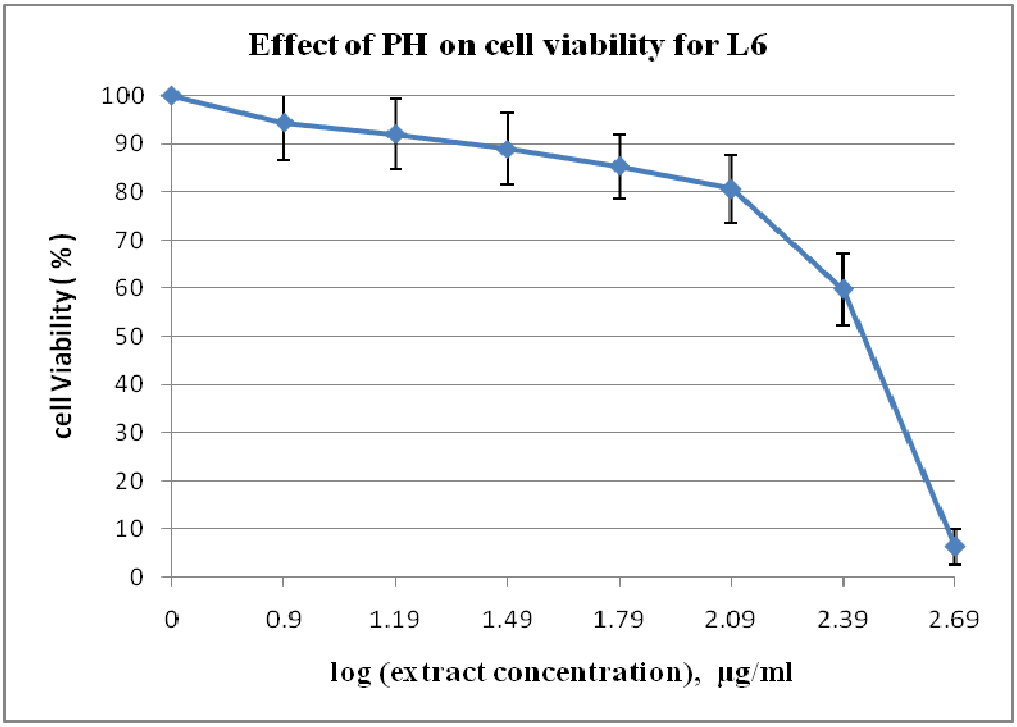
Figure 3.2: HCT-116 cell line, (A) Effect of different concentrations, in logarithmic scale, (0-500 µg/ml) of *P. haemala* extract on cell survival of human colon cancer cell line (HCT-116) obtained by MTT colorimetric assay. Results are expressed as the percentage of surviving cells. (B) Apoptotic effect of P. *haemala* extracts(250 µg/ml) after using Annexin v-cy3 under the florescent microscope .

3.1.3 Muscle normal cell line (L6)

MTT assay shows that there were no toxic effect of *P. harmala* on normal muscle cell line at different concentrations except at 500 µg/ml it was very toxic, 93% of cells were dead, IC50 is 276 µg/ml (Figure 3.3, Table 3.2). MTT assay shows that at 62.5 µg/ml 15% of cells were dead and 40% of cells were apoptotic. Plant extract were not toxic at 250 µg/ml, 40% of cells were dead while 90% of cells were induced apoptosis (Table 3.1). When cells are dead, there is no metabolic activity for the cells, hence in the MTT test there were less dead cell than the apoptosis test.

We conclude that plant extract may has an apoptotic effect on muscle normal cells at non toxic concentration of the plant, and it is not toxic at concentrations less than 300 µg/ml.

A



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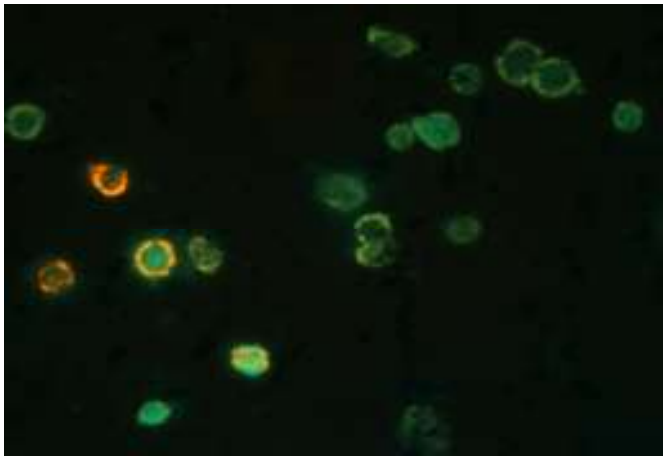


L6- 250 µg/ml

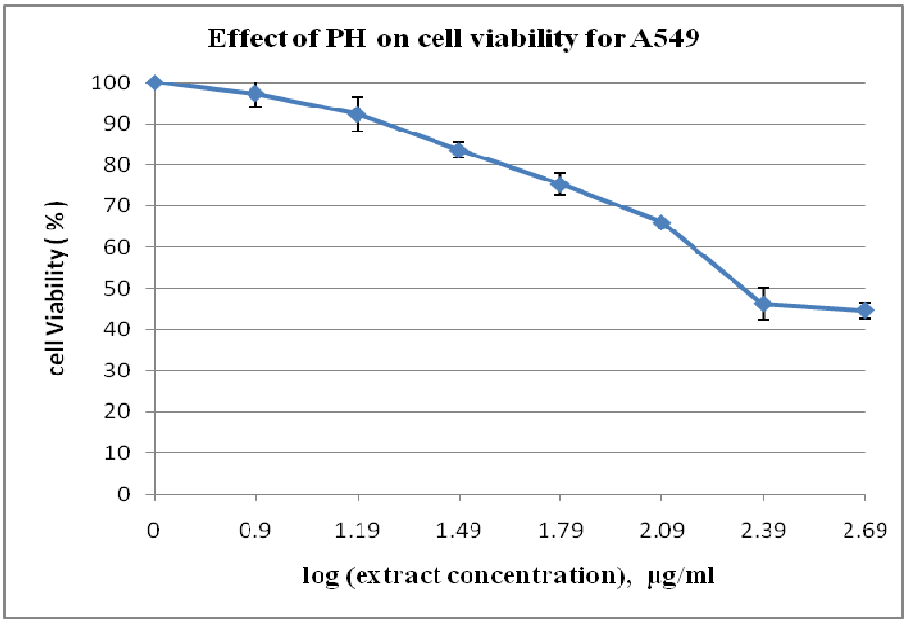
Figure 3.3 :L6 cell line, (A) Effect of different concentrations, in logarithmic scale, (0-500 µg/ml) of *P. haemala* extract on cell survival of human normal muscle cell line (L6) obtained by MTT colorimetric assay. Results are expressed as the percentage of surviving cells, (B) Apoptotic effect of P. *haemala* extracts(250 µg/ml) after using Annexin v-cy3 under the florescent microscope .

3.1.4 Lung cancer cell line (A549)

P. haemala extract has less toxicity on lung cancer cell line(A549) compared with the effect of the plant extract on the previous cell lines, especially the highest two concentrations tested, 250 and 500 µg/ml. IC50 is 219 µg/ml (table 3.2). MTT assay shows that 53% of cells were dead at 250 µg/ml (toxic), while at 62 µg/ml only 22% of cells were dead (figure 3.4). However, at 250 µg/ml apoptosis was evident (85%) and at 62.5 µg/ml there was a very low apoptosis effect (10% of cells were apoptotic, table 3.1).

We conclude that *P. haemala* extract has minimal toxic effect on lung cancer cell line (A549) at concentrations less than 219 µg/ml, and has apoptotic effect at non toxic concentrations.

A



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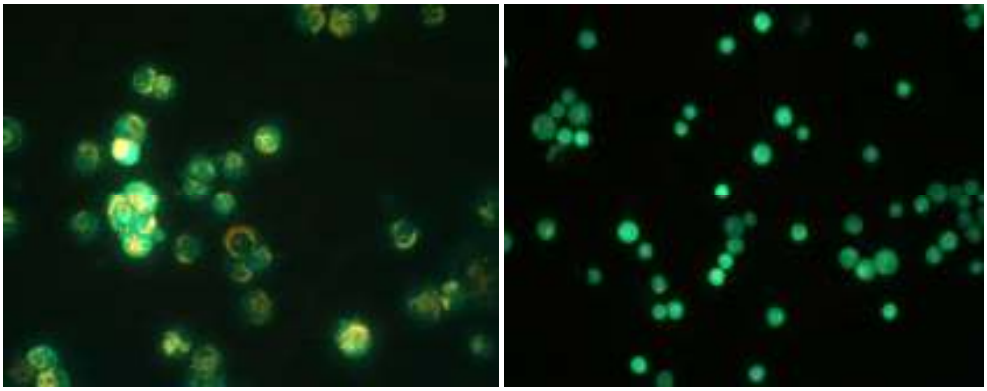
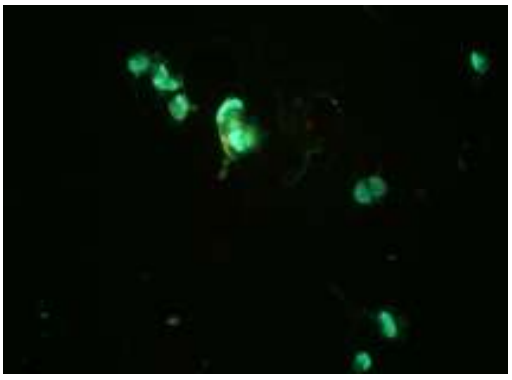


A549-250 µg/ml

A549-62 µg/ml



A549-control

Figure 3.4 :A549 cell line, (A) Effect of different concentrations, in logarithmic scale, (0-500 µg/ml) of *P. haemala* extract on cell survival of human lung cancer cell line (A549) obtained by MTT colorimetric assay. Results are expressed as the percentage of surviving cells, (B) Apoptotic effect of P. *haemala* extracts(0, 62, 250 µg/ml) after using Annexin v-cy3 under the florescent microscope .

Table 3.1 Apoptosis induction by *A. palaestinum* and *P. harmala* detected by Annexin V-CY3 and Acridin Orange dye.

Cell line		*Arum palaestinum* Apoptosis %				*Peganum Harmale* Apoptosis %	
200,000 cell/well	Control	62 (µg/ml)	250 (µg/ml)	250 (µg/ml)	250 (µg/ml)	62 (µg/ml)	250 (µg/ml)
L6-wt	3%	__	__	__	__	40%	90%
A459	1%	__	__	__	__	10%	85%
PC-3	3%	__	__	__	__	30%	90%
HCT-116	0%	__	__	__	_	5%	70%

Table 3.2 LD50 for the cytotoxic effect of *Peganum harmala* on PC3, HCT-116, L6 and A549.

L6-wt	PC3	HCT-116	L6	A549
IC50	174	155	276	219

3.2 Cytotoxic and apoptotic effect of *Arum palaestinum*

A. palaestinum is widely reported and used as anti cancer agent (95). Surprisingly, Our Results shows that *A. palaestinum* has no cytotoxic effect, at all concentrations tested (Figure 3.5), on the selected cell lines (PC3, HCT116, L6 and A549). Moreover, it did not induce apoptosis at any of these cell lines (Table 3.1). In addition, figure 3.6 shows that there were no morphological changes on the cells treated with *A. palaestinum* compared to the control. These unexpected results, encouraged us to double check the reported articles dealing with *A. palaestinum*. We found that the papers dealing with the anticancer effect of *A. palaestinum* in the literature are mostly ethanobotanical reviews. However, Abu Dahab and Afifi (2007) reported that low concentration (50 µg/ml) of ethanolic *A.*

palaestinum extract did not have a cytotoxic effect on breast adenocarcinoma cell line (MCF7) treated for 72 h; but they did not test its effect on apoptosis induction (55). Concomitantly, El-Desouky and colleagues (96) reported that proliferation of breast carcinoma and lymphoblastic leukemia, treated with ethyl acetate fraction of *A. palaestinum* was suppressed at IC50 of about 55 μg/ml. However, they found no effect of the same extract on the growth of hepatocellular carcinoma cells (HepG2).

According to our best knowledge, this is the first reported research that had tested water extracted *A. palaestinum* cytoxicity (the survival of the cells by MTT assay) or its apoptotic effect (by the annexin v-cy3). So we conclude that water extracted *A. palaestinum* has no cytotoxic or apoptotic effects up to 1 mg/ml.

Yet we cannot exclude the possibility that *A. palaestinum* might has anticancer properties in a distinct way such as anti angiogenesis activity. Hence, more experiments are needed, especially in vivo tests in tumurgenic animal models.

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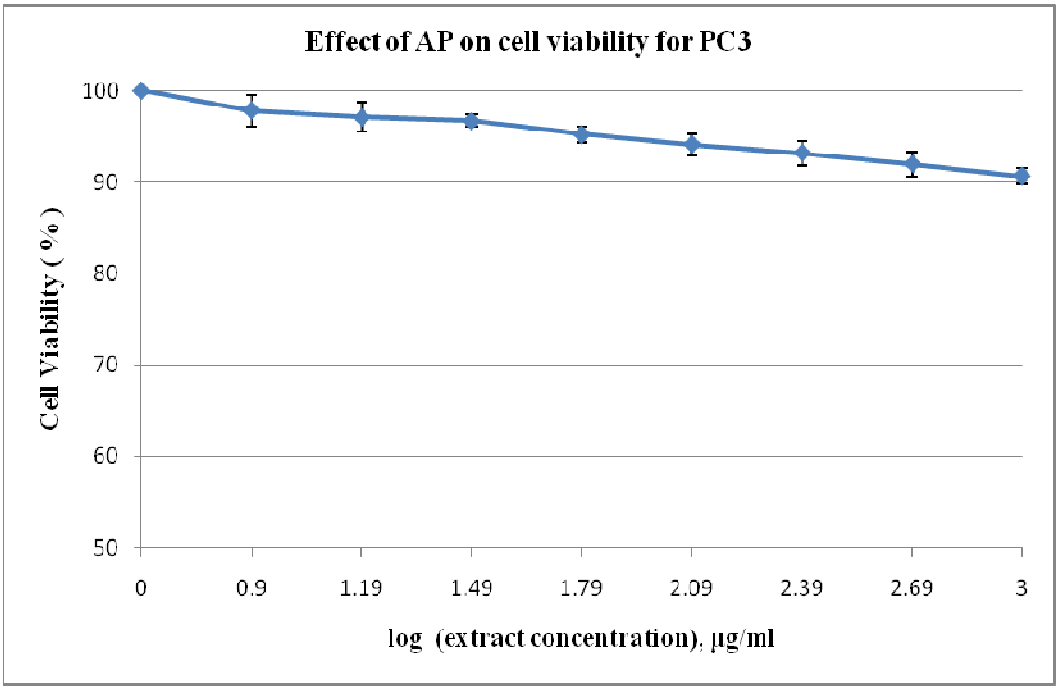
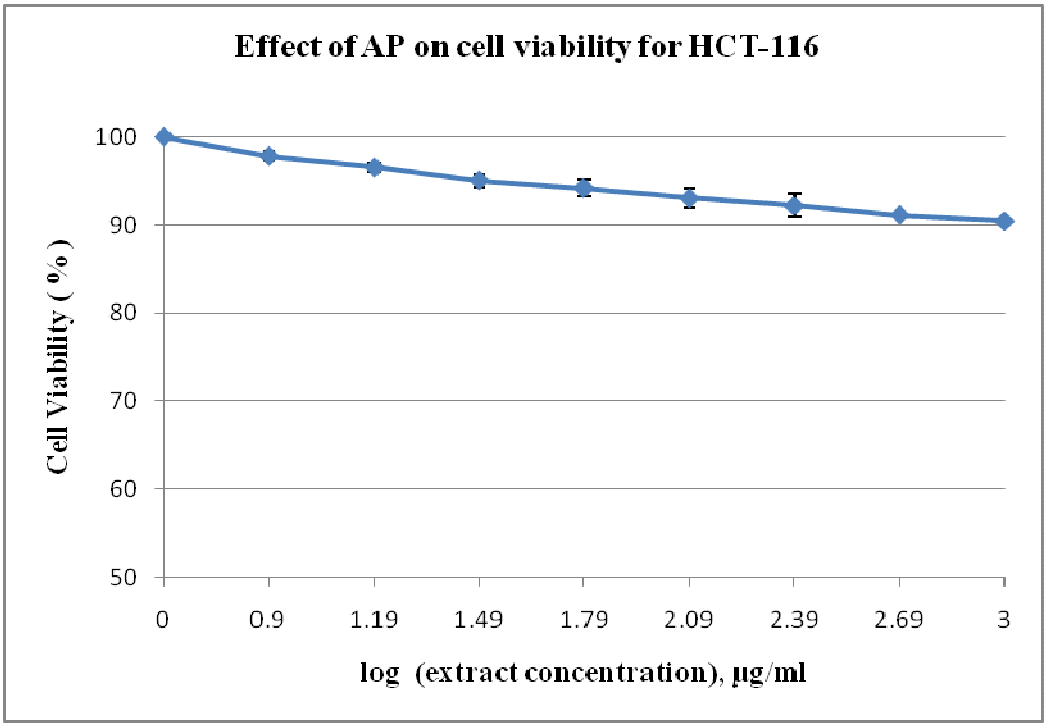
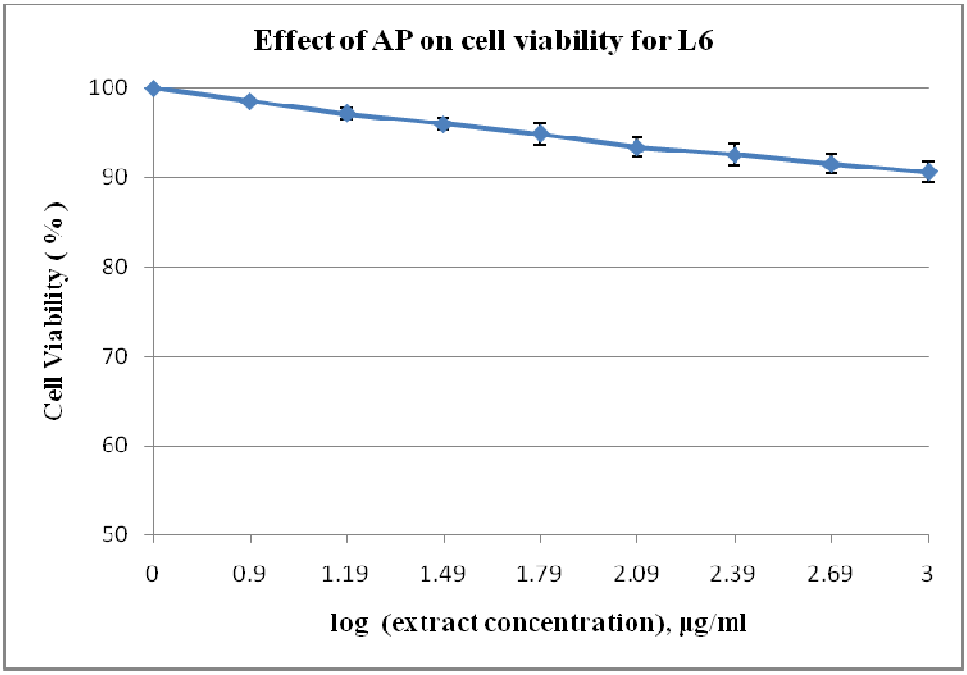

Effect of AP on cell viability for PC3
Cell Viability (%)
100
90
80
70
60
50
0
0.9
1.19
1.49
1.79
2.09
2.39
2.69
3
log (extract concentration), µg/ml

B


Effect of AP on cell viability for HCT-116
Cell Viability (%)
100
90
80
70
60
50
0
0.9
1.19
1.49
1.79
2.09
2.39
2.69
3
log (extract concentration), µg/ml

C



D

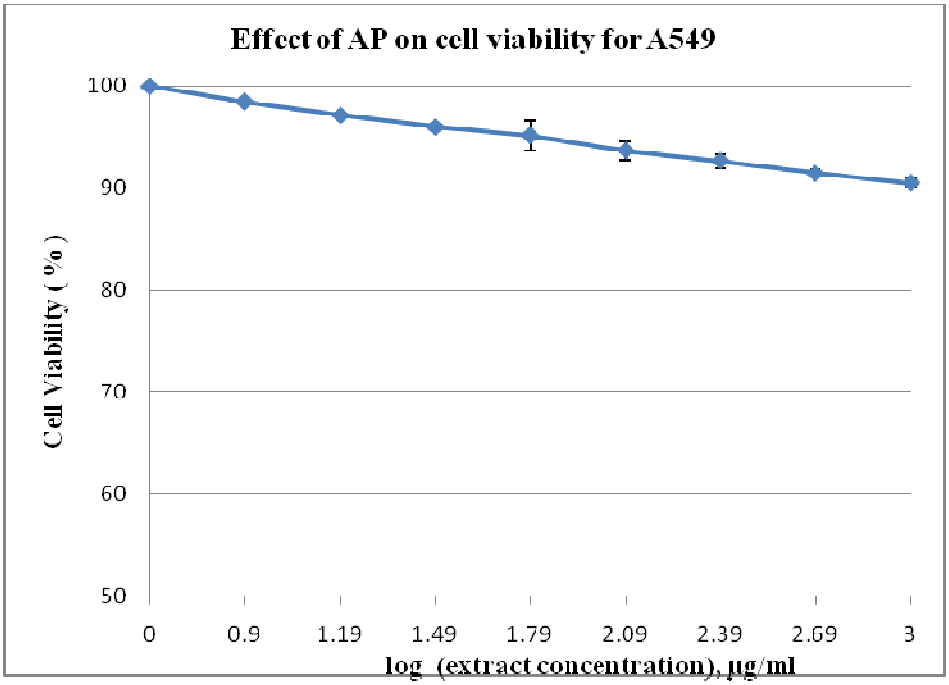
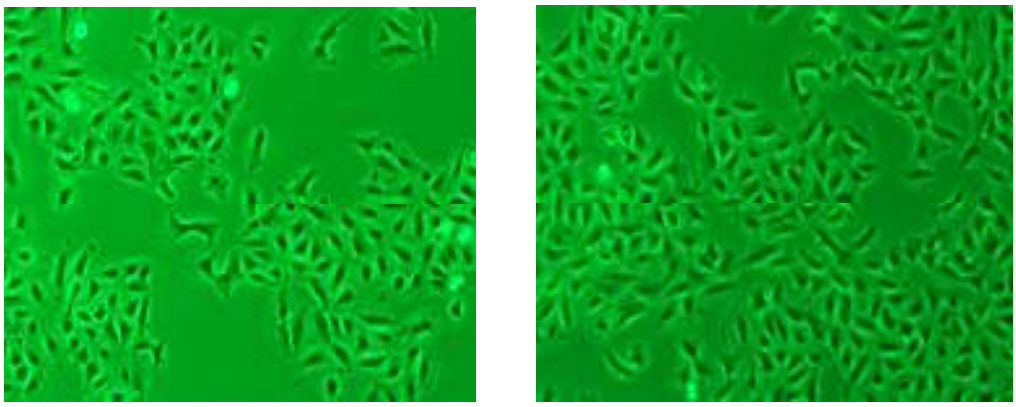
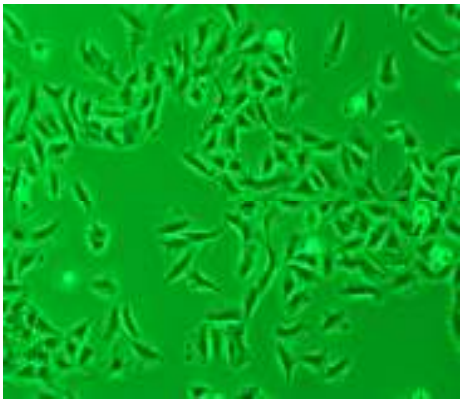


Figure 3.5: The effect of different concentrations, in logarithmic scale, (0-1000 µg/ml of *A. palaestinum* extract on cell survival of human different cell lines (A: PC3, B: HCT-116, C: L6 and D: A549) obtained by MTT colorimetric assay. Results are expressed as the percentage of surviving cells.



Control (0 mg/ml) **0.5 mg/ml**



1mg/ml

Figure 3.6: The morphology of the prostate cancer cell line (PC3) obtained by microscope with different *A. palaestinum* concentrations (0, 0.5, and 1 mg/ml).

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جامعة النجاح الوطنية
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قدمت هذه الاطروحة استكمالا لمتطلبات درجة الماجستير في العلوم الحياتية بكلية الدراسات العليا في جامعة النجاح الوطنية، نابلس، فلسطين.
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الملخص

ان مخاطر حدوث السرطان بتزايد في الدول النامية وحتى ايضا في الدول المتقدمة. السرطان هو عبارة عن حدوث انقسام للخلايا بشكل غير مسيطر عليه مما يؤدي إلى تكوين كتلة من الخلايا. تلعب الميتوكندريا دورا هاما في حدوث الموت المبرمج للخليلة, حيث أنها تفرز مواد تؤدي إلى الموت المبرمج للخلية مثل مادة سيتوكروم سي من منطقة الفراغ البين غشائي للميتوكندريون. هذه المواد تلعب دور مهم في بدء سلسلة تفاعلات تؤدي الى الموت المبرمج للخلية. المراحل المتقدمة للسرطان يتم علاجها بالعلاج الكيميائي. على الرغم من ان هذه المواد الكيميائية فعالة الا ان لها اعراض جانبية خطيرة. هنالك دراسات عديدة تؤكد ان المواد الطبيعية لها فوائد بيولوجية عديدة مثل تحفيز جهاز المناعة,مضاد للبكتيريا والفيروسات, مضاد للاكسدة, مضاد للالتهاب, مضاد للسرطان وكذلك تحفيز الموت المبرمج للخلية. ان العلاج بالطب العربي الاسلامي التقليدي عن طريق النباتات يمكن ان يكون علاج فعال ضد السرطان، حيث ان المواد الطبيعية لها درجة سمومية قليلة واثارها الجانبية قليلة، وبما انها طبيعية تجعل المريض يشعر بالراحة. بناءا على ذلك تم اختيار نباتين طبيين من اجل فحص تاثيرهما على السرطان (اللوف الفلسطيني والحرمل). تم معالجة ثلاثة أنواع من الخلايا السرطانية (البروستاتا، الرئة والكولون) بتراكيز متزايدة من مستخلص النباتات (0، 8، 16، 32، 62، 125، 250، 500 ميكروغرامِ/مل). وتم استخدام صبغة (MTT) لفحص سمية النبات وكذلك صبغة (Annexin v-cy3) من اجل فحص الموت المبرمج للخلية. اظهرت النتائج ان نبات الحرمل ليس له تاثير

سمي على جميع انواع الخلايا المستخدمة بتركيز اقل من 250 ميكروغرامِ/مل, لكن لها ناثير على تحفيز الموت المبرمج للخلية لكل من الخلايا السرطانية للبروستات، الرئة والخلايا العضلية الطبيعية. اما بالنسبة لخلايا الكولون فقد كان التاثير قليل. اما نبات اللوف الفلسطيني لم يكن له ناثير سمي او تحفيز للموت المبرمج للخلايا، حتى على تركيز 1000 ميكروغرامِ/مل.