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Antitumor and Antioxidant Activity of Different *Pelargonium graveolens* Crude Leaves Extracts

A Thesis

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By

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In the name of "Allah" who deserves all praise and thanks for inspiring me with the willingness , strength, and patience to establish this work, mercy and peace to Prophet Mohammed and His Relatives and Companions.

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Fatimah

Dedication

I dedicate this thesis to my husband :

Faisal

My constant source of love, care, support and encouragement. I am truly thankful for having you in my life.

Summary

Pelargonium graveolens is an important medicinal plant which is traditionally used to staunch bleeding, heal wounds, ulcers and skin disorders as well as treat diarrhea and dysentery. The present study was conducted to evaluate the anti-oxidant and anti-tumor properties of *P. graveolens* leaves collected from Baghdad, Iraq.

Methanol, di-ethyl ether and hexane extract from *P. graveolens*. The antioxidant activity of *P. graveolens* extracts from methanol, di-ethyl ether and hexane were assessed using DPPH at concentrations ranged from 75 to 1000µg/ml. The methanolic extract of *P. graveolens* reduced the concentration of DPPH free radicals in a dose-dependent manner with IC_{50} up to 484µg/ml, while di-ethyl ether and hexane extracts showed a lower scavenging activity with IC_{50} of 872 and 883µg/ml respectively.

The anti-tumor activity of methanol, di-ethyl ether and hexane extracts of *P. graveolens* on MCF-7 and PC-3 tumor cell lines were investigated *in vitro* using MTT assy. The methanolic extract showed the highest anti-tumor activity and reduction in cell viability against MCF-7 in a dose-dependent pattern in which the methanolic extract significantly exhibit the most potent anti-tumor activity with IC_{50} value of 288µg/ml, whereas IC_{50} values of di-ethyl ether and hexane were 822 and 415µg/ml, respectively. No significant anti-tumor activity was recorded against PC-3 cell line.

The effect of *P. graveolens* methanolic extract on MCF-7 cell line regarding viability, cell nucleus morphology, membrane permeability, potential mitochondrial permeability, cytochrome C release and ROS generation were further verified using High Content Screening array scan with multi-parametric kit and ROS kit. Results indicated that the

methanolic extract of *P. graveolens* dose-dependently inhibited the cell growth of MCF-7 cells with a maximum inhibition of 53.92% at 200µg/ml. The intensity of cell membrane permeability was also increased in a dose-dependent pattern by increasing the extract concentration, while only higher concentrations caused alterations in nucleus morphology.

On the other hand, only at higher concentration (200µg/ml) of *P. graveolens* methanolic extract caused reduction in mitochondrial membrane potential by 40.3% in which the disruption of mitochondrial membrane potential in consequence resulted in the release of the mitochondrial cytochrome C, which was significantly detected by 0.7 fold increases in the average fluorescent intensity of the treated MCF-7 cells than that of untreated cells. Methanolic extract treatment at higher concentrations (100 and 200µg/ml) also resulted in reactive oxygen species(ROS)generation,0.53 fold and 1.32 fold increase, respectively, as compared with untreated MCF-7 cells.

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DPPH	2,2-Diphenyl-1-Picrylhydrazyl
DMSO	Dimethyl sulfoxide
EDTA	Ethylenedi-aminetetraacetic acid
HSC	High Screening Content
MCF-7	Michigan Cancer Foundation-7
MTT	3-(dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
PC-3	Prostate cancer cells
PCR	Polymerase Chain Reaction
ROS	Reactive Oxygen Species

Chapter one

Introduction and literature Review

1.1 Introduction

For centuries plants considered one of the most abundant sources of medicines and drugs. Due to the vast diversity of plant species in all around the world as well as their constituents and active compounds, this made their applications ranged from simple traditional rituals into a disease specific treatment after extensive research on chemical composition, mode of action and toxicity.

One of these plants is *Pelargonium graveolens*. It's an aromatic plant belong to the Geraniaceae family that grows in temperate area of the world (Charlwood and Charlwood, 1991). Essential oils are the major components of *P. graveolens* which are mainly β-citronellol, geraniol, δ-selinene, l-menthone and others (Gomes *et al*., 2007). Besides to its benefits in food and beverage industries (Džamić *et al*., 2014), many researches and studies on the importance of *P. graveolens* were highly concentrated on essential oils and their wide array of biological activities, for example: antimicrobial and antimalarial activity (Lalli, 2005), antiasthmatic, antiallergic, antidiarrhoeic and antihepatotoxic (Boukhris *et al*., 2012). Extracts of *P. graveolens* were shown to have antioxidant properties and the ability to scavenge free radicals and reduce reactive oxygen species (Singh *et al*., 2009;Ben Slima *et al*., 2013), in addition some reports referred that extracts of *P. graveolens* exhibit antitumor activity (Fayed, 2009;Zhuang *et al*., 2009). However few studies revealed the exact cytotoxic effect of *P. graveolens* extract on tumor cells and cells morphological changes after exposure to the extract.

Identifying the toxicity of new drugs considered to be an important step before start marketing and consuming the new drugs. Many procedures are currently used to detect the level of anti-tumor activity of compounds or drugs. Multi-parametric analysis is one of the valued

approaches using flow cytometry and cellular imaging–based techniques such as high-content screening (HCS) to detect the level of compound toxicity and classification of compounds based on observed patterns of reversible and irreversible cellular injury (O'Brien *et al*., 2006;Abraham *et al*., 2008). It provides an easy and rapid method to identify substances with apoptosis inducing characteristics with high throughput analysis. The standard measurements that multi-parametric analysis provides are (a) nuclear morphology and intensity, (b) mitochondrial trans-membrane potential, (c) plasma membrane permeability, (d) cell proliferation, and (e) cytochrome C release (Looi *et al*., 2013).

According to the information mentioned above, this study aims to evaluate the antitumor and antioxidant properties of *P. graveolens* methanol, di-ethyl ether and hexane crude extracts *in vitro* using two tumor cell lines PC-3 and MCF-7, through the following applications:

- Detecting the potential antioxidant activities through the DPPH-scavenging assay, and reactive oxygen species induction capacity.
- Investigation the anti-tumor properties by employing regular MTT assay and HCS for detecting cellular parameters: cell viability, total nuclear intensity, membrane permeability, mitochondrial membrane potential changes, cytochrome C release and reactive oxygen generation.

1.2 Literature Review:

1.2.1 Medicinal Plants

Over the past decades, herbal medicine has become a topic of global importance making an impact on both world health and international trade (Ahmad and Husain, 2008). Medicinal plants continue to play a central role in the health care system of large proportions of the world's population (Ahmad and Husain, 2008).

Medicinal plants have been found as important contributors to the pharmaceutical, agriculture and food industries. With the onset of the synthetic era, pharmaceutical industries are producing a lot of synthetic drugs that help to alleviate the chronic diseases. With the passage of time many problems associated with frequent use of synthetic drugs become prominent like severe side effects and resistance of microbes against these drugs.(Ahmad and Husain, 2008).

In recent times there is an emerging trend in research to support the biological activities of medicinal plants. Many scientific researchers have been reported about the efficacious and chemotherapeutic role of medicinal plants in the treatment of diverse diseases. Cancer considered to be the most disease in which scientists are expecting new molecules and compounds from herbs that can provide an important implement for fighting against this dreaded disease, for example: *Terminalia arjuna* (Vaidya *et al*., 2008) and flavonoids extracted from different sources have shown significant inhibiting effect on cancer cells (Jiangrong and Jiang 2007; Zhao *et al*., 2007).

For a long time plants have provided a source of emerging modem medicines and drug compounds, as plant derived medicines have made large contributions to human health (Prasad *et al*., 2012). Their role is

twofold in the development of new drugs and/or a phyto-medicine to be used for the treatment of diseases(Prasad *et al*., 2012).

The uses of traditional medicinal plants for primary health care have steadily increased worldwide in recent years. The goals of using plants as sources of therapeutic agents are:

- Isolating bioactive compounds for direct use as drugs example: digoxin, morphine, vinblastine and vincristine.
- Producing bioactive compounds of novel or known structures as lead compounds for semi synthesis to produce patentable entities of higher activity and/or lower toxicity example: metformin, oxycodone and other narcotic analgesics.
- Using agents as pharmacologic tools example: lysergic acid and diethylamide.
- Using the whole plant or part of it as herbal remedy example: cranberry, feverfew and garlic.

1.2.2 *Pelargonium graveolens*

1.2.2.1 The Scientific Classification of *Pelargonium graveolens* (Demarne, 2002):

Kingdom: *Plantae*
Division: *Magnoliophyta*
Class: *Magnoliopsida – Dicotyledons*
Subclass: *Rosidae*
Order: *Geraniales*
Family: *Geraniaceae*
Genus: *Pelargonium* L'Hér
Species: *Pelargonium graveolens*L'Hér

Figure (1-1): *Pelargonium graveolens* leaves. (Miller D., 2002)

Pelargonium graveolens L'Hér (Fig. 1.1) is an aromatic and hairy herbaceous shrub with up to 1 m height. Leaves are prickly and carved, about 4 x 6 cm. They are soft and velvety to the touch, due to the presence of many glandular hairs.*Pelargonium graveolens* (geranium) is found in Iraq (Röschenbleck *et al*., 2014). Also in South Africa and Russia, Egypt, Algeria, Morocco, Congo, Japan, Central America and Europe (Spain, Italy, France) (Lawless, 2001). The plant is an evergreen flowering plant commonly known for its essential oil, with rose like aroma which is commonly called Rose geranium or Rose scented geranium and there are about 300 geranium species. It has several medicinal and aromatic values of commercial importance (Verma *et al*., 2010; Brian *et al*., 2010). Traditionally, geranium was used to staunch bleeding, heal wounds, ulcers and skin disorders as well as treat diarrhea, dysentery and colic (Matthews, 1995).

The extracts of *Pelargonium graveolens* are reported to be used as antibacterial and insecticidal agents (Jeon *et al*., 2008; Ooshiro *et al*., 2009; Tabanca *et al*., 2013).

1.2.2.2 Active Components of *Pelargonium graveolens*

Pelargonium graveolens is growing in many parts of the globe for the production of essential oil by steam or hydro-distillation of the plant leaves with a yield of 0.19%, (v/w) (Verma *et al*., 2010). Upon GC/MS analysis, the essential oil was found to contain 47 constituents (Table 1-1), 96.23% of which were identified. The volatile oil contained 67.39% monoterpenoids, 25.4% sesquiterpenoids, and 3.44 other compounds. The major components were β-citronellol (16.24%) followed by geraniol(15.30%), δ-selinene (8.69%), citronellyl formate (7.39%), geranyl formate (6.47%), l-menthone (4.13%) and linalool-l (3.8%). Twenty nine components of the oil amounted to <1% (Boukhris *et al*., 2012).

There are variations in the composition of these volatile oils in comparison to the volatile oilsof other countries. These differences seem to depend on climate changes and conditions and types and methods of distillation.

1.2.2.3 The Biological Significance of *Pelargonium graveolens*

The efficacy of the oil is exploited in many major food, alcohol and beverage industries. Commercially, geranium is widely used for scenting soaps and high-grade perfumes due to the presence of low molecular weight aroma compounds (Verma *et al*., 2010).

The activity of the oils would be expected to relate to the composition of the plant essential oils and possible synergistic interaction between components. High proportions of *β*-citronellol and caryophyllene oxide showed an interesting and valuable significant for food, medicine, aromatherapy and cosmetics industries where an antiseptic, clean and fresh characteristics flavor and fragrance is desired. The food protective

and antimicrobial properties of *β*-citronellol as prominent part of *P. graveolens* volatile oil (Jeon *et al*., 2009).

The observed antibacterial properties show thatthey have a good potential for use as antimicrobial agents and natural preservatives in different products. Antimicrobial and antimalarial activity of *P. graveolens* extracts can be attributed to significant cytotoxic effectwhich this extracts provided and probably flavonoid derivativeshave positive contribution to this biological activity (Lalli, 2005).Antioxidant and antitermitic activity of *P. graveolens* has been reported as well (Yang *et al*., 1999;Džamić *et al*., 2014).

Table (1-1): Composition (%) and Retention Indices (RI) of the Essential Oil of *Pelargonium graveolens*

N°	Composant	RT	KI[a]	%
1	α-pinene	8.870	930	0.66
2	β-myrcene	10.633	997	0.18
3	phellandrene	10.010	1007	0.09
4	β-phellandrene	11.766	1023	0.21
5	cis-ocimene	12.063	1030	0.16
6	β-ocimene	12.378	1037	0.13
7	linalool l	14.003	1073	3.80
8	rose oxide-trans	14.772	1079	0.54
9	l-menthone	15.908	1141	4.13
10	3-p-menthanol	16.418	1186	0.14
11	α-terpineol	16.646	1197	0.24
12	β-citronellol	17.894	1237	16.24
13	z-citral	18.106	1244	0.71
14	nerol	18.495	1259	3.08
15	geraniol	18.706	1266	15.30
16	e-citral	18.981	1277	0.84
17	citronellyl formate	19.113	1282	7.39
18	geraniol formate	19.834	1309	6.47
19	propanoic acid	21.087	1357	0.39
20	lavandulyl acetate	21.893	1388	0.76
21	β-bourbonene	22.014	1393	1.01
22	trans-caryophyllene	22.912	1427	1.63
23	β-cubebene	23.129	1434	0.64
24	citronellyl propionate	23.393	1444	0.14
25	azulene	23.473	1449	0.40
26	α-cubebene	23.662	1458	0.96
27	α-humulene	23.759	1460	0.51
28	aromadendrene	23.942	1467	0.68
29	germacrene-d	24.457	1486	2.87
30	β-selinene	24.577	1492	0.17
31	β-cuvebene	24.709	1496	0.32
32	ledene	24.806	1500	3.10
33	α-muurolene	24.880	1504	0.18
34	elemol	25.029	1510	0.40
35	α-amorphene	25.229	1519	0.29
36	δ-cadinene	25.447	1528	1.36
37	epizonaren	25.504	1530	0.24
38	α-gurjunene	25.790	1545	0.55
39	α-agarofuran	26.030	1553	0.57
40	neryl acetate	26.236	1563	1.06
41	phenylethyl tiglate	26.894	1591	2.07
42	δ-selinene	27.816	1631	8.69
43	agarospirol	28.090	1643	0.46
44	isoledene	28.474	1660	1.18
45	propanoate	28.657	1667	0.43
46	geranyl tiglate	29.475	1703	3.05
47	mintsulfide	30.373	1746	1.81

1.2.2.4 The Uses of *Pelargonium graveolens* in Medicine

1. Antioxidant activity:

The antioxidant activity of geranium essential oil assigned to the presence of monoterpenes. The geraniol and β-citronellol were the major monoterpenes detected in the chemical composition of geranium oils. The

Measured antioxidant activities could be due to the synergistic effects of two or more compounds present in the oils (Singh *et al*.,2009).

2. Antimicrobial:

The pure and neat essential oils showed the most extensive inhibition zones and they were very effective antimicrobial system against (*Listeria monocytogenes ,Salmonella enteritidis, Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis*) comparing to chloramphenicol and amoxicillin(Ghannadi *et al*.,2012).

3. Antitumor:

Most of the principle components present in geranium essential oils are monoterpenes. Monoterpenes have shown prevention ofmammary, lung, skin, and liver and forstomach cancers inrat models (Haag*et al*., 1992).

4. Medicinal Uses:

It is very effective for menopausal problems, diabetes, blood disorders, and throat infections and as anerve tonic and works well as a sedative (Petrie and Peck, 2000; Karato *et al*., 2006; Bussmann *et al*., 2013).

1.2.3 Oxidative Damage and Diseases

Oxidative stress is suspected to be important in neurodegenerative diseases including Lou Gehrig's disease , Parkinson's disease, Alzheimer's disease,Huntington's disease and Multiple sclerosis (Patel and Chu, 2011). Indirect evidence via monitoring biomarkers such as reactive

oxygen species, and reactive nitrogen species production, antioxidant defense indicates oxidative damage may be involved in the pathogenesis of these diseases (Nunomura *et al.,* 2005;Bošković *et al.,* 2011), while cumulative oxidative stress with disrupted mitochondrial respiration and mitochondrial damage are related with Alzheimer's disease, Parkinson's disease, and other neurodegenerative diseases (Ramalingam and Kim, 2012).

Oxidative stress is thought to be linked to certain cardiovascular disease, since oxidation of low density lipoprotein inthe vascular endothelium is a precursor to plaque formation. Oxidative stress also plays a role in the ischemic cascade due to oxygen reperfusion injury following hypoxia. This cascade includes both strokes and heart attacks. Oxidative stress has also been implicated in chronic fatigue syndrome (Nijs*et al.,* 2006) and contributes to tissue injury following irradiation and hyperoxia, as well as in diabetes.

Oxidative stress is likely to be involved in age-related development of cancer. The reactive species produced in oxidative stress can cause direct damage to the DNA and are therefore mutagenic, and it may also suppress apoptosis and promote proliferation, invasiveness and metastasis (Halliwell, 2007). Infection by *Helicobacter pylori* which increases the production of reactive oxygen and nitrogen species in human stomach is also thought to be important in the development of gastric cancer. (Handa *et al.,* 2011).

1.2.3.1 Antioxidants

Antioxidants are compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidizing chain reactions. In general, there are two basic categories of

antioxidants, natural and synthetic. Recently, interest has increased considerably in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants, which are being restricted due to their carcinogenicity (Osawa, 1994).

Herbs have been used for a large range of purposes including medicine, nutrition, flavorings, beverages, dyeing, repellents, fragrances, cosmetics, charms, smoking, and industrial uses. Since prehistoric times, herbs were the basis for nearly all medicinal therapy until synthetic drugs were developed in the nineteenth century. Today, herbs are still found in 40% of prescription drugs (Ito *et al*., 1984).Various herbs along with vegetables and fruits contain numerous phytochemicals in addition to phenolic compounds, such as nitrogen compounds, carotenoids, and ascorbic acid (Kahkonen *et al*., 1996). Many of these phytochemicals possess significant antioxidant capacities that are associated with lower incidence and lower mortality rates of cancer in several human cohorts (Velioglu *et al*., 1998).

The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Velioglu *et al*., 1998).

1.2.3.2 Classification of Antioxidants:

Antioxidants are grouped into two categories:

(1) Primary or natural antioxidants.

(2) Secondary or synthetic antioxidants.

Primary or Natural Antioxidants

They are the chain breaking antioxidants which react with lipid radicals and convert them into more stable products. Antioxidants of this group are mainly phenolic in structures and include the following (Hurrell., 2003):

(1) Phytochemicals: These are phenolic compounds that are neither vitamins nor minerals. These include: Flavonoids, Catechins …etc.

(2) Antioxidants vitamins: It is needed for most body metabolic functions. They include vitamin C,vitamin E and vitamin B.

(3) Antioxidants minerals: These are co-factor of antioxidants enzymes. Their absence will definitely affect metabolism of many macromolecules such as: carbohydrates. Examples: selenium, copper, iron, zinc and manganese.

Secondary or Synthetic Antioxidants (Hurrell., 2003)

These are phenolic compounds that perform the function of capturing free radicals and stopping the chain reactions, the compounds include:

i. Butylated hydroxyl anisole (BHA).
ii. Butylatedhydroxyrotoluene (BHT).
iii. Propyl gallate (PG) and metal chelating agent (EDTA).
iv. Tertiary butyl hydroquinone (TBHQ).
v. Nordihydroguaretic acid (NDGA).

1.2.3.3 Types of Antioxidants

A- Ascorbic acid

Ascorbic acid or "vitamin C" is a monosaccharide antioxidant found in both animals and plants. As one of the enzymes needed to make ascorbic acid has been lost by mutation during human evolution, it must be obtained from the diet and is a vitamin. Most other animals are able to produce this compound in their bodies and do not require it in their diets. Ascorbic acid is a reducing agent and can reduce and thereby neutralize reactive oxygen species such as hydrogen peroxide (Ortega, 2006).

B- Glutathione

Glutathione is a cysteine-containing peptide found in most forms of aerobic life. It is not required in the diet and is instead synthesized in cells from its constituent amino acids (Meister and Anderson, 1983).

C- Melatonin

Melatonin is a powerful antioxidant that can easily cross cell membranes and the blood-brain barrier. Melatonin, once oxidized, cannot be reduced to its former state because it forms several stable end-products upon reacting with free radicals. Therefore, it has been referred to as a terminal (or suicidal) antioxidant (Reiter *et al*., 1997; Tan *et al*., 2000).

D-Tocopherols and tocotrienols (vitamin E)

Vitamin E is the collective name for a set of eight related tocopherols and tocotrienols, which are fat-soluble vitamins with antioxidant properties. Of these, tocopherol has been most studied as it has the highest bioavailability, with the body preferentially absorbing and metabolizing this form (Herrera and Barbas, 2001).

It has been claimed that the tocopherol form is the most important lipid-soluble antioxidant and that it protects membranes from oxidation by reactingwith lipid radicals produced in the lipid peroxidation chain reaction. This removes the free radical intermediates and prevents the propagation reaction from continuing (Herrera and Barbas, 2001; Packer *et al*., 2001).

1.2.3.4 The Antioxidant Properties of *Pelargonium graveolens*

The antioxidant activity of phenolic acids is related to the number and position of hydroxylgroups within the molecule (Jung*et al*., 2003).

The most abundant components(> 4%) of the *Pelargonium graveolens* leaves essential oil were β-citronellol (29.3%) followed by geraniol (10.53%), linalool(10.42%) and citronellyl formate (9.54%). The presence of essential oils in *Pelargonium graveolens* was proved that the antioxidantactivity could be attributed in part tothe presence of compounds such as β-citronellol and geraniol and its ability to decompose free radicals byquenching reactive oxygen species and trapping radicalsbefore reaching their cellular targets (Ben Slima *et al*., 2013)

1.2.4 Mammalians Cell Cycle:

1.2.4.1 Control and Regulation:

The development of mammals from single fertilized egg into a complete adult requires many round of division. In each division, cell will enter a series of events, in general called “cell cycle” (Bashir *et al*., 2004).

This cycle includes accurate duplication of the genome during the DNA synthesis phase (S phase), and segregation of complete sets of

chromosomes to each of the daughter cells in M phase (Figure 1.2). The somatic cell cycle also contains "Gap"phases, known as G, which connects the completion of M phase to initiation of S phase in the next cycle, and G2,which separates the S and M phases. Dependent on environmental and developmental signals, cells in G1 maytemporarily or permanently leave the cell cycle and enter reversibly or irreversibly a quiescent or arrested phase known as G0 and (Bertoli*et al*., 2013).

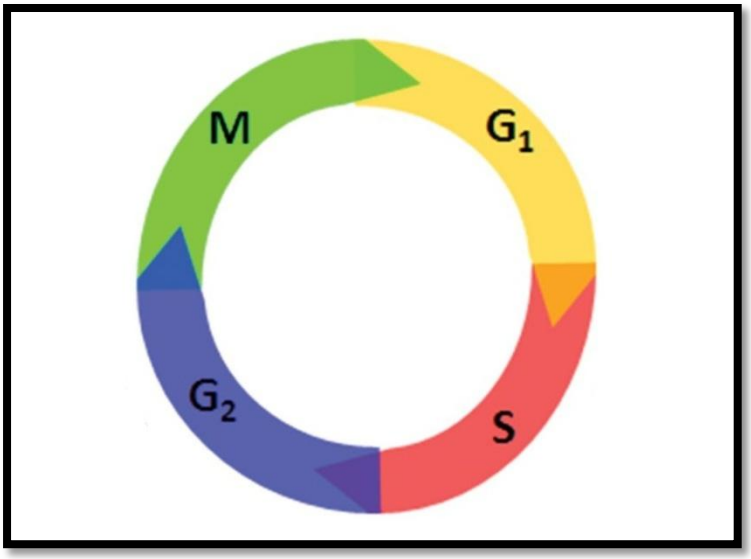


Figure (1-2): Somatic Cell Cycle (Suryadinata R *et al*., 2010).

As the eukaryotic cell cycle is a complicated process, eukaryotes have evolved a network of regulatory proteins, known as the cell cycle control system, which monitors and dictates the progression of the cell through the cell cycle (Bertoli *et al*., 2013).

1.2.4.2 Check Points

During eukaryotic cell cycle progression, signaling pathways monitor the successful completion of upstream events prior to proceeding to the next phase. These regulatory pathways are commonly referred to as

cell cycle checkpoints (Kliein *et al*., 2007). Cells can arrest at cell cycle checkpoints temporarily toallow for: (i) cellular damage to be repaired; (ii) the dissipation of an exogenouscellular stress signal; or (iii) availability of essential growth factors, hormones,or nutrients. Defects in cell cycle checkpoints canresult in gene mutations, chromosome damage, and aneuploidy, all of which cancontribute to tumorgenesis (Chen *et al*., 2009).

G1 (Restriction) Checkpoint:

The G1 checkpoint, also known as the restriction point in mammalian cells. As the cell progresses through G1, depending on internal and external conditions, it can either delay G1 or enter a quiescent state known as G0, or proceed past the restriction point. The decision to commit a new round of cell division occurs when the cell activates cyclin-CDK-dependent transcription which promotes entry into S phase (Bertoli *et al*., 2013).

When DNA damage occurs, the rapid response involves phosphorylation events that initiate with kinases which act as the sensors, depending on the type of damage. These kinases phosphorylate and activate the effector kinases Chk2 and Chk1, respectively, which in turn phosphorylate the phosphatase Cdc25A, thus marking it for ubiquitination and degradation. To maintain the arrest, another response is initiated, by which Chk2 or Chk1 phosphorylate p53, a tumor suppressor gene, the p53 then acts a transcriptional activator of several target genes, including p21, an inhibitor of the G1 to S promoting complex cyclin E-CDK21 (Bartek and Lukas, 2001; Bertoli *et al*., 2013).

G2 Checkpoint

The biochemical pathways involved in the DNA damage-induced G2 arrest are thought to involve signaling cascades that converge to inhibit the activation of cyclin dependent cyclase (Cdc2) (Calonge and O'Connell, 2008). After DNA damage, members of the phosphatidyl inositol 3-kinase family (PI-3K) become activated and initiate signal transduction pathways that regulate DNA repair andcell cycle progression. Several members of the PI-3K family can directlyphosphorylate p53 (Ling *et al*., 2010).

Metaphase checkpoint

The mitotic spindle checkpoint occurs at the point in metaphase where all the chromosomes should have aligned at the mitotic plate and be under bipolar tension. The tension created by this bipolar attachment is what is sensed, which initiates the anaphase entry. To do this, the sensing mechanism ensures that the anaphase-promoting complex (APC/C) is no longer inhibited, which is now free to degrade cyclin B, which harbors a D-box (destruction box), and to break down securin. (Peters *et al*., 1998). The latter is a protein whose function is to inhibit separase, which in turn cuts the cohesins, the protein composite responsible for cohesion of sister chromatids. (Ciosk *et al*., 1998).Once this inhibitory protein is degraded via ubiquitination and subsequent proteolysis, separase then causes sister chromatid separation. (Karp and Gerald, 2005). After the cell has split into its two daughter cells, the cell enters G_1.

1.2.4.3The Cell Cycle and Cancer

Under normal conditions, growth regulating mechanisms maintain homeostasis. Homeostasis within a cell is regulated by the balancebetween proliferation, growth arrest and apoptosis. Disturbance in

the balance between cell growth and death may result in hyperplasia or neoplasia (Erenpreisa and Errag, 2007).

Once the stimulus is removed, however, the process of hyperplasia is reversible, whereas in cancer cells, it is irreversible (Houtgraaf *et al*., 2006). Cancer cells are characteristically independent of growth stimulus due to mutation of intracellular signal pathways. Such independence facilitates re-entry into the cell cycle, irrespective of positive or negative external stimulus (Pietenpol and Stewart, 2002).

It is evident therefore, that target cells which are predisposed to genetic lesions, may lead to malignancy in the early stages of carcinogens is. However, efficient control mechanisms are shown to exist, which detect damage to cells. Destruction of the damaged cells help to minimize malignant progression, occurring in two distinct ways: the first is by necrosis, which is characterized by cell swelling and rapid degeneration. The second type, apoptosis is characterized by cell shrinkage with blistering of plasma membrane and DNA fragmentation (Agami and Bernards, 2002)**.**

The cell cycle is a specific sequence of events which enables cells to grow and replicate. The two gene types which play a major role in the cell cycle, and therefore in the development of cancer, are the oncogenes (e.g. HER2/neu, Ras, c-Myc, etc.) and tumor suppressor genes (e.g. p53 and Rb) (Jeggo and Löbrich, 2006;Foster, 2008).

DNA damage may result from some environmental insult, leading to disruption of cell cycle control mechanisms. Cell cycle checkpoints are these controlling mechanisms of cell growth and development, regulating the complex and intricate network of interactions. Mutations of these checkpoints are evidenced in all types of cancer (Yasuda, 2008).

1.2.5 Apoptosis and Cancer

Apoptosis is a physiological process that eliminates harmful and severely damaged cells and maintains tissue homeostasis in multicellular organisms (Young *et al.,* 1991). The process is defined based on changes in cellular morphology and biochemical features, including chromatin condensation, DNA fragmentation, cytoplasm vacuolation, plasma membrane blebbing, and cell shrinkage. Eventually, the cells breaks into small membrane surrounded fragments (apoptotic bodies), which are cleared by phagocytosis without inciting an inflammatory response (Kerr,1994).

In mammalian cells, apoptosis occurs through two distinct molecular pathways, the intrinsic or mitochondrial pathway which activated by intracellular events and depends on the release of proapoptotic factors from the mitochondria. Standard chemotherapy and radiotherapy for cancer predominately initiate apoptosis via the intrinsic pathway and thus may positively select for cancer cells that can evade intrinsic apoptosis signaling (Estaquier *et al.*, 2012).

By contrast, the extrinsic apoptosis pathway receives signals through the binding of extracellular protein death ligands to proapoptotic death receptors (DRs). In some cancer cells following extrinsic apoptosis signaling, cell death can still occur in the absence of intrinsic apoptosis. Both pathways lead to the hierarchical activation of specialized proteases called caspases (Sayers, 2011).

Thornberry and Lazebnik (1998) stated that the apoptotic process is executed mainly by a family of cysteine proteases called caspases. The activation of a family of intracellular cysteine proteases which cleave their substrates at aspartic acid residues, known as caspases for Cysteine Aspartyl specific Proteases (Alnemri *et al.*, 1996). These proteases are

present as inactive zymogens in essentially all animal cells, but can be triggered to assume active states, generally involving their proteolytic processing at conserved aspartic acid (Asp) residues.

Suppression of apoptosis in carcinogenesis plays a central role in the development and progression of cancer. Tumor cells use a variety of molecular mechanisms to suppress apoptosis. Hence, induction of apoptosis in tumor cells is a specific therapeutic approach towards cancer therapy (Elmore, 2007).

1.2.6 Caspases

Apoptosis is executed by a subfamily of cysteine proteases known as caspases. In mammalian cells, a major caspase activation pathway is the cytochrome initiated pathway. Caspases are normally inactive in their zymogen form or pro-form. During apoptosis, a procaspase is proteolytically cleaved to generate a small subunit and a large subunit, and two cleaved caspase molecules form a heterotetramer, which is the active form of the enzyme(Plas and Thompson, 2002).

The proteolytic cleavage of a caspase can induce a dramatic conformational change that exposes the catalytic pocket of the enzyme, and therefore results in its activation. The proteolytic activation of caspases can be achieved either by autocatalysis or by an upstream protease (Liu *et al*., 2010).

A caspase that cleaves and activates itself is called an initiator caspase. Once an initiator caspase is activated, it can trigger a cascade to activate downstream executioner caspases. Subsequently, the activated executioner caspases cleave numerous cellular targets to destroy normal cellular functions, activate other apoptotic factors, inactivate

antiapoptotic proteins, and eventually lead to apoptotic cell death (Yoshida, 2007).

The central role of caspase activity in apoptosis is further underscored by the observation that inhibition of caspase activity can block apoptosis and all classical morphological changes associated with the process (Krysko *et al*., 2008).

Therefore, understanding caspase activation is essential for apoptosis research and development of therapies for apoptosis-related diseases.

1.2.7 Cytochrome Complex

The Cytochrome complex or cytochromec is a small heme-protein found loosely associated with the inner membrane of the mitochondrion. Cytochrome c is a highly water soluble protein, unlike other cytochromes, with a solubility of about 100 g/L and is an essential component of the electron transport chain, where it carries one electron. It is capable of undergoing oxidation and reduction, but does not bind oxygen. It transfers electrons between complexes III (Coenzyme Q - Cyt C reductase) and complexes IV (Cyt C oxidase). In humans, cytochrome c is encoded by the CYCS gene (Tafani *et al*., 2002)

Cytochrome c is a component of the electron transport chain in mitochondria. The heme group of cytochrome c accepts electrons from the bc1 complex and transfers electrons to the complex IV. Cytochrome c is also involved in initiation of apoptosis. Upon release of cytochrome c to the cytoplasm, the protein binds apoptotic protease activating factor-1 (Apaf-1) (Er *et al*., 2006).

Cytochrome c can catalyze several reactions such as hydroxylation and aromatic oxidation, and shows peroxidase activity by oxidation of various electron donors such as 2,2-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), 2-keto-4-thiomethyl butyric acid and 4-aminoantipyrine and Cytochrome c is also involved in one form of nitrite reductase (Schneider and Kroneck ,2014).

1.2.7.1 Cytochrome C and the Role of Mitochondria in Apoptosis

Cytochrome C released from the mitochondria during the early stages of apoptosis, although the precise mechanisms regulating this event remain elusive (Ott *et al*., 2002).Ott *et al*., (2002), proposed that mitochondrial Ca^{2+} overload promotes the opening of the permeability transition pore. This increased permeability of the inner mitochondrial membrane leads to matrix swelling, rupture of the outer mitochondrial membrane, and the release of cytochrome C. Ca^{2+}-independent cytochrome c release seems to be governed by different members of the Bcl-2 family of proteins. In particular, the oligomeric form of the pro-apoptotic protein Bax stimulates cytochrome c release.

Cytochrome C binds to cardiolipin in the inner mitochondrial membrane, thus anchoring its presence and keeping it from releasing out of the mitochondria and initiating apoptosis. While the initial attraction between cardiolipin and cytochrome c is electrostatic due to the extreme positive charge on cytochrome c, the final interaction is hydrophobic, where a hydrophobic tail from cardiolipin inserts itself into the hydrophobic portion of cytochrome c (Orrenius and Zhivotovsky, 2005).

The sustained elevation in calcium levels precedes cytochrome c release from the mitochondria. The release of small amounts of cytochrome c leads to an interaction with the IP3 receptor on the endoplasmic reticulum which causing endoplasmic reticulum calcium release. The overall increase in calcium triggers a massive release of cytochrome c (Boehning *et al*., 2003). This explains how the endoplasmic reticulum calcium release can reach cytotoxic levels. This release of cytochrome c in turn activates caspase 9. Caspase 9 can then go on to activate caspase 3 and caspase 7, which are responsible for destroying the cell from within (Logue *et al*., 2008).

1.2.8 The role of Plant Natural Product and Cell Cycle Arrest

Naturally based compounds play an essential role in the primary health care of the majority of the world's population. Of the 250,000 species of plants, more than one thousand have been found to contain agents with significant anticancer properties (Muhtasib, 2006).

A drug development new approach includes the discovery of small molecules that are specifically able to attack the aberrant genetic alterations and deregulated biochemical pathways that are responsible for cancer while sparing healthy tissues. By targeting cancer cells this new generation of anticancer agents promises to be more selective and less toxic than current drugs that were used for cancer prevention and treatment (Dhorajiya *et al*., 2012).

In selecting drug targets for novel therapies, interest has been focused on cell cycle molecular targets particularly in those pathways that are most frequently deregulated in cancers. The Cyclin-dependent Kinases (CDKs) are frequently deregulated in cancers; mutations and

overexpression of these kinases, mainly CDK4, have been reported and proposed to be oncogenic events (Shah and Schwartz, 2001).

Some of CDK inhibitors derived from plant natural products and flavopiridol is one of the very promising small molecules that are currently one of the most advanced CDK inhibitors in clinical development. Flavopiridol is a flavonoids related to rohitukine, an alkaloid isolated from a plant in India. Because of its promising anticancer effects in many cancer models it is the subject of extensive investigations (Muhtasib, 2006). This flavonoid can cause cell cycle arrest at G1 or G2 and can inhibit the activation and activity of several CDKs, specifically CDK1, CDK2, CDK4 and CDK6 (Schang, 2004). This inhibition occurs through its ability to interact with ATP-binding site of all CDKs. Moreover, flavopiridol inhibits CDK7, a CAK, leading to the loss of the activation phosphorylation of most CDKs (Dong *et al*, 2011).

1.2.9Mammalian Cell Viability:

Cell viability is often defined as the number of healthy cells in a sample. Cell viability methods loosely can be categorized into those which analyze whole populations and those which involve analysis of individual cells. One of the earliest methods for assessing cell viability was trypan blue dye exclusion assay, which is still widely used today. It is based on the principle that viable cells have an intact cell membrane which can therefore exclude the trypan blue dye. Dead cells take up trypan blue, and appear blue as a consequence, as their membrane is no longer able to control the passage of macromolecules (Louis and Siegel, 2011).

Over time the assays for viability have become more complex.Increasingly, dyes which rely on the metabolic activity of cells are gaining favor as they can be performed on adherent cells and therefore lend themselves to high-throughput analysis (Riss and Moravec, 2004).

The best known, metabolic dye is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and the development of a 96-well assay format dramatically increased the capacity (Mosmann, 1983). This assay relies on the conversion of soluble tetrazolium into insoluble blue formazan crystals by reduction (Berridge *et al*., 2005). Other similar dyes have become increasingly popular, such as XTT and WST derivatives. Recently, there are wide arrays of protocols and techniques have been frequently used in detecting the viability of mammalian cells, ranging from the most routine trypan blue technique to the highly complex analysis like Flow Cytometer and High Screening Microscopy (Stoddart, 2011).

Cell viability and cytotoxicity assays are used for drug screening and cytotoxicity tests of chemicals usually by determining the number of viable cells remaining after a defined incubation period. Figure (1-3) indicates various reagents used for cell viability detection. They are based on various cell functions such as enzyme activity, cell membrane permeability, cell adherence, ATP production, co-enzyme production, and nucleotide uptake activity.Many have established methods such as Colony Formation method, Crystal Violet method, Tritium-Labeled Thymidine Uptake method, MTT, and WST methods, which are used for counting the number of live cells (Pegg, 1989).

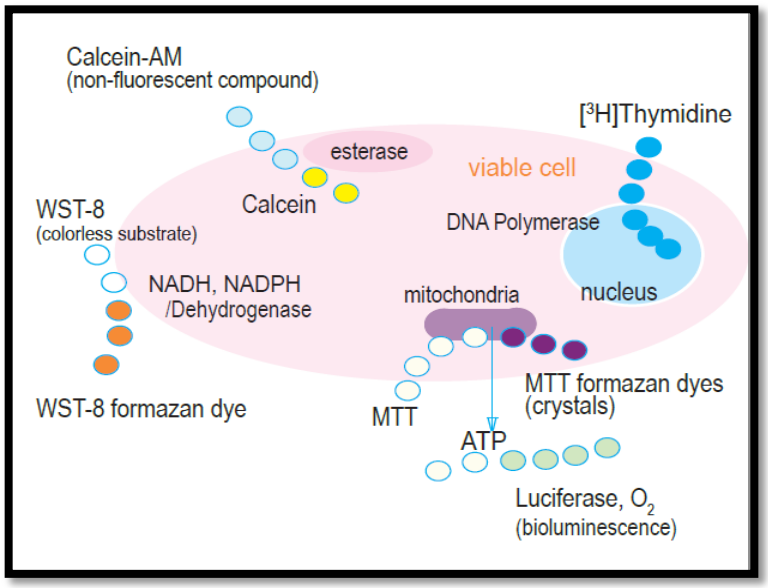


Figure (1-3): Reagents and Chemicals Used for Cell Viability Detection(from Dojindo Cell Counting Kit-8 instructions).

The desired approach is to use a convenient and cost-effective method that predicts *in vivo* toxicity by measuring a surrogate marker to indicate the viable cell number compared to untreated controls. For example, The MTT tetrazolium assay was the first convenient 96-wellmethod developed for screening large numbers of samples (Mosmann, 1983); however the major disadvantages of the tetrazolium and resazurin reduction assays are that they require a 1–4 hours period of incubation with viable cells for conversion into the colorimetric or fluorescent indicator; chemical interference is caused by reducing compounds; and perhaps most importantly, the tetrazolium and resazurin compounds used to indicate cell viability are themselves toxic to cells.

On the other hand, detection of ATP has become the gold standard cell viability assay for high-throughput screening. The level of ATP is closely regulated by viable cells. The process of cell death results in a

loss of ability to synthesize new ATP along with a rapid depletion of cytoplasmic ATP by endogenous ATPases. These properties combine to make ATP a valid marker of viable cells in culture (Severson *et al*., 2007; Riss *et al*., 2006).

1.2.10High-Content Screening (HCS) Analysis

High- Content Screening (HCS) is a cellular imaging- based approach that played a key role in the detection of toxicity and classification of compounds based on observed patterns of reversible and irreversible cellular injury. HCS provide multi-parametric analysis of compound toxicity at the level of individual cells (Abraham *et al*., 2008).

The ArrayScan high-content screening System, Cellomics, Pittsburgh, PA (Fig. 1-4), is a cytometer based on a fully automated fluorescence microscope that acquires images from cells seeded in multi-well plates (Giuliano *et al*., 1997). This instrument performs automated measurements, including the intensity and localization of the fluorescence signals within single cells and within subcellular compartments over a wide cell population. The image analysis is performed in real time for up to4 fluorescence channels. The ArrayScan has been used to analyze the execution of several cellular events that can be tracked by fluorescence immunocytochemistry, such as variations in cellular morphology (Simpson *et al*., 2001) and intracellular trafficking (Ghosh *et al*., 2000) or to characterize NF-kB translocation (Ding *et al*., 1998).

The "cell- centric" approach opens a new frontier in the high-throughput screening (HTS) of pharmaceutical compounds (Giuliana and Taylor, 1998;Taylor *et al*., 2001).

The main advantage of such approach resides in the possibility of acquiring both intrinsic data (for individual cells) and population data within a single analysis (Gasparri *et al*., 2004).

Chapter two

Materials and Methods

2. Materials and Methods

2.1 Materials

2.1.1 Equipment and Apparatuses

The following equipments and apparatus were used throughout this study:

Equipment	Company
Balance	Ohans (France)
Blander	K and K (Korea)
Centrifuge	Eppendorf(Germany)
Inverted Microscope	Olympus (Japan)
Cooling Centrifuge	HemleLabortechnink(Germany)
Co2 incubator	Gallenkamp(England)
High Content Screening Array Scan	Thermo Scientific(USA)
Incubator	Memaret (Germany)
Laminar Air Flow	K and K (Korea)
Lyophilizer	Labcanco (USA)
Magnetic stirrer	K and K (Korea)
Micropipette	Witey (Germany)
Micro-titer Plate Reader	Bio-Rad (Germany)
Oven	Memaret (Germany)
Rotary Evaporator	Buchii (Switzerland)
Sensitive Balance	Sartorius(Germany)
U.V-Visible Spectrophotometer	Shimadzu(Japan)
Vortex	Griffin(England)
Water Bath	Gallenkamp(England)

2.1.2 Chemicals and Biological Materials

Chemicals and biological materials used in this study were classified according to their manufacturer as follow:

Material	Company
Di-ethyl ether, Hexane	BDH(England)
DMSO, EDTA, Trypsin, Fetal Bovine Serum, DPPH, RPMI-1640media, DMEM media,Ascorbic acid, Doxorubicin.	Sigma-Aldrich(USA)
Absolute Methanol,Formaldehyde	Hemadia (India)
Streptomycin, Benzyl Penicillin	Ajenta Pharm (India)
NaCl, KCl, NaH_2PO_4	Merck (Germany)
Na_2HPO_4, $NaHCO_3$	Analar (England)

2.1.3 Kits

Kits used in the study are listed as follow:

Kit	Company
Cytotoxicity3 Kit	ThermoScientific(USA)
ROS Kit	Thermo Scientific(USA)
MTT kit	Intron Biotech (Korea)

2.1.4 Cell lines

The following cell lines were from Biotechnology Research Center in Al- Nahrain University:

1. MCF7 (Breast Cancer Cells): Cell line was isolated in 1970 from a 69 year old Caucasian woman.MCF-7 is the acronym of Michigan Cancer Foundation-7, referring to the institute in Detroit where the cell line was established in 1973. The passage number used was 176(Lacroix and Leclercq, 2004).
2. PC3 (Human Prostate Cancer Cells):Cell line was established in 1979 from bone metastasis of grade IV of prostate cancer in a 62-year old Caucasian male. These cells do not respond to androgens, glucocorticoids, or epidermal or fibroblast growth factors,and the passage number used was142(Kaighn *et al*., 1979).

2.1.5 Reagents, Solutions and Solvents

2.1.5.1 Solvents used in *Pelargonium graveolens* extraction (Alexander-Lindo *et al*., 2007):

- **Absolute Methanol99.9%**

Absolute methanol was added to 100 g of *Pelargonium graveolens* leaves and stirred for 4 hours before the filtration, centrifugation and evaporation.

- **Di-ethyl ether 99.5%**

Di-ethyl ether was added to 100 g of *Pelargonium graveolens* leaves and left for 4 hours before the filtration, centrifugation and evaporation.

- **Hexane 95%**

Hexane was added to 100 g of *Pelargonium graveolens* leaves and left for 4 hours before the filtration, centrifugation and evaporation.

2.1.5.2 Solutions Used in Antioxidant Activity Test:

The following solutions were prepared according to Rajesh and Natvar, (2011).

- **Methanol-DMSO Mixture (9:1 v/v) Solution:**

Methanol-DMSO mixture (9:1 v/v) solution was prepared by adding 9 volumes of methanol to 1 volume of DMSO.

- **DPPH Radical Solution:**

DPPH radical was dissolved in DMSO-Methanol mixture to prepare (0.1mg/ml) DPPH radical stock solution.

C-Vitamin C Solution:

Ascorbic acid powder was dissolved in DMSO-Methanol mixture to prepare a concentration of (0.1mg/ml) vitamin C stock solution.

2.1.5.3Media, Reagents and Solutions Used in Tissue Culture Technique:

Media, reagents and solutions used for cell culture were prepared according to Freshney, (2010).

A-antibiotic solution:

1. Streptomycin (1g/vial):

It was prepared by dissolving vial contents in 5 ml of sterile distilled water to prepare a stock solution (200,000µg/ml). The stock was stored at -18ºC.

2. Benzyl Penicillin:

It was prepared by dissolving the contents of one vial which has 10^6IU 5 ml of sterile distilled water to prepare a stock solution (200,000 IU/ml). The stock was stored at -18ºC.

B. Sodium Bicarbonate Solution:

The solution was prepared by dissolving 4.4 g of $NaHCO_3$ in 100 ml distilled water. The solution was sterilized by autoclaving and kept at 4ºC until use.

C. Phosphate Buffer Saline (PBS):

This buffer was prepared by dissolving 8 g NaCl, 0.2 g KCl, 1.15 g NaH_2PO_4 and 0.2g Na_2HPO_4 in 1 litter of distilled water, pH was adjusted to 7.2. The solution was sterilized by autoclaving and stored at 4ºC until use.

D. Trypsin Solution:

It was prepared by dissolving 1 g of trypsin powder in 100 ml PBS and sterilized by filtration. The solution was dispensed into 10 ml aliquots and stored at -20ºC.

E. EDTA Solution:

It was prepared by dissolving 1 g of ethylene-diamine-tetra Acetic Acid (EDTA) in 100 ml of PBS and sterilized in autoclave for 10 minutes. The solution was dispensed in 10 ml aliquots and stored at 4ºC.

F. Trypsin-EDTA Solution:

It was prepared by mixing 20 ml of trypsin solution, 10 ml EDTA solution and 370 ml PBS. The mixture was stored at 4ºC.

G. Dulbecco's Modified Eagle Complete Medium (DMEM):

A ready to use package (100 ml) DMED was used throughout this study. The medium was already supplied with 4-(2-hydroxyethyl)-1piperazine-ethane sulfonic acid (HEPES) and L-glutamine as illustrated by manufacturer.

The medium was completed by adding the following ingredients:

Penicillin G 10^3IU

Streptomycin 0.001 g

Sodium Bicarbonate 1%

Fetal Bovine Serum 10 %

H. Roswell Park Memorial Institute – 1640 Medium (RPMI):

A ready to use package (100 ml) DMED was used throughout this study. The medium was already supplied with 4-(2-hydroxyethyl)-1piperazine-ethane sulfonic acid (HEPES) and L-glutamine as illustrated by manufacturer.

The medium was completed by adding the following ingredients:

Penicillin G	10^3 IU
Streptomycin	0.001 g
Sodium Bicarbonate	1%
Fetal Bovine Serum	10 %

I. Serum Free Medium:

Serum free medium is DMEM or RPMI-1460excluded from fetal calf serum.

G. Doxorubicin:

Medication used in cancer chemotherapy. It is derived by chemical semi-synthesis from a bacterial species. It is an anthracycline antitumor antibiotic. (Tacar *et al.*,2013).

2.2 Methods

2.2.1 Sterilization Methods (Atlas *et al*., 1995)

A. Moist Heat Sterilization

Solutions and some laboratory utensils were sterilized by autoclaving at 121ºC, 15 psi for 15 minutes.

B. Dry Heat Sterilization

Electric oven was used to sterilize glassware and others by heating at 180ºC for 2 hours.

C. Filtration (Membrane Sterilization)

Solutions that sensitive to heat were sterilized by filtration using Millipore's 0.22μm in diameter filters.

2.2.2 Sample collection

Leaves of *Pelargonium graveolens* were collected from the local plant nurseries in Baghdad, Iraq. They were authenticated by Professor Dr. Ali Al-Mosawy, Ph.D. in Plant Taxonomy, Department of Biology / College of Sciences / Baghdad University, Baghdad, Iraq.

2.2.3 Extraction of *Pelargonium graveolens*

2.2.3.1 Preparation of *Pelargonium graveolens* Leaves for Extraction:

The collected *Pelargonium graveolens* leaves were washed thoroughly under tap water to remove traces and dust. After that *Pelargonium graveolens* leaves were dried by incubation overnight at 37°C. After incubation the fully dried leaves were blended in order to obtain a fine powder for extraction purpose.

2.2.3.2 Alcoholic, di-ethyl ether and hexane Extraction of *Pelargonium graveolens* (Alexander-Lindo *et al*., 2007).

1- The dried powder (100g) of *Pelargonium graveolens* were treated with 500ml of the solvent (Methanol, Di-ethyl ether and Hexane).

2- The mixture was homogenized with magnetic stirrer for 4 hours at room temperature.

3-The mixture was filtrated by Whatman filter paper No.1.The filtrate solvent was cooled centrifuged (15°C) at 1500 rpm for 30 minutes.

4-The supernatant was evaporated at 40°C using rotary evaporator under vacuum to obtain the final *Pelargonium graveolens* crude extract.

5- The pellet resulted from filtration and centrifugation was subjected to further extraction by repeating the same procedure.

The above procedure was applied by using three different solvents: methanol, di-ethyl ether and hexane in order to obtain three different types of *Pelargonium graveolens* crude extracts.

6- The crude extracts for each of methanol, di-ethyl ether and hexane were lyophilized overnight and the lyophilized powder was stored in refrigerator at $4^{\circ}C$.

2.2.4 Antioxidant Activity

Antioxidant activity of methanol, di-ethyl ether and hexane *Pelargonium graveolens* leaves crude extracts was detected by using DPPH radical scavenging assay according to the procedure described by Rajesh and Natvar, (2011) and as follow:

1. Aliquots (0.5ml) of two fold serial dilution from *Pelargonium graveolens* crude extracts and ascorbic acid (75, 125, 250, 500, 1000 µg/ml) were added in reaction test tubes.
2. Simultaneously, 3ml of methanol-DMSO mixture and 0.3ml of DPPH solution were added to each concentration.
3. Samples were shaken few seconds and allowed to stand at room temperature for 60 minutes.
4. Radical scavenging activity of samples against the stable DPPH radical was determined spectrophotometrically. The colorimetric changes (from deep violet to light yellow) when DPPH is reduced were measured at 517 nm .The % inhibition of radical by the samples was calculated according to the following formula:

$$\%\text{inhibition} = \frac{\text{Absorbance of – ve control - Absorbance of sample}}{\text{Absorbance of – ve control}} \times 100$$

The negative control consists of methanol-DMSO mixture and DPPH solution, while ascorbic acid was used as reference.

The experiment was done in triplicate for each substance and the IC50 value of the sample (the concentration of sample required to inhibit 50% of the DPPH free radical) was calculated using log dose inhibition curve.

2.2.5 The Cytotoxic Effect of *Pelargonium graveolens* Crude Extracts:

This *in vitro* method was performed to investigate the effect of methanol, di-ethyl ether and hexane crude extracts of *Pelargonium graveolens* on two types of cell lines (MCF7 and PC3).

2.2.5.1 Cell Line Maintenance (Freshney, 2010).

When the cells in the vessel formed confluent monolayer, the following protocol was performed:

A- The growth medium was aspirated and the cell sheet washed with PBS.

B- Two to three ml Trypsin-EDTA solution was added to the cell. The vessel was turned over to cover the monolayer completely with gentle rocking. The vessel allowed incubating at 37°C for 1 to 2 minutes. until the cells had detached from the vessel.

C- Fresh complete RPMI medium (15-20 ml) was added and cells were dispersed from the wedding surface into growth medium by pipetting.

D- Cells were redistributed at required concentration into culture vessels, flasks or plates whatever needed and incubated at 37°C in 5% CO2 incubator.

E- Cell concentration was achieved by counting the cells using the haemocytometer and applying the formula:

Total Cell Count/ml: cell count x dilution factor (or sample volume) $x10^4$

2.2.5.2 MTT cytotoxicity Assay (Freshney, 2010).

The cytotoxic effect of *Pelargonium graveolens* crude extracts was performed by using MTT kit:

A- Kit contents:

- MTT solution 1 ml x 10 vials.
- Solubilization solution 50 ml x 2 bottle.

B- Protocol:

- Tumor cells ($1x10^4$ – $1x10^6$ cells/ml) were grown in 96 flat well micro-titer plates, in a final volume of 200 µl complete culture medium per each well. The microplate was covered by sterilized parafilm and shacked gently.
- The plates were incubated at 37°C, 5% CO_2 for 24 hrs.
- After incubation, the medium was removed and two fold serial dilutions of *Pelargonium graveolens* crude extracts (25, 50, 100, 200, 400 µg/ml) were added to the wells.
- Triplicates were used per each concentration as well as the controls (cells treated with serum free medium). Plates were incubated at 37°C, 5% CO_2 for selected exposure time (24 hours).
- After exposure, 10 µl of the MTT solution was added to each well. Plates were further incubated at 37°C, 5% CO_2 for 4 hours.

- The media were carefully removed and 100 µl of solubilization solution was added per each well for 5 min.
- The absorbance was determined by using an ELISA reader at a wavelength of 575 nm. The data of optical density was subjected to statistical analysis in order to calculate the concentration of compounds required to cause 50% reduction in cell viability for each cell line.

2.2.6 Multi-parameter Cytotoxicity Assay on MCF-7 Cell Line:

The multi-parameter cytotoxicity assay was performed to measure five orthogonal MCF7 cell health parameters after the exposure to *Pelargonium graveolens* crude extract *in vitro*. The parameters were: viability cell count, total nuclear intensity, cell membrane permeability, mitochondrial membrane permeability and cytochrome c. The mutli-parameters were quantitatively measured using high content screening analyzer (ArrayScan XTI, ThermoScientific).

2.2.6.1CellomicsMultiparameter Cytotoxicity Kit 3 Contents:

Cytochrome C Primary Antibody	75 µl
DyLight ™ 649 Conjugated Goat-Mouse Antibody	72 µl
Mitochondrial Membrane Potential Dye	1 ea
Permeability Dye	25 µl
Hoechst Dye	30 µl
Wash Buffer (10 X Duldeccos PBS)	100 ml
Permeabilization Buffer (10 X Duldeccos PBS with 1% Triton X-100)	100 ml
Blocking Buffer (10 X)	85 ml
Thin Plate Seal Assembly	7/ pack

2.2.6.2 Reagents and Solutions Preparation:

The following reagents and solutions were prepared according to the manufacturer instructions supplied with the kit:

1- Wash Buffer (1X): it was prepared by mixing 20 ml of 10X wash buffer with 180 ml ultrapure distilled water. The buffer was stored at 4°C for up to 7 days.

2- Fixation Solution: it was prepared by mixing 3ml of 16% formaldehyde solution with 9 ml of 1X wash buffer. The solution was prepared just before the assay.

3- Permeabilization Buffer (1X): it was prepared by mixing 1.5ml of 10X permeabilization buffer with 13.5 ml of the 1X wash buffer. The buffer was stored at 4°C for up to 7 days.

4- Blocking Buffer (1X): it was prepared by mixing 5 ml of 10X blocking buffer with 44 ml of 1X wash buffer. The buffer was stored at 4°C for 7 days.

5- Primary Antibody Solution: it was prepared by mixing 15µl of the cytochrome c primary antibody with 6 ml of 1X blocking buffer. The solution was prepared just before the assay.

6- Secondary Antibody/Staining Solution: it was prepared by adding 0.6 µl of Hoechst dye and 12µl of the DyLight 649 Goat Anti-Mouse to 6 ml of 1X blocking buffer. Solution was prepared just before the assay.

7- Live Cell Staining Solution: it was prepared by adding 117µl of DMSO to the Mitochondrial Membrane Potential dye to make a 1mM stock solution. Then 2.1µl of permeability dye and 21µl of Mitochondrial Membrane Potential dye were added to 6ml complete culture medium pre-warmed to 37°C.

8- Doxorubicin (0.1M): A stock solution was prepared by dissolving 0.05g of doxorubicin in few drops of DMSO and then diluted up to 1ml with 1X wash buffer. The solution was stored at 4°C until use.

2.2.6.3 MCF-7 Cell Preparation

When MFC7 cells formed a confluent monolayer in the flask, the growth media decanted off and the cell sheet washed twice with PBS.

The cells were harvested following trypsinization by adding 2-3 ml Trypsin-EDTA solution and incubation at 37°C for few minutes until the cells had detached. The cells were diluted to 7.5 x 10^4cells/ml in DMEM complete medium.

Aliquots of 100 µg of cell suspension were added to each well of a 96-well plate. Cells were incubated overnight at 37°C in 5% CO2.

2.2.6.4 Procedure:

1. Twenty five µl from each concentrations of *Pelargonium graveolens* extract (12.5, 25, 50, 100 and 200µg/ml) was added to the cells in addition 25µl of doxorubicin (20µM) was added as positive control. Cells were incubated at 37°C for 24 hours.
2. Cell staining solution (50µl) was added to each well. The cells were incubated at 37°C for 30 minutes.
3. The medium and the staining solution were gently aspirated and 100µl/well of fixation solution was added, plate was incubated for 20 minutes at room temperature.
4. The fixation solution was gently aspirated and 100µl/well of 1X wash buffer was added.
5. The wash buffer was removed and 1X permeabilization buffer was added. Plate was incubated for 10 minutes at room temperature, protected from light.

6. Permeabilization buffer was removed and the plate was washed twice with 100µl/well of 1X wash buffer.
7. Wash buffer was aspirated and 100µl of 1X blocking buffer was added and plate was incubated for 15 minutes at room temperature.
8. Blocking buffer was aspirated and 50µl/well of primary antibody solution was added. Plate was incubated for 60 minutes protected from light at room temperature.
9. Primary antibody solution was aspirated and plate was washed three times with 100µl/well 1X wash buffer.
10. Wash buffer was aspirated and 50µl/well of secondary antibody/staining solution was added. Plate was incubated for 60 minutes at room temperature.
11. Secondary antibody/staining solution was aspirated and plate was washed three times with 100µl/well of 1X wash buffer.
12. Aliquots of 100µl/well of 1X wash buffer were added.
13. Plate was sealed and evaluated on the ArrayScan HCS Reader.

2.2.7 Reactive Oxygen Species (ROS) Assay

ROS assay was performed in order to measure the production of oxidative damage using a fluorogenic probe designed to measure ROS in living cell (Chen *et al*., 2013). The results were optimized with ArrayScan (ThermoScientific) Reader.

2.2.7.1 ROS Kit Contents

Dihydroethidium	10 x 1mg
Hoechst 33324 Dye	10mg

2.2.7.2Reagents and Solutions Preparation

The following reagents and solutions were prepared according to the manufacturer instructions supplied with the kit:

1- Staining Solution: It was prepared by mixing 2.5 µg/ml of dihydroethidium and 500 nM of Hoechst 33324dye in DMEM serum free medium. The solution was stored at 4°C until use.
2- Doxorubicin (0.1M): A stock solution was prepared by dissolving 0.05g of doxorubicin in few drops of DMSO and then diluted up to 1ml with DMEM serum free medium. The solution was stored at 4°C until use.
3- Fixative Solution: It was prepared by diluting 3.5ml formaldehyde in 96.5ml PBS.

2.2.7.3 Cell Preparation:

When MFC7 cells formed a confluent monolayer in the flask, the growth media decanted off and the cell sheet was washed twice with PBS.

The cells were harvested following trypsinization by adding 2-3 ml Trypsin-EDTA solution and incubation at 37°C for few minutes until the cells had detached. The cells were diluted to 1 x 10^4cells/ml in DMEM complete medium.

Aliquots of 100 µg of cell suspension were added to each well of a 96-well plate. Cells were incubated overnight at 37°C in 5% CO2.

2.2.7.4 Procedure:

1. After incubation for 24 hours, the medium was removed and 100µl of two-fold serial dilutions of *P. graveolens* crude extract (25,

50,100, 200µg/ml serum free medium final concentration) were added to the wells. Triplicates were used for each concentration in addition 100 µl of doxorubicin (20µM final concentration) was added as positive control. Untreated cells treated only with fresh serum free medium.

2. The plate was incubated at 37°C, 5% CO_2 for 16 – 20 hrs.
3. Staining solution (50µl) was added to each well. Plate was incubated at 37°C for 30 min.
4. Staining solution was aspirated and 100µl /well of fixative solution was added. Plate was incubated for 15 minutes at room temperature.
5. Fixative solution was aspirated and then the plate was washed with 100µl / well of PBS.
6. PBS buffer was aspirated and replaced with 150µl / well of PBS.
7. The plates was sealed and evaluated on the ArrayScan high content screening reader.

2.3Statistical Analysis:

A one way analysis of variance ANOVA (Duncan) was performed to test whether group variance was significant or not. Data were expressed as mean± standard error and statistical significances were carried out using SPSS program version 20 (Susan *et al*., 1997) and drowned using GraphPad Prism version 6.

Chapter three

Result and discussion

3. Result and discussion

3.1The Alcoholic and Organic Extraction of *Pelargonium graveolens* Leaves

An amount of 100g of *P. graveolens* dried leaves was successively extracted three times per each solvent (methanol, di-ethyl ether and hexane) to obtain a *P. graveolens* crude extract. After final extraction step and the evaporation of the solvent the dry material was weighted and the percentage yields (w/w) of the dry extracts were calculated (Table 1-3).

Table (3-1): The percentage yields (%) of *P. graveolens* dried crude extracts (100 gram of p. graveolens dried leaves was taken).

Solvent	Dry Weight of Crude Extract (gram)	Percentage Yields %
Methanol	1.4	1.4
Di-ethyl Ether	1.6	1.6
Hexane	1.2	1.2

The resulted crude dried extracts showed the appearance of dark green color with odor which most probably attributed to the presence of phenolic compounds within the constituents of the dried extracts (Cherevatiy *et al*., 1980). Many studies reported that the yields percentage ranged between 5% to 15% depending on the successive repeats of the extraction methods in addition it was reported that the alcoholic (mostly ethanol and methanol) considered being the most suitable solvent used for extraction of essential oils with high phenolic contents (Ardestani and Yazdanparast,2007 ; Hsouna and Hamdi, 2012).

3.2 Antioxidant Activity of *Pelargonium graveolens* Crude Extract

The scavenging activity of methanol, di-ethyl ether and hexane extracts of *P. graveolens* leaves was evaluated using stable DPPH free radical scavenging assay. Results in Fig. (3.1) revealed that the more increasing in concentration of the extract the more scavenging percentage of the free radicals. The concentrations that were used ranged from 75 to 1000µg/ml, nevertheless the methanolic extract of *P .graveolens* significantly showed the highest antioxidant activity (87.7% at 1000µg/ml) among di-ethyl ether and hexane extracts which were 56.1% and 52.4% respectively at 1000µg/ml.

The above results were compared with free radicals scavenging activity of ascorbic acid as a positive antioxidant, in which according to the Fig. (3.1), the methanolic extract showed almost the same pattern of ascorbic acid free radical scavenging activity especially at concentrations 500 and 1000µg/ml, while di-ethyl ether and hexane extracts showed approximately half the scavenging activity that of the ascorbic acid.

These results indicated that the DPPH free radical scavenging activity of methanol extract was dose dependent in which the higher concentration result in higher antioxidant activity and the methanolic extract had a significant higher scavenging activity than the di-ethyl ether and hexane extracts which showed lower antioxidant activity.

The IC_{50} for each treatment was calculated and it was showed that the inhibitory effects of methanol, di-ethyl ether, hexane and ascorbic acid for DPPH were 484, 872, 883 and 292µg/ml respectively (Fig. 3.2, 3, 4 and 5).

Comparing between the IC_{50} values it was obvious that methanolic extract significantly with the lowest concentration that causing 50% scavenging activity of the DPPH among di-ethyl ether, hexane extracts. This scavenging activity is somewhat similar to the inhibitory capacity of the positive control ascorbic acid (292µg/ml).

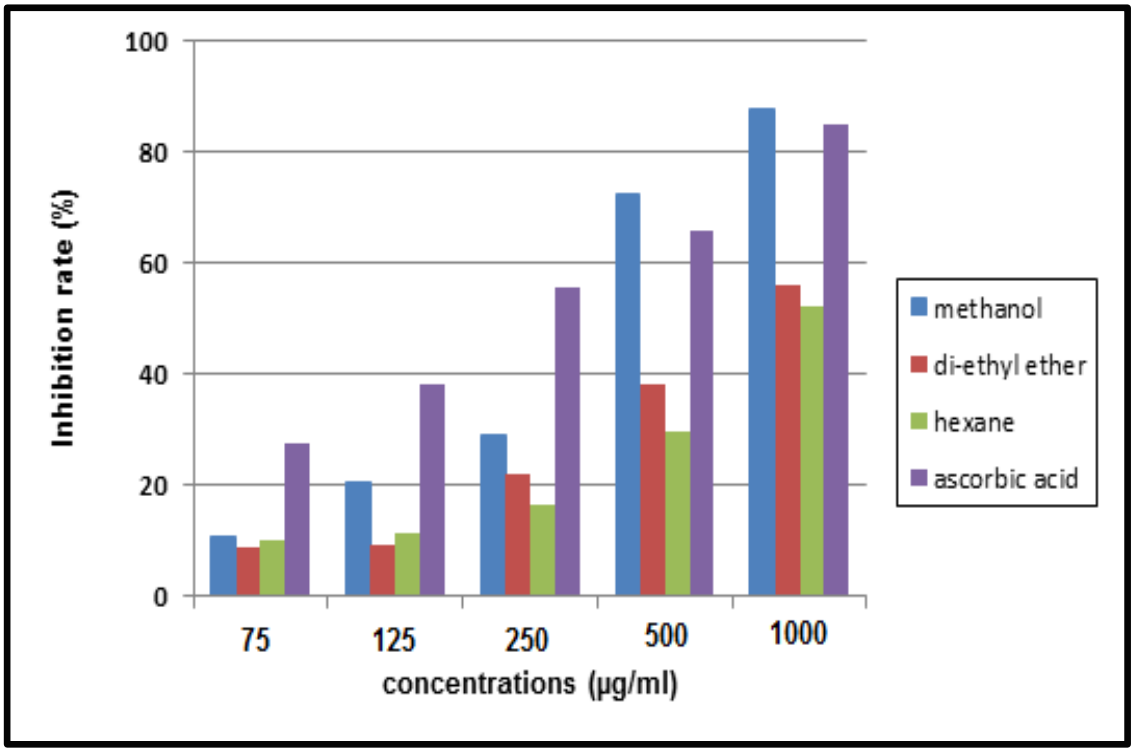


Figure (3-1): DPPH free radical scavenging activity of methanol, di-ethyl ether and hexane extracts of *Pelargonium graveolens* depending on concentrations.

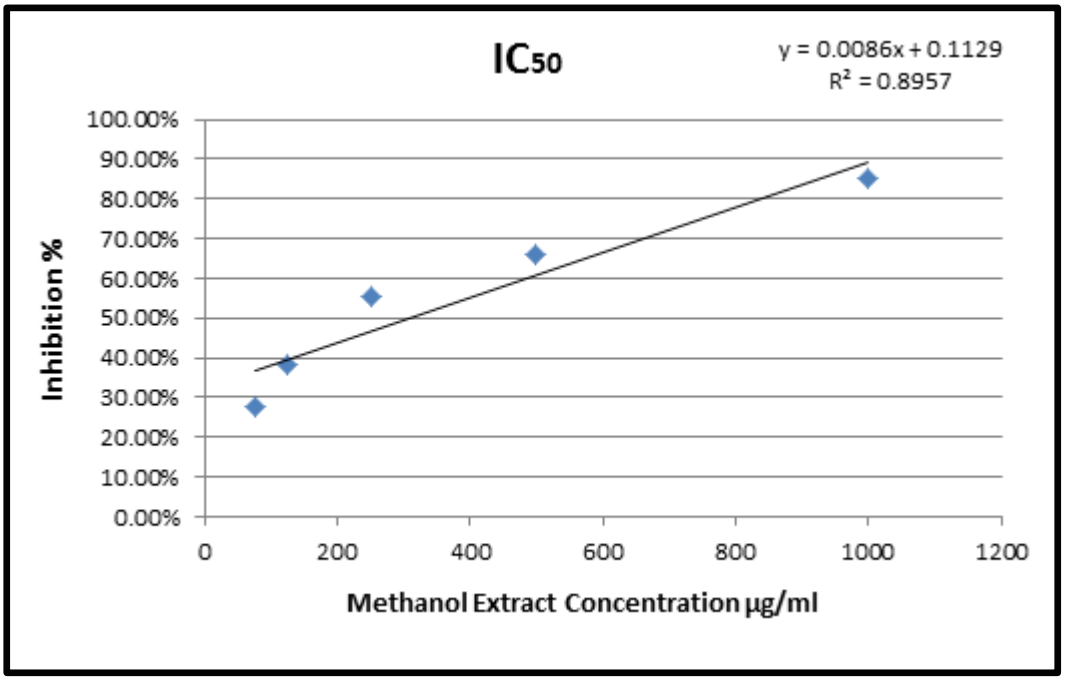


Figure (3-2): IC50 equation (linear regression) of *P. graveolens* methanol extract DPPH scavenging activity.

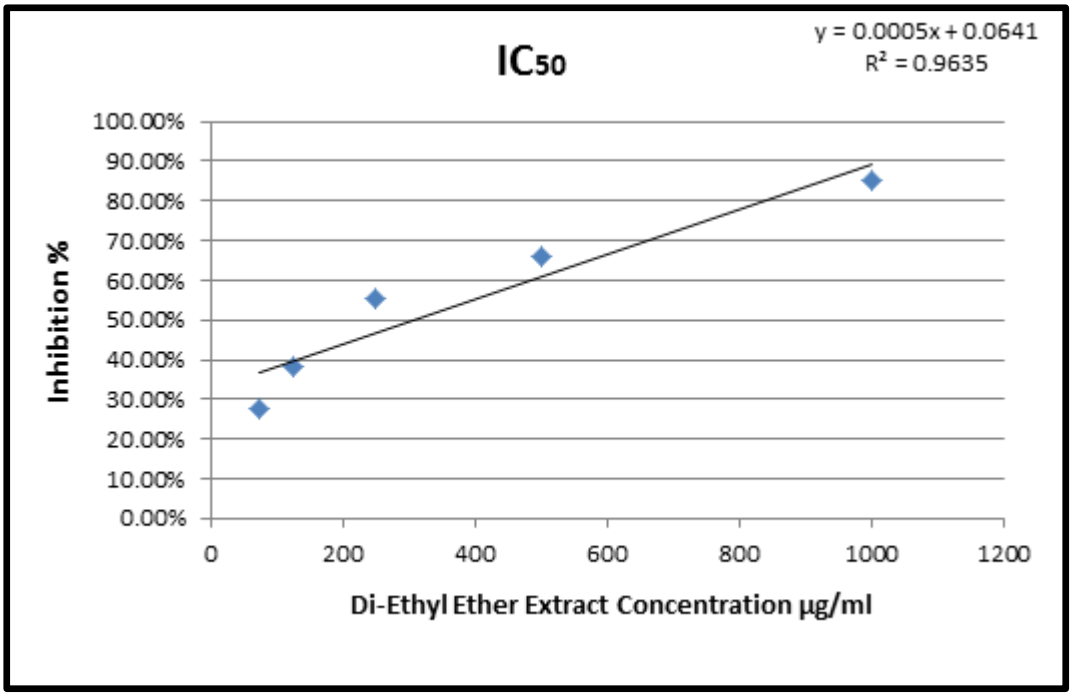


Figure (3-3):IC50 equation (linear regression) of *P. graveolens* di-ethyl ether extract DPPH scavenging activity.

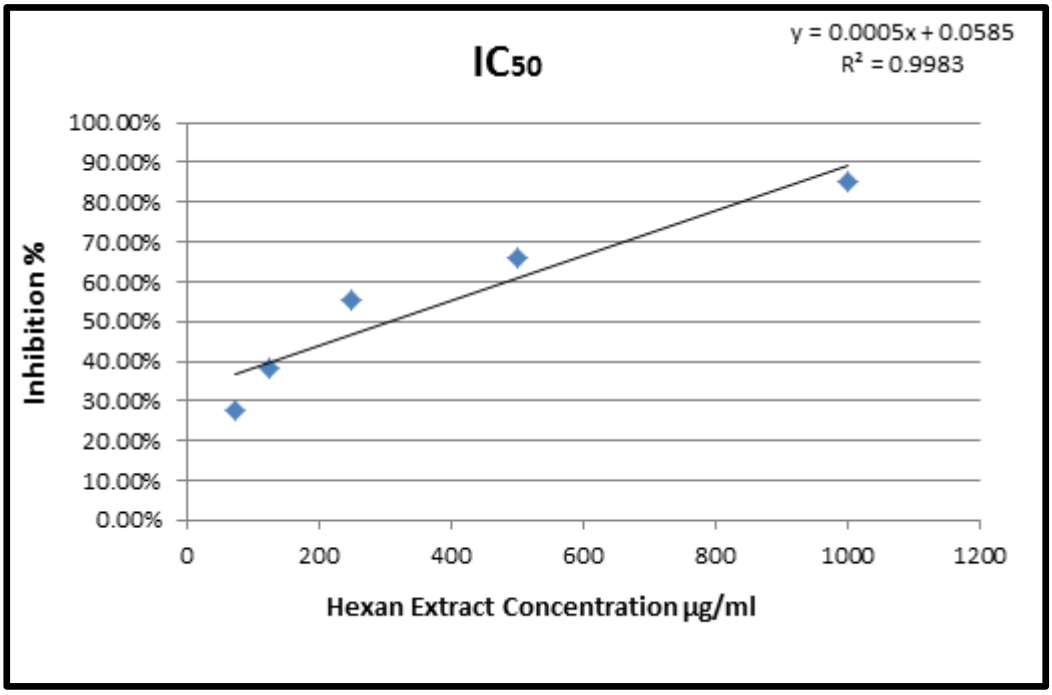


Figure (3-4):IC50 equation (linear regression) of *P. graveolens* hexane DPPH scavenging activity.

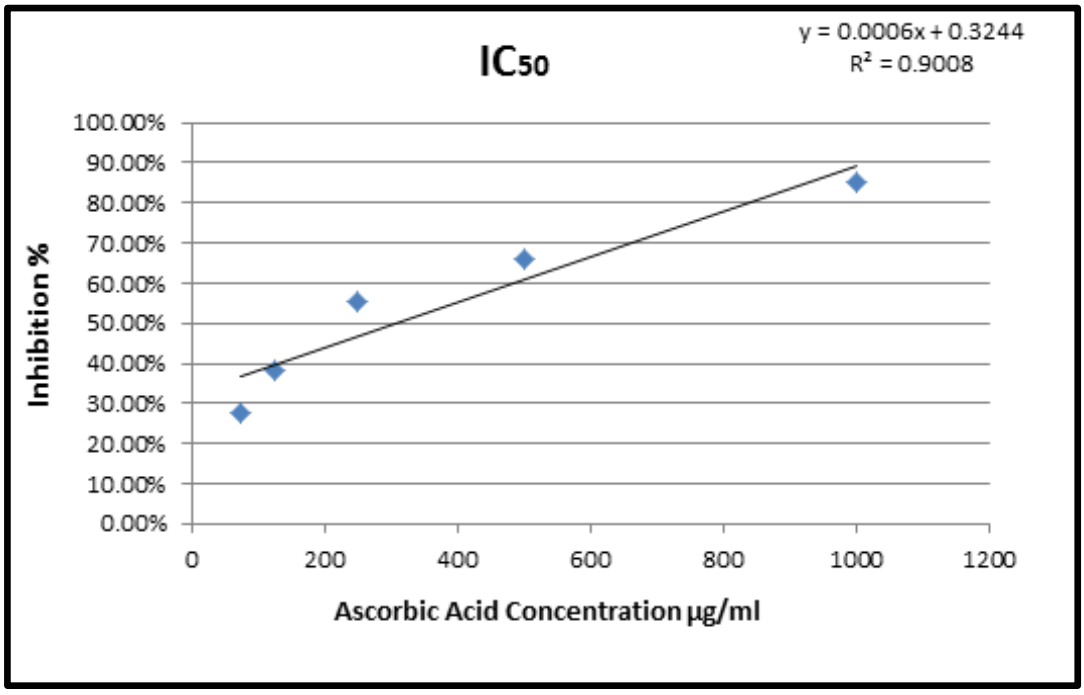


Figure (3-5):IC50 equation (linear regression) of *P. graveolens* ascorbic acid DPPH scavenging activity.

The DPPH is characterized as a stable radical owing to the delocalization of the spare electron with maximum absorption at about 517 nm, and it has been frequently used to determine the antioxidant activity of different substances including plant extracts (Jean-Ping *et al*., 2012).

The antioxidant activity of the *P. graveolens* methanolic extract could be attributed in part tot he presence mainly of essential oil compounds such as β-citronellol and geraniol and its ability to reduce free radicals by quenching reactive oxygen species and trapping radicals before reaching their cellular targets (Boukhris *et al.,* 2012). Many studies exposed the radical-scavenging activity of extracts and essential oils of *P. graveolens* using DPPH method, with values ranged from 63.70 mg/ml (leaves) to 64.88 mg/ml (stems) for essential oils (Čavar and Maksimović, 2012), in addition Marangoni and Fernandes de Moura, (2011), mentioned that the use of essential oil extracted from *Coriandrum sativum* presented stronger synthetic antioxidant effect than that of butyl hydroxytoluene on the delay of lipid oxidation.

Di-ethyl ether and hexane (organic solvents) extracts were showed very low DPPH scavenging activity and this could be resulted from the absence of an appropriate scavenging compound within the extracts. The free radical scavenging activity is highly dependent on the type of the solvent that used in extraction process due to the differences in polarities and extract ability of the potentially antioxidant phytochemicals (Ghatak *et al*., 2014). Since the methanol is an amphiphilic compound it helps to extract most of the various chemical groups from the plant material. Therefore the methanol extract of *P. graveolens* had the highest activity among the other two extracts (Djeridane *et al*.,2005).

3.3 Cytotoxic Effect of *P. graveolens* Extracts on Tumor Cell Line *in vitro* Using MTT Assay:

The assay of 3-(dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) was achieved to determine the cytotoxic effect of methanol, di-ethyl ether and hexane extracts of *P. graveolens* on two different types of tumor cell lines: breast cancer cell line(MCF-7) and prostate cancer cell line (PC-3). This assay was performed to measure the cell viability and inhibition rate by applying different concentrations of *P. graveolens* extracts on the tumor cell lines.

Data analysis carried out in µg/ml and log values of µg/ml is being plotted in Graphpad Prism 6 using log (Inhibitor) versus response curve. Best values were chosen for the most significant IC_{50} values. Cell viability at each time-point was determined by MTT colorimetric assays.

The cytotoxic effect of the three different extracts of *P. graveolens* on MCF-7 cells was shown in Fig. (3.6). Results indicated that the incubation of MCF-7 cells with methanol extract at concentrations ranged from 25 to 400µg/ml for 24 hours showed a reduction in cell viability in a dose-dependent pattern by which the cell viability decreased by increasing the concentration of the methanolic extract. The lowest MCF-7 cell viability (%) was recorded by methanol extract (38%) at concentration 400µg/ml; however it was 73.6% and 53.9% for di-ethyl ether and hexane extracts, respectively at 400µg/ml. It was observed significant differences in the rate of MCF-7 cell inhibition between the different extracts by calculating the IC_{50}. The methanolic extract showed significantly the most potent cytotoxic activity with IC_{50} value of 288µg/ml, whereas IC_{50} values of di-ethyl ether and hexane were 822 and 415µg/ml, respectively.

On the other hand, Figure (4.7) shows the PC-3 cell viability after exposure to methanol, di-ethyl ether and hexane extracts of *P. graveolens* at concentrations ranged from 25–400µg /ml for 24 hours. Results indicated that the cell PC-3 viability was not significantly affected by the application of different extracts concentrations as compared with the viability of MCF-7 cells, with a cell viability (68.8%) of methanolic extract at 400µg/ml, while the inhibition rate recorded for di-ethyl ether and hexane extracts were 73.7% and 72.2%, respectively. The IC_{50} of methanol, di-ethyl ether and hexane on PC-3 treated cells were 736, 900 and 747µg/ml, respectively.

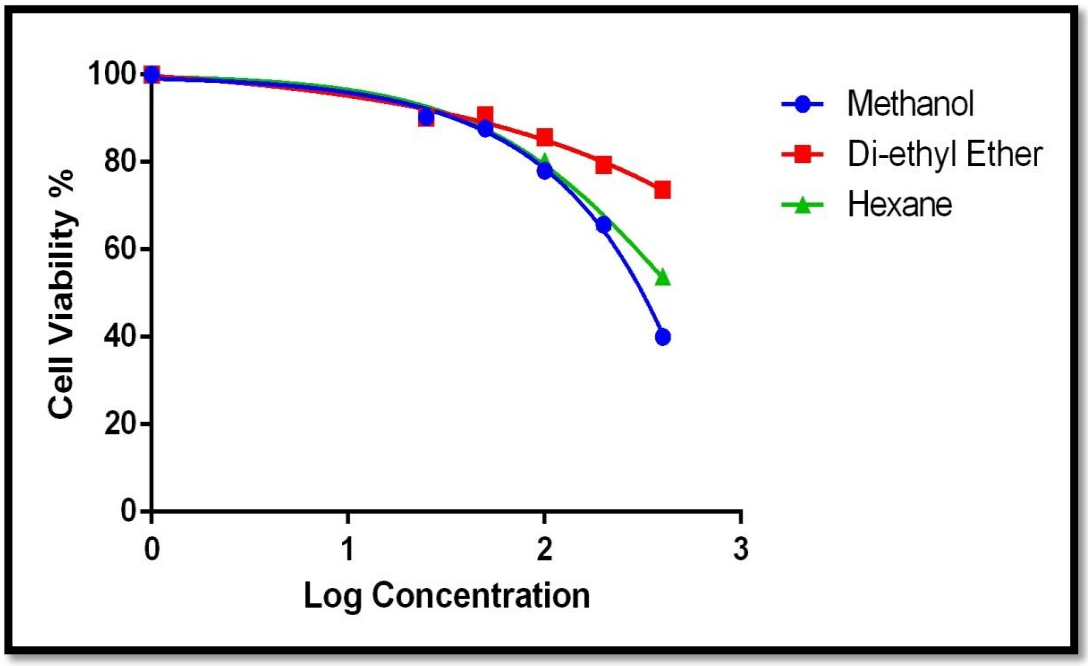


Figure (3-6): Cytotoxicity effect of methanol, di-ethyl ether and hexane extracts on MCF7 cells after 24 hours incubation at 37ºC.

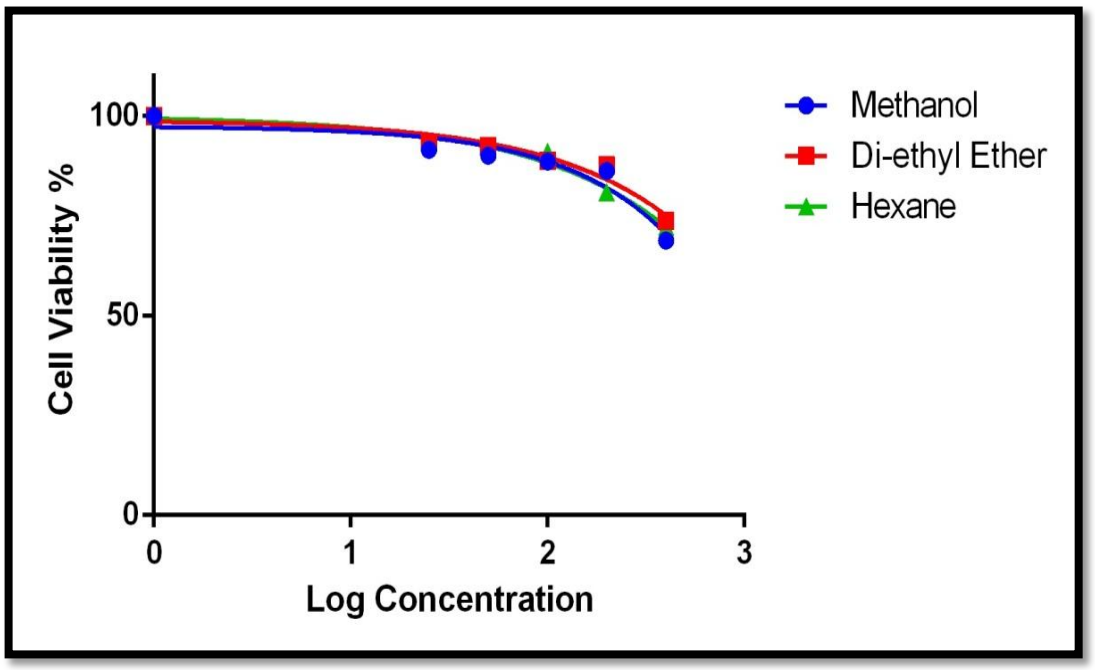


Figure (3-7): Cytotoxicity effect of methanol, di-ethyl ether and hexane extracts on PC3 cells after 24 hours incubation at 37ºC.

P. graveolens is often used as medicinal herbs in different areas of the world due to its biological activities such as bactericidal, antifungal, and antiviral as well as antioxidant activity (Bakkali *et al*., 2008), however, very few researches were reported the antitumor activity of *P. graveolens* extracts. The cytotoxic effect mainly resulted by the methanolic extract on MCF-7 cells may be attributed to the presence of essential oils which considered to be the major constituent that possess a wide variety of biological activity. Such indication is in agreement with Fayed, (2009), which reported that the essential oils yielded from the aerial parts of *P. graveolens* showed a potent cytotoxic effect against HL-60 and NB4 human leukemic cell lines with an IC_{50} value up to 86.5µg/ml. In addition, a significant inhibition (60% -90%) of MIAPaCa2

pancreatic tumor cells was reached with geraniol, one of the major essential oil constituents of *P. graveolens* (Burke *et al*., 1997).

Some studies correlated between the anti-inflammatory activity and cytotoxic activity (anti-tumor activity) of *P. graveolens* essential oils, in such a way that the essential oils can provoke the immunity of patients suffering from cancer diseases. Zhuang *et al*., (2012), reported an approved trail on 66 breast cancer patients (stage I – IV) were treated with surgery followed chemotherapy or radiotherapy and were enrolled in administrating herb complex including essential oils from *P. graveolens*, which they suggested that using herb complex have the capacity to delay the reduction in levels of leucocytes and neutrophils that are experienced by patients during cancer treatment.

The MCF-7 cell line was affected by *P. graveolens* methanolic extract but not the di-ethyl ether and hexane *P. graveolens* extracts. The inhibition may resulted from synergistic relationship between the combined abundant components that found in the methanolic extract while these components could be not be extracted by di-ethyl ether and hexane. On the other hand, PC-3 cell line was not significantly affected by any type of *P. graveolens* extracts. Many researches have been reported that not all the types of tumor cells showed the same sensitivity toward a particular type of plant extracts, for example the essential oils from roots/rhizomes of *Anemopsis californica* was used to screen its anticancer bioactivity against number of tumor cell lines, which showed no activity against PC-3 and HCT116, while it was an anti-proliferative active against the AN3CA and HeLA cells (Medina-Holguín *et al*., 2008).

Certain chemical compounds within alcoholic extract may have a specific effect on MCF-7 cell viability and that agreed with Kailasapathy and Chin (2000) and Lee *et al.*, (2005) who demonstrated that certain anticancer drugs can affect in different ways on different tumor cell types, and for this reason only the methanolic extract was used for multi-parameter cytotoxicity assay and Reactive Oxygen Species generation assay ROS on MCF-7 cell line.

3.4The Multi-parameter Cytotoxic Activity of *P. graveolens* Methanolic Extract on MCF-7 Cell Line.

The Multi-parameter cytotoxic activity of the *P. graveolens* methanolic extract was implemented in High Content Screening (ArrayScan XTI, ThermoScientific) using MCF-7 cells. Five different measurements (cell count viability, nuclear intensity, cell membrane permeability, mitochondrial membrane potential and cytochrome C) were detected in this assay and images of treated (extract and doxorubicin) and untreated MCF-7 cells were resulted.

Results in Figure (3-8), shows that the viable count of MCF-7 cells was decreased by increasing the concentration of *P. graveolens* extract as compared with untreated cells. The reduction in cell counts (% inhibition rate) was (10.20%, 15.12%, 29.5%, and 53.92%) treated with 25, 50, 100 and 200µg/ml of *P. graveolens* methanolic extract, respectively (Table 3-2). This result is highly support that of the MTT regarding the cytotoxic of methanolic extract against MCF-7 cells, in which the reduction of the cell count was a dose dependent and the most significant reduction appeared ($p< 0.0001$) after applying 200µg/ml of the extract.

Table (3-2): The count of viable cell after exposure to different concentrations of methanolic extract of *P.graveolens* on MCF-7 cells

Treatment (µg/ml)	Viable Cell Count (Mean ± SD)	Inhibition %
Untreated Cells	3975 ± 35.35	0
25	3567 ± 55.15	10.20
50	3377 ± 94.04	15.12
100	2839 ± 172.45	29.5
200	1908 ± 63.36	53.92
Doxorubicin 20 µM	1050 ± 98.99	72.27

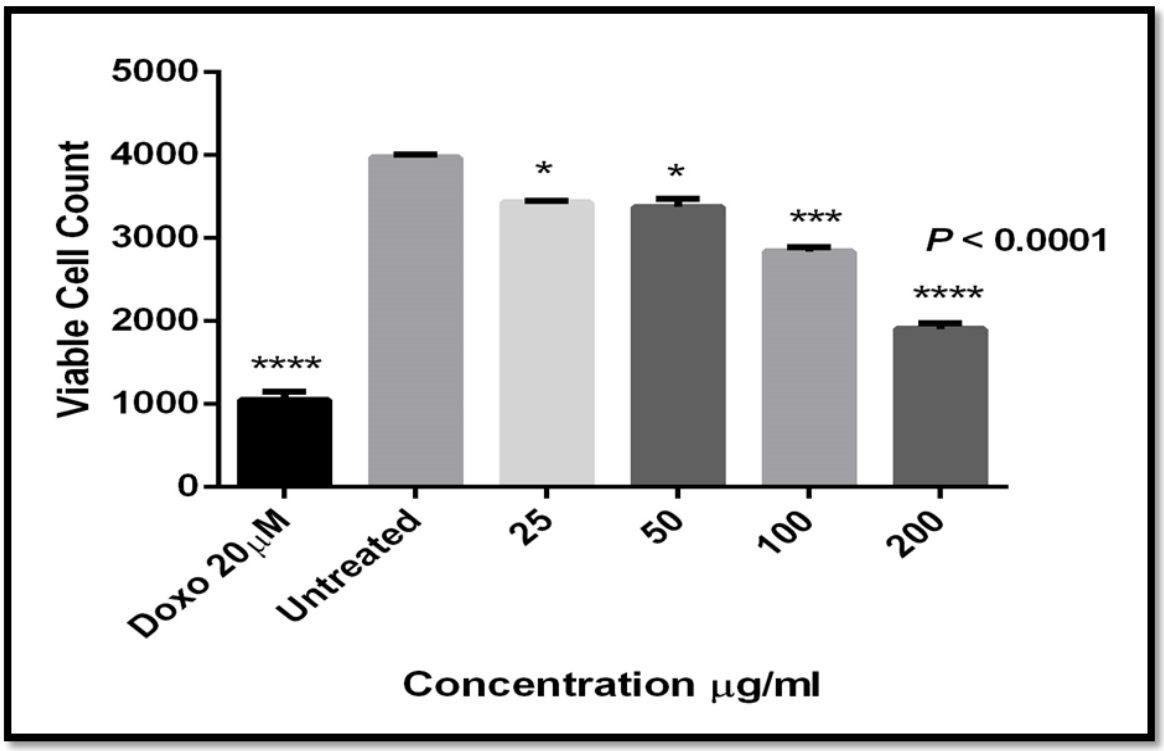


Figure (3-8): Reduction in MCF-7 cell count after 24 hours exposure to different concentrations of methanolic extract of *P.graveolens* at 37° C and evaluated on the ArrayScan HCS Reader, mean differences for signifiacance were made between the untreated cells and the whole group.

Figure (3-9), shows the representative images acquired from a multi-parametric (nuclear intensity, cell membrane permeability, mitochondrial membrane potential and cytochrome C releasing) cytotoxic effect on MCF-7 cell nucleus and mitochondria. The exposure of MCF-7 cells to *P.graveolens* extract at higher concentrations cause increased in nuclear size due to nuclear swelling and increasing in cell membrane permeability. The morphology of the nucleus (Hoechst blue) showed a nuclear condensation which was mostly appear at higher concentration of *P.graveolens* extract (100 and 200µg/ml), both were similar to cells treated with doxorubicin and significantly different ($p< 0.1$ and $p< 0.0001$ respectively) from that of untreated cells (Figure 3-10A) such events were not induced at lower concentrations of *P.graveolens*.

On the other hand, Figure (3-10 B) Shows that the intensity of MCF-7 cell membrane permeability (green) was increased gradually in a dose dependent pattern and the significant increases observed after exposure to 100 and 200µg/ml of *P.graveolens*. Data analysis (Table 3-3) for the resulted fluorescent intensity of extract and doxorubicin treated cells compared with that of untreated cells revealed that the nuclear intensity significantly increased in about 1.3 fold after exposing to 200 µg/ml of the extract which was the nearest result to cells treated with 20µM doxorubicin, a strong antitumor chemotherapy that intercalating DNA molecule and cause massive DNA damage (Pang *et al*., 2015) who showed a 2 fold increase in intensity.

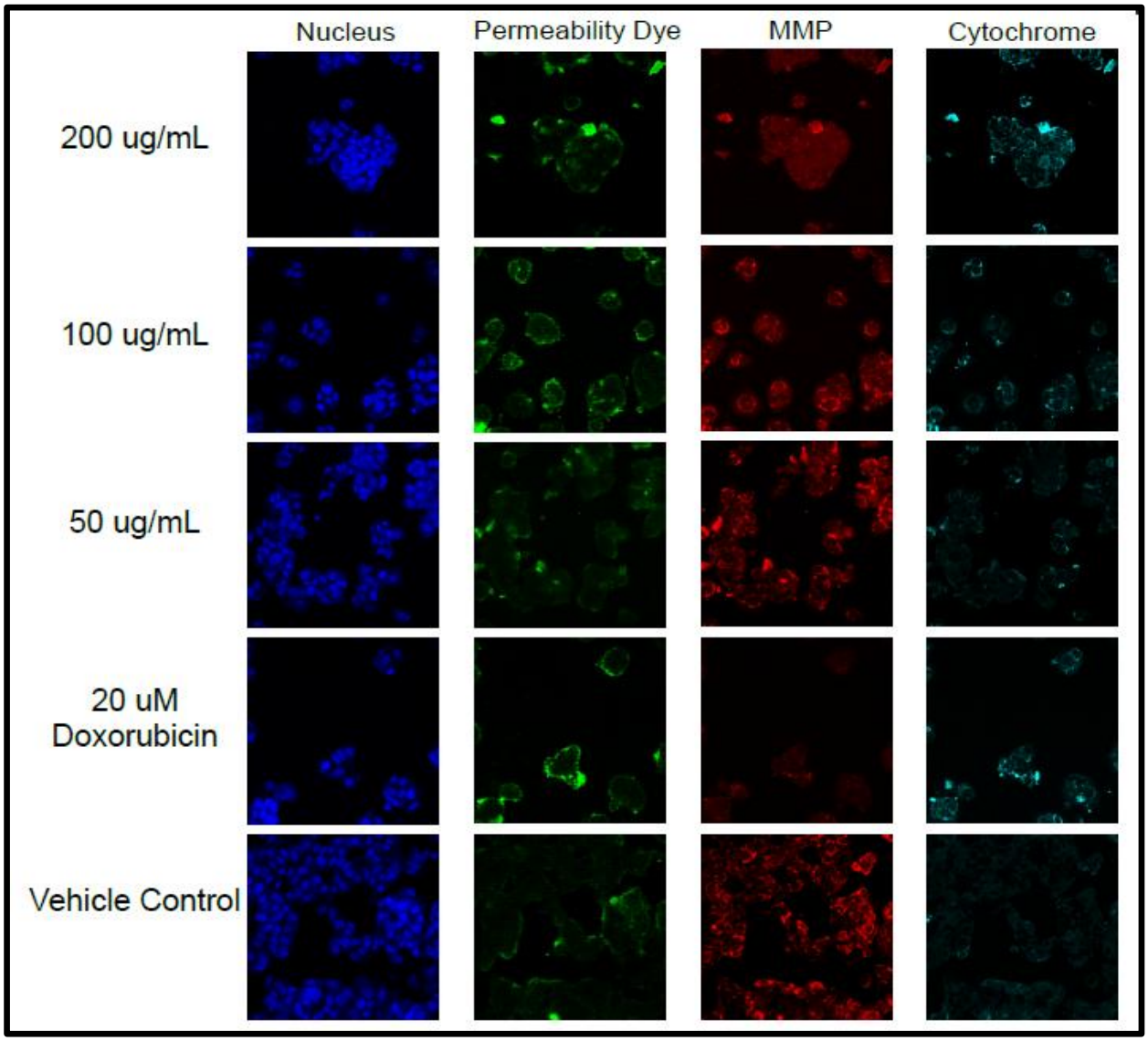
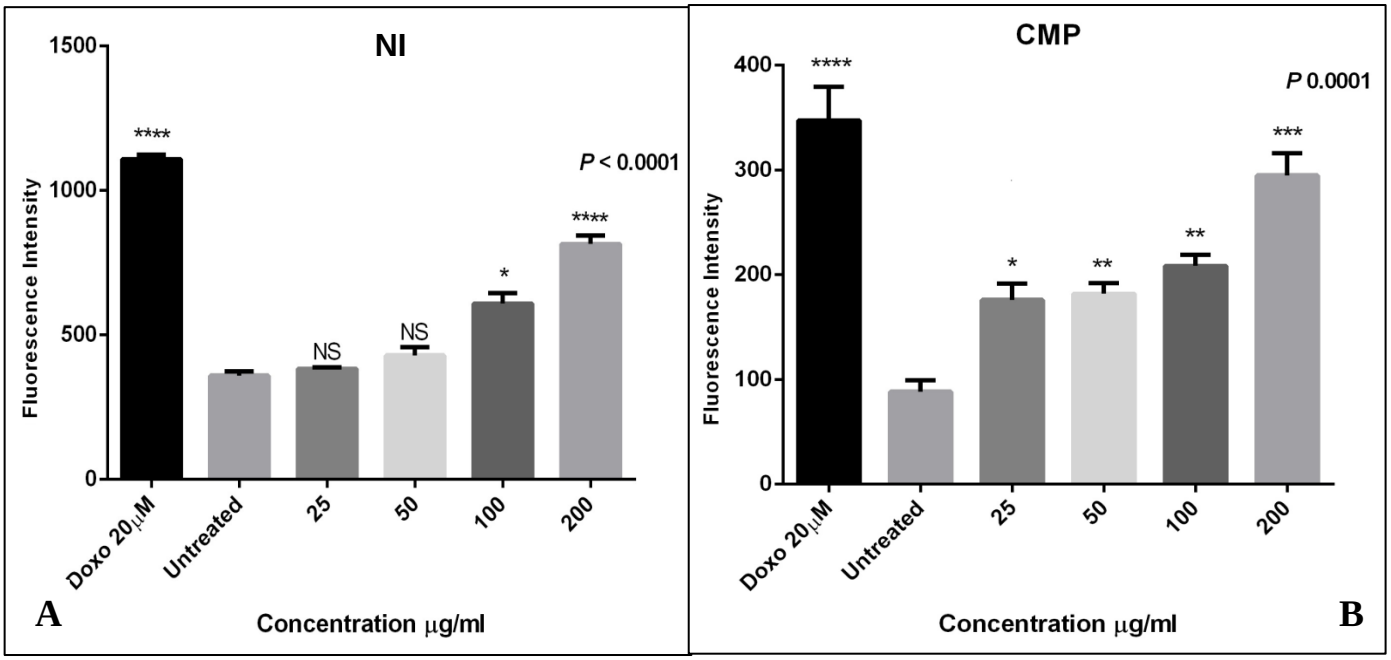


Figure (3-9): Multiparameter cytotoxicity (HCS) analysis of *P. graveolens* extract-treated MCF-7cell line after 24hrs of incubation at 37ºC. Cells were stained with Hoechst 33342 (Blue) (*Ex* 330 nm / *Em* 420 nm) Dye which enables monitoring of cell loss, nuclear morphology changes and DNA content, Permeability Dye (Green) (*Ex* 491nm /*Em* 509 nm) for membrane permeability monitoring, MMP Dye (Red) (*Ex*552 nm /*Em* 576 nm) for mitochondrial membrane potential changes, and with goat anti-mouse secondary antibody conjugated with DyLight™for Cytochrome C releasing.

However, the average fluoresce data for the cell membrane permeability showed a dose dependent pattern started from 1 fold at 25µg/ml to 2.3 fold increase at 200µg/ml and with significant in differences (ranged from *p* 0.01 - *p* 0.0001) as compared with untreated MCF-7 cells. Only high concentrations of extract showed similar effect to doxorubicin on MCF-7 cells.



Figur(3-10):The effect of *P.graveolens* methanolic extract exposure on MCF-7 cell line for 37°C. A: Neuclear intensity effect. B: Cell membrane permeability effect. The effect was evaluated on the ArrayScan HCS Reader, mean differences for signifiacnce were made between the untreated cells and the whole group.

Table (3-3): Effect of different concentrations of *P. graveolens* methanolic extract on nuclear intensity and cell membrane permeability.

Treatment (µg/ml)	Nuclear Intensity (Mean ± SD)	CMP (Mean ± SD)
Untreated Cells	360 ± 15.50	88 ± 10.60
25	383 ± 4.90	176 ± 15.55
50	430 ± 28.20	182 ± 9.89
100	609 ± 36.99	208 ± 10.60
200	815 ± 28.76	292 ± 17.67
Doxorubicin 20 µM	1109 ± 14.10	347 ± 31.81

Another two parameters, mitochondrial membrane potential and cytochrome C release were measured. In comparison, as shown in Figure (3-11A),only higher concentration of *P. graveolens* extract (200µg/ml) induced a significant ($p < 0.0001$) reduction in mitochondrial membrane potential by 40.3% while the positive doxorubicin control cause a reduction up to 71.9%. The measurement of mitochondrial membrane potential depended on the average intensity of MMP dye that punctuate the mitochondria within the nuclear cytoplasmic region (Nannan *et al*., 2007)and the less fluorescent intensity the more effect against the mitochondria. On the other hand, the release of cytochrome C was only significantly ($p = 0.0005$) induced after MCF-7 cell exposed to 200µg/ml of the extract (Figure 3-11B). The release of cytochrome C was detected by 0.7 fold increases in the average fluorescent intensity of the treated MCF-7 cells than that of untreated cells, where as doxorubicin recorded 1.5 fold increases in intensity (Table 3-4).

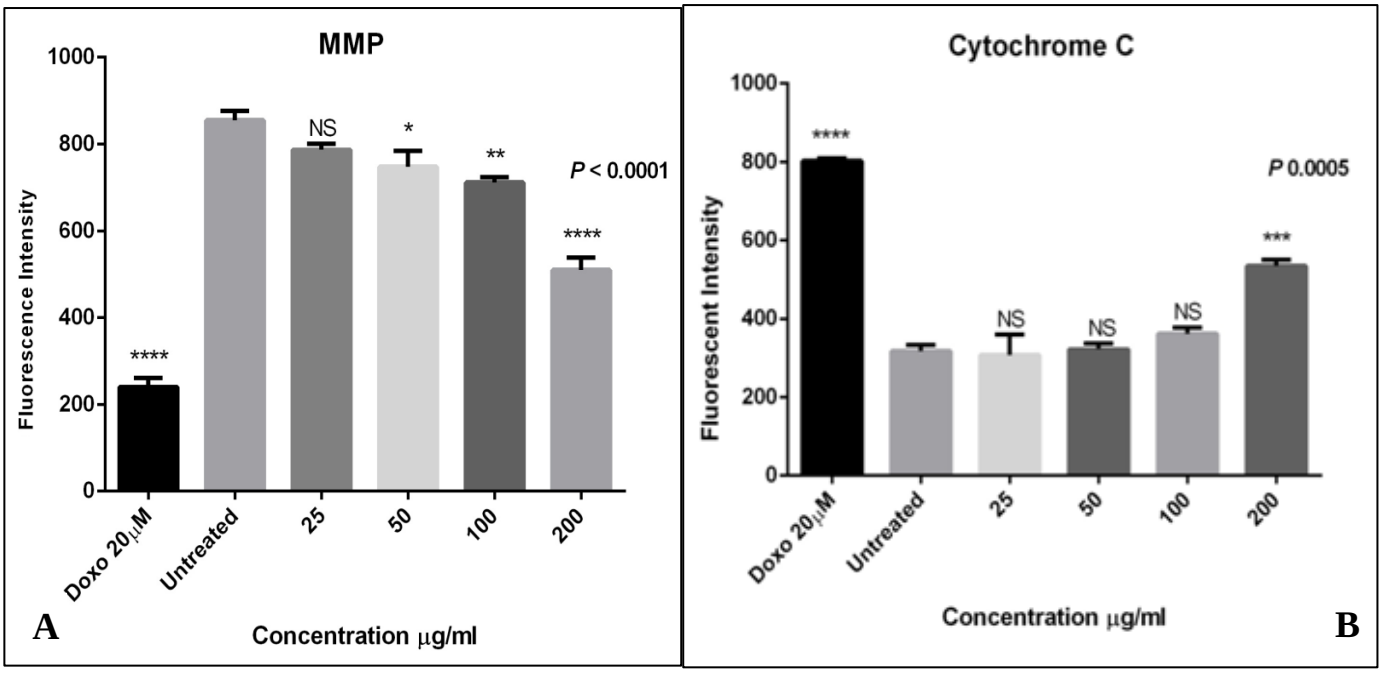


Figure (3-11):The effect of *P.graveolens* methanolic extract expouser on MCF-7 cell line for 37°C. A: Mitochondrial membrane potential. B: Cytochrome C release. The effect was evaluated on the ArrayScan HCS Reader, mean differences for signifiacnce were made between the untreated cells and the whole group.

Table (3-4): Effect of different concentrations of *P. graveolens* methanolic extract on MMP and Cytochrome C release.

Treatment (µg/ml)	Nuclear Intensity (Mean ± SD)	Cytochrome C (Mean ± SD)
Untreated Cells	855 ± 21.21	318 ± 12.63
25	383 ± 4.90	176 ± 15.55
50	748 ± 36.55	322 ± 14.84
100	712 ± 11.32	362 ± 15.55
200	510 ± 28.46	535 ± 13.17
Doxorubicin 20 µM	240 ± 20.50	802 ± 6.39

Cell – based – high content screening considered to be more predictive assay comparing with conventional MTT cytotoxicity. It covers a broad range of effects, monitoring multiple and independent toxicity in the same cell with a quantitative measurement of many parameters related to toxicity (O'Brien and Haskins, 2007; Abraham *et al*., 2008). Therefore this assay was adopted to monitor the different parametric effects of *P. graveolens* methanolic extract on MCF-7 cells, and depending on results the significant reduction of MCF-7 cells by the higher concentrations of the extract may be attributed to the induction of cellular apoptosis. The condensed and bright intensity of Hoechst blue stain was contributed to the nuclear condensation and such observations are the typical features of apoptotic cell morphology: nuclear condensation, nuclear fragmentation, cell shrinkage, formation and aggregation of apoptotic bodies (Antczak *et al*., 2009; Martin *et al*., 2014). In addition the use of membrane permeability dye and the increasing intensity of this dye especially at the highest exposure concentration supporting the fact that the extract could induce apoptosis of MCF-7 cells since this can only stain the cells when plasma membrane permeability increase due to the loss of plasma membrane integrity (Nannan *et al*., 2007; Xu *et al*., 2015).

One of the most distinctive feature of cell death or cell undergoes apoptosis is the disrupting of active mitochondria (Finke, 2001).The MMP dye was used to detect the functionality of active mitochondria; it has the ability to accumulate in mitochondria that maintain their inner membrane potential (Kumar, 2008). Compared with the doxorubicin exposure, results indicated that only 200µg/ml of *P. graveolens* methanolic extract triggered a significant reduction of mitochondrial brightness which might be due to the changes of mitochondrial

transmembrane potential and triggering apoptosis of MCF-7 cells. It was suggested that changes in the membrane potential are due to the opening of the mitochondrial permeability transition pore, allowing transition of ions and small molecules like calcium ions and in consequence this leads to the decoupling of the respiratory chain and then the release of cytochrome C into the cytosol (Susin *et al*., 1998). Finally the release of cytochrome C activates a series of caspases, cysteine proteases, which are mainly responsible for the degradation and digestion of the cell from inside (Susin *et al*., 1998; Tafani *et al*., 2002).

3.5 The Detection of Reactive Oxygen Species Generation Using ROS Kit:

Previous results indicated that the methanolic extract of *P. graveolens* showed an antioxidant activity which was 87.7% of DPPH scavenging activity at concentration 1000µg/ml, therefore the ability of *P. graveolens* to affect the intracellular redox parameters related to oxidative stress was detected by measuring the ROS generation using MCF-7 tumor cells for 24 hours and the level of ROS was determined using ROS Kit (ThermoScientific).

Results in Figure (3-12) shows that only higher concentrations of *P. graveolens* extract exhibited a significant increases in the level of ROS which were 0.53 fold ROS generation (at 100µg/ml) and 1.32 fold ROS generation (at 200 µg/ml) as compared with untreated MCF-7 cells, while lower concentrations of *P. graveolens* extract showed no significant alteration in redox balance as also compared with non-treated cells (Table 3-5).

ROS are constantly generated and eliminated in a biological regulatory pathway, and normal cells control the levels of ROS by balancing their generation and elimination by scavenging system (Dickinson and Chang, 2011).

However, cells under chemical or environmental stress, ROS overproduced and lead to modification of cell morphology, structure and cell apoptosis (Looi *et al*., 2013).

Figure (3-13) indicates the level of ROS in MCF-7 treated cells by staining with DHE dye and viewed using High Content Screening system. Results indicated that ROS generation was strongly induced in MCF-7 treated cells *P. graveolens* extract, mostly at concentration (200µg/ml) after 24 hours exposure as compared with the untreated cells

The generation of ROS in increased levels is associated to the control of multiple interacting molecules including antioxidant enzymes, in which cells injuries or cells exposure to chemicals will dramatically affect the intracellular balance between antioxidant enzymes and lead to overproduction of ROS (Hussain *et al*., 2004).

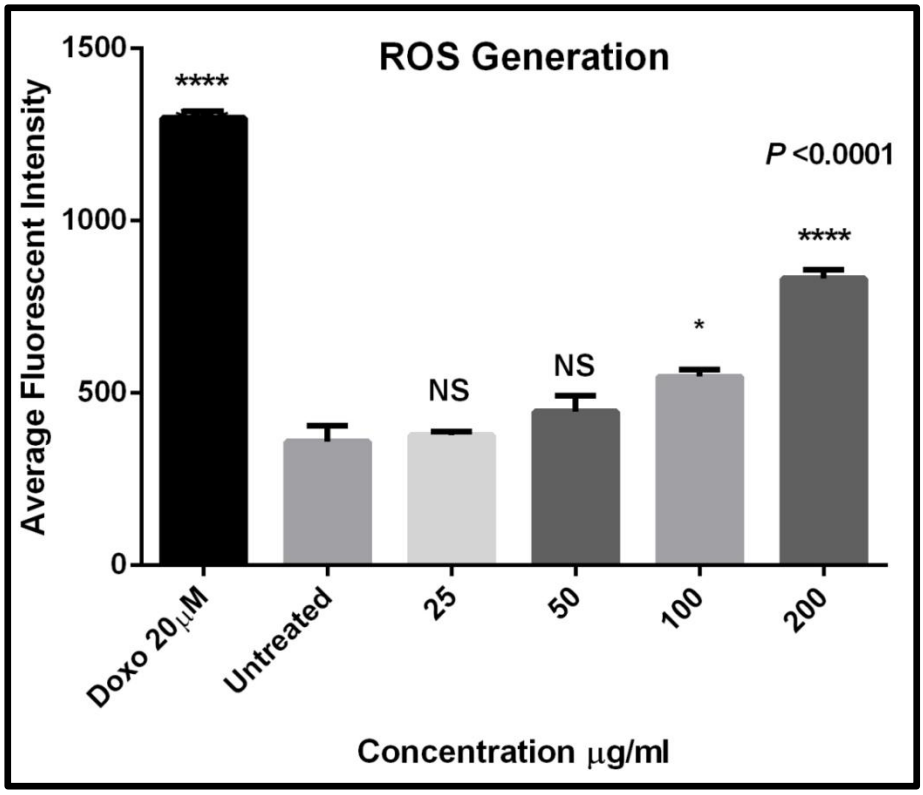


Figure (3-12): Effect of different concentrations of *P. graveolens* on ROS generation in MCF-7 cells. The assay was optimized with the Thermo Scientific Arrayscan HCS Reader.

Table (3-5): The mean intensity of different *P. graveolens* extract concentration on ROS induction in MCF-7 cells.

Treatment (µg/ml)	Intensity (Mean ± SD)
Untreated Cells	359±14.67
25	367 ± 11.58
50	453±20.50
100	552±13.34
200	934±22,62
Doxorubicin 20 µM	1312±38.18

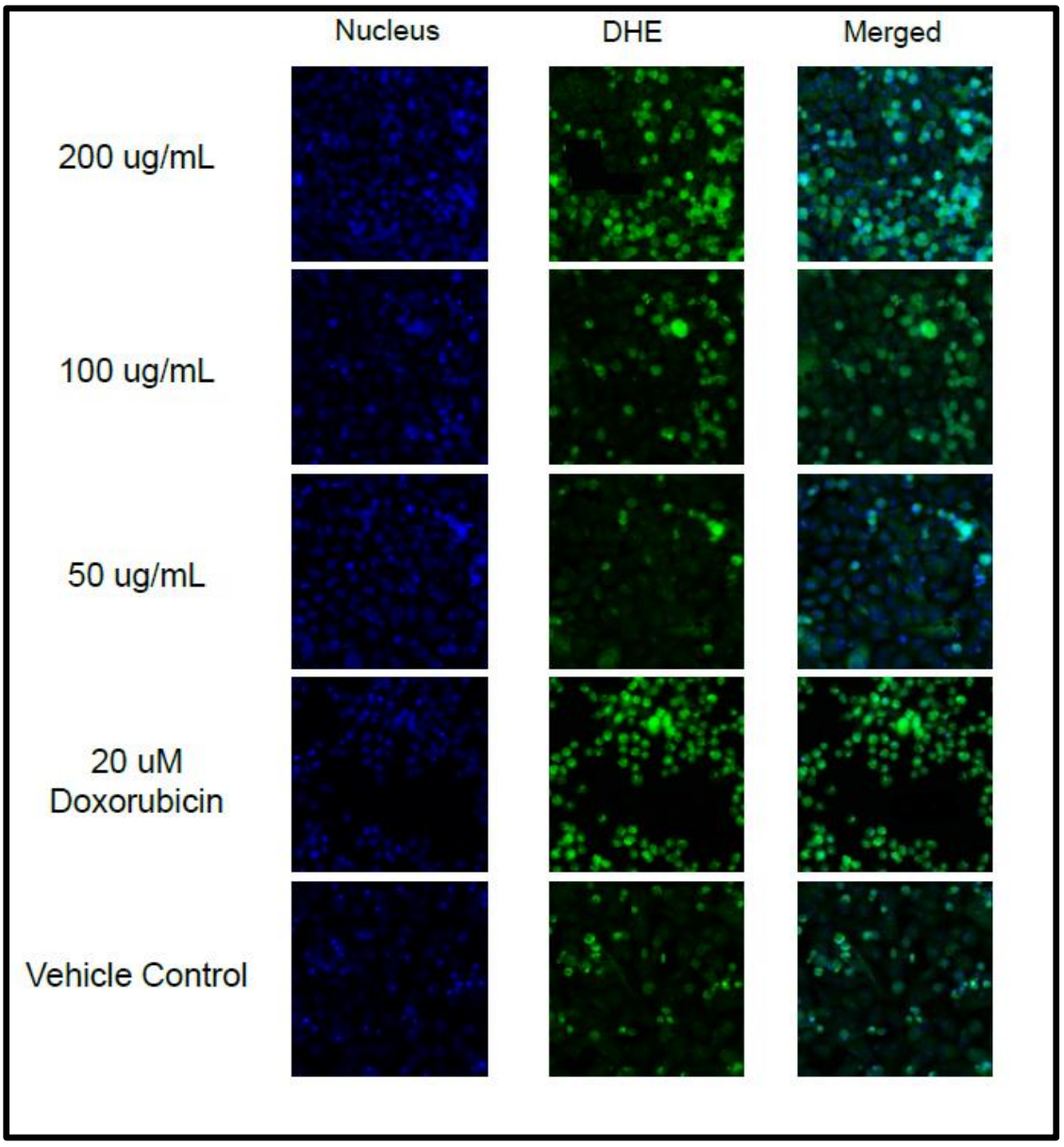


Figure (3-13): The fluorescent intensity of DHE dye in MCF-7 cells treated with different concentrations of *P. graveolens* extract and doxorubicin after 24hrs of incubation at 37°C.

Some natural compounds as well as doxorubicin can induce the generation of ROS and trigger cells to undergo apoptosis (Looi *et al*., 2013). The excessive production of ROS after exposure to the higher concentration of *P. graveolens* extract may attribute to the destruction of mitochondrial membrane potential and eventually resulted in releasing of apoptotic cytochrome C. Furthermore, Gibellini *et al*., (2010), were

described that higher levels of ROS can destroy the cell DNA and plasma membrane, resulting in higher permeability of plasma membrane in addition to DNA fragmentation. These findings were previously observed throughout this study.

ROS are either free radical or non-radical oxygen atoms which formed as a result of oxygen partial reduction, such as superoxide anion (O_2^-) or hydrogen peroxide (H_2O_2). Cellular ROS are generated endogenously from normal aerobic respiration "mitochondrial oxidative phosphorylation" or exposure to toxic drugs or UV irradiation (Trachootham *et al*., 2009). When ROS accumulate and overwhelm the cellular antioxidant defense system oxidative stress occurs and results in disrupting the cellular metabolism and damaging DNA and proteins (Ray *et al*., 2012). The accumulation of the oxidative stress has been reported to associate with different diseases like aging, cancer, rheumatoid arthritis and Alzheimer's (Trachootham *et al*., 2009; Haigis and Yankner, 2010; Shukla *et al*., 2011).

P. graveolens is well-known plant in traditional medicine and pharmacy, in addition to its activity as antioxidant (Saraswathi *et al*., 2011), however little information are available for the cellular and molecular mechanism for reducing ROS and scavenging the oxidants, especially the cellular redox not only influenced by one factor or one molecule. The reduction in oxidative stress may be attributed to the important role of the essential oils like geraniol and β-citronellol as antioxidants. In addition, geraniol was reported to have significant antioxidant properties by stabilizing and protecting the cell membrane against oxidative stress (Maruyama *et al*., 2008).

Chapter Four

Conclusions and Recommendations

4.1 Conclusions

1. Crude extraction of *P. graveolens* with methanol as a solvent gives the higher antioxidant activity by scavenging DPPH, more than using hexane and di-ethyl ether as solvent.
2. A dose dependent cytotoxic activity was significantly recorded after applying *P. graveolens* methanolic extract on MCF-7 cells with IC_{50} of 288µg/ml; however PC-3 cells were not significantly affected by the exposure to the extract.
3. The multi-parametric cytotoxic analysis on MCF-7 showed induction in apoptosis, DNA damage, increased membrane permeability and activation of a mitochondria-dependent proapoptotic cytochrome C release after MCF-7 cells exposure to *P. graveolens* methanolic extract.
4. Higher concentration of *P. graveolens* methanolic extract (200µg/ml) can cause a significant increase in ROS generation due to alteration in redox balance in MCF-7 cells after exposure for 24 hours. Which may be atribute to present to phenols and terpenes.

4.2 Recommendations

1. Quantitative and qualitative study of the active components from *P. graveolens*
2. More studies concerning the apoptosis mechanism induced by *P. graveolens* active component using flow cytometer with suitable fluorescent conjugate.
3. Detecting the expression of Bcl-2 and P^{53} genes in tumor cells treated with *P. graveolens* methanolic extract using Western Blotter.

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الخلاصة:

يعتبر نبات العطرة (*Pelargonium graveolens*) من النباتات الطبية المهمة التي تستخدم عادة لعلاج النزيف الحاد والجروح والقرحو يستخدم ايضا لعلاج حالات الاسهال و الزحار. هذه الدراسة هدفت الى تقييم الخصائص المضادة للتأكسد و المضادة للسرطان لاوراق نبات *Pelargonium graveolens* و التي تم جمعها من بغداد – العراق.

تم الحصول على مستخلصات الميثانول و ثنائي اثيل ايثر و الهكسان من نبات *Pelargonium graveolens*.وتم التحري عن الفعالية المضادة للتأكسد لمستخلصات الميثانول و الداي اثيل ايثر و الهكسان بأستخدام طريقة DPPH و بتراكيز متدرجة ابتداء من 75 الى 1000 مايكروغرام/مل. اشارت النتائج الى ان مستخلص الميثانول كانت له فعالية عالية في اختزال الجذور الحرة لمركب DPPH و كانت الفعالية تدريجية اعتمادا على زيادة تركيز المستخلص حيث ان قيمة IC_{50} هو 484 مايكروغرام/مل ، بينما قيمته لكل من مستخلص ثنائي اثيل ايثر و الهكسان هي 872 مايكروغرام / مل و 883 مايكروغرام/مل على التوالي.

اما الفعالية السمية للمستخلصات الثلاثة على خط خلايا سرطان الثدي البشري MCF-7 و خط خلايا سرطان البروستات البشري PC-3 فقد تم التقصي عنها بأستخدام طريقة MTT. اكدت النتائج ان مستخلص الميثانول اظهر اعلى فعاليةسمية ضد خط خلايا سرطان الثدي البشريMCF-7و كذلك كمختزل لحيوية الخلايا،حيث ان الفعالية كانت تدريجية اعتمادا على تركيز المستخلص بقيمة معنوية للIC_{50} هي 288 مايكروغرام/مل، بينما كانت قيمةIC_{50} هي 822 مايكروغرام/مل لمستخلص الداي اثيل ايثر و415 مايكروغرام/مل لمستخلص الهكسان. ولم تكن هناك تأثير معنوي لكافة المستخلصات ضد خط خلايا سرطان البروستات البشري PC-3.

تمت دراسة ثاثير مستخلص الميثانول في ما يتعلق بحيوية الخلية،هيئة نواة الخلية، نفاذية غشاء الخلية، نفاذية غشاء المايتوكوندريا و cytochrome c و ROSعلى خط خلايا سرطان الثدي البشري MCF-7عن طريق تقنية High Content Screening بأستخدام القياسات المتعددة و ROS. اظهرت النتائج امكانية مستخلص الميثانول على تثبيط نمو خلايا سرطان الثدي البشري MCF-7بنسبة53.92%و بتركيز 200 مايكروغرام/مل للمستخلص. و كانت الفعالية تدريجية اعتمادا على تركيز المستخلص، كما اظهرت النتائج ايضا امكانية زيادة نفاذية الغشاء الخلوي

بزيادة تركيز المستخلص. بينما حصلت تغييرات في شكل نواة الخلية فقط عند التراكيز العالية للمستخلص.

اما بالنسبة لنفاذية غشاء المايتوكوندريا فقد بينت النتائج أمكانية التركيز العالي (200 مايكروغرام/مل) فقط من مستخلص الميثانول بأختزال جهد غشاء المايتوكوندريا و بنسبة 40.3% و الذي بدوره تسبب بأطلاقcytochrome cو زيادة التركيز معنويا و بقدر 0.7 ضعف في معدل كثافة الضوئية المفسفرة بالنسبة للخلايا MCF-7 المعاملة بالمستخلص مقارنة بالخلايا غير المعاملة. كما اكدت النتائج ان التراكيز العالية من مستخلص الميثانول 100 و 200مايكروغرام/مل ادت الى زيادة تخليق اجناس الاوكسجين التفاعلية (ROS) بنسبة 0.53 و 1.32 ضعف على التوالي مقارنة بالخلايا غير المعاملة.

" نَرْفعُ دَرَجَاتٍ مَّن نَّشَآءُ

وَفوْقَ كُلِّ ذِي عِلْمٍ عَلِيمٌ "

من سورة يوسف/ جزء من الاية (76)

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الفعالية المضادة للسرطان والاكسدة لمستخلصات عدة لاوراق نبات العطرة *Pelargonium Graveolens*

رسالة

مقدمة الى كلية العلوم-جامعة النهرين وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الاحيائية

من قبل

فاطمة احمد صبري

بكالوريوس تقانة احيائية / جامعة النهرين 2013

بأشراف

الاستاذ المساعد الدكتور

علي زيد الصفار

حزيران 2016 **شعبان 1437**