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United Arab Emirates University

College of Science

Department of Biology

ANTI-COLON CANCER ACTIVITY OF *ORIGANUM MAJORANA*

Nedaa Faisal Al Tamimi

This thesis is submitted in partial fulfilment of the requirements for the degree of
Master of Science in Molecular Biology and Biotechnology

Under the Supervision of Dr. Rabah Iratni

December 2015

Declaration of Original Work

I, Nedaa Faisal Al Tamimi, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled “*Anti-Colon Cancer Activity of Origanum Majorana*”, hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Dr. Rabah Iratni, in the College of Science at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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Abstract

In the present project, we extend the previous studies of *Origanum majorana* by evaluating the anti-cancer potential of this plant extract on proliferation of colon cancer. Toward this, we first evaluated the effect of the plant extract on the proliferation and DNA damage, *in vitro*, of the human colon cancer HT-29 cell line. We also determine the mechanism by which *Origanum majorana* induced cell death by this extract using biochemical approach. Our results revealed that *O. majorana* inhibited the growth of HT-29 cells by effecting the cells viability. Further work showed the ability of the extract to cause DNA damage, cell cycle arrest and apoptotic death through activation of caspase 8 and 3 pathways. In conclusion our data recommend *Origanum majorana* is an effective inhibitor for colon cancer progression and more investigation has to explore the *Origanum majorana* extract *in vivo*.

Keywords: *Origanum majorana*, HT-2, proliferation, cell viability, DNA damage, cell cycle, apoptotic cell death.

Title and Abstract (in Arabic)

المخلص

تأثير مستخلص نبتة الأوريغانوماجورانا على خلايا سرطان القولون

تعتبر هذه الأطروحة امتداداً لدراسات سابقة أجريت على نبتة الأوريغانوماجورانا (*O. majorana*)، بحيث تهدف هذه الدراسة إلى تقييم مضادات السرطان في هذه النبتة على نمو سرطان القولون (colon cancer). لقد تمت دراسة تأثير هذا المستخلص النباتي على نمو وتلف الحمض النووي (DNA damage) في المختبر على خلايا سرطان القولون للإنسان. كما تم تحديد كيفية آلية عمل هذه النبتة على قتل الخلايا السرطانية باستخدام طرق الكيمياء الحيوية (biochemical approaches). وأظهرت نتائج هذه الدراسة أن نبتة الأوريغانوماجورانا (*O. majorana*) تثبّت نمو سرطان القولون من خلال التأثير على حيوية الخلايا (cells viability) وإضافة إلى ذلك أظهرت هذه النبتة القدرة على إتلاف الحمض النووي، و إيقاف دورة الخلية (cell cycle arrest) و قتل الخلايا المبرمج (programed cell death- apoptosis) عن طريق تفعيل كاسباز 8 و 3 (Caspase8 & 3). وفي الختام فإن هذه النتائج توصي بنبتة الأوريغانوماجورانا كمثبط مؤثر على تقدم ونمو سرطان القولون (colon cancer progression) وسيتم إجراء المزيد من الأبحاث لاستكشاف تأثير هذا المستخلص النباتي على جسم الكائن الحي.

مفاهيم البحث الرئيسية: نبتة الأوريغانوماجورانا، سرطان القولون، حيوية الخلايا، تلف الحمض النووي، دورة الخلية، قتل الخلايا المبرمج.

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Dedication

This work is dedicated to my family, my parents and specially my husband Ali who always encouraged me. And this project is a dedication to my lovely children.

Table of Contents

Title	i
Declaration of Original Work	ii
Copyright	iii
Approval of the Master Thesis	iv
Abstract	vi
Title and Abstract (in Arabic)	vii
Acknowledgements	viii
Dedication	ix
Table of Contents	x
List of Figures	xii
List of Abbreviations.....	xiii
Chapter 1: Introduction	1
1.1 Cancer	1
1.2 Colorectal Cancer	4
1.2.1 Functions of the Colon.....	4
1.2.2 Understanding Colorectal Cancer	4
1.3 Apoptosis.....	5
1.3.1 Programmed Cell Death or Apoptosis	5
1.3.1.1 The Extrinsic Pathway	6
1.3.1.2 The Intrinsic Pathway	7
1.4 Mitotic Cell Cycle.....	9
1.5 Treatments of Cancer	10
1.5.1 Phytochemicals.....	10
1.5.1.1 <i>Origanum majorana</i>	11
1.5.1.2 <i>Origanum majorana</i> and Cancer	13
Chapter 2: Materials and Methods	15
2.1 Preparation of <i>O. majorana</i> Extracts	15
2.2 Cell Culture	15
2.3 Cell Viability.....	15
2.4 Western Blot	16
2.5 Statistical Analysis.....	17
Chapter 3: Results	19
3.1 <i>O. Majorana</i> Extract Inhibits the Viability of the HT-29 Colon Cancer Cells.....	19

3.2 <i>O. majorana</i> Extract Induces Morphological Changes in HT-29 Colon Cancer Cells.....	21
3.3 <i>Origanum majorana</i> extract induces cell death (apoptosis) in HT-29 colon Cancer Cells through caspase 3 activation	23
3.4 Significant induction of apoptosis by <i>O. majorana</i> extract in HT-29 cells.	25
3.5 <i>Origanum majorana</i> Extract Activates the Extrinsic Pathway for Apoptosis via down regulation of Pro-caspase 8.	27
3.6 <i>Origanum majorana</i> Leads to Upregulation of p21 in treated HT-29 cells.....	29
3.7 Detection of γ H2AX, a Marker of DNA fragmentation, in Treated HT-29 Cells with <i>O. majorana</i> Extract.....	31
Chapter 4: Discussion	33
Chapter 5: Conclusion.....	38
Bibliography.....	39

List of Figures

Figure 1: Cancer hallmarks (Hanahan & Weinberg, 2011).	3
Figure 2: Extrinsic and intrinsic apoptotic pathways (Favaloro et al., 2012).	8
Figure 3: Effect of <i>O. majorana</i> on cell viability of colon cancer HT-29. Cell viability in percent after incubation with the indicated concentrations of OME.	20
Figure 4: Morphological changes in HT-29 cells treated with OME. Morphological changes observed in the treated HT-29 cells after 48 hours. Images taken at 40x and 400x magnifications. Cells were examined under EVOS XL Core Cell Imaging System (Life Technologies).....	22
Figure 5 : An increase in the level of caspase 3 in cells treated with OME treated cells. Cells were treated with or without increasing concentrations of OME for 48 hr. Western blot analysis was carried out using anti-cleaved caspase 3.	24
Figure 6: Induction of apoptosis by <i>O.majorana</i> extract in HT-29 cells. Cells were treated for 48 hr. with different concentrations of the OME extract and proteins were extracted. Western blot analysis was carried out using anti- cleavedPARP antibodies.	26
Figure 7: A decrease in the expression of pro-caspase 8 in cells treated with OME treated cells. Cells were treated with or without increasing concentrations of OME. Western blot analysis was carried out using anti-pro-caspase 8.	28
Figure 8: Expression levels of p21 in <i>O.majorana</i> -treated HT-29 cells. Cells were treated with or without increasing concentrations of OME. Western blot analysis was carried out using anti-p21 antibody.....	30
Figure 9: <i>O. majorana</i> extract induces a dose-dependent activation of γ H2AX, a marker of DNA double-strands breaks in HT-29 colon cancer cells. Western blot analysis of γ H2AX in HT-29 cells exposed for 48h with the indicated concentrations of OME or equal volume	32
Figure 10: The proposed model shows the effect of <i>Origanum majorana</i> on HT-29 human colon cancer cells.....	37

List of Abbreviations

OM	<i>Origanum majorana</i>
OME	<i>Origanum majorana</i> Extract
IARC	International Agency for Research on Cancer
FAP	Familial Adenomatous Polyposis
APC	Adenomatous Polyposis Coli
HNPCC	Hereditary Non Polyposis Colon Cancer
ECM	Extracellular Matrix
TNF	Ligand Necrosis Factor
FADD	Fas-associated death Domain Protein
TRADD	Receptor Associated Death Domain protein
DEDs	Death Effector Domains
Apaf1	Activating Factor1 Apoptotic Protease
CDK	Cyclin Dependent Kinases
PARP	Poly ADP Ribose Polymerase
TNBC	Triple Negative Breast Cancer
P53	Tumor Suppressor Protein
P21	Cyclin Dependent Kinase Inhibitor
HUVECs	Human Umbilical Vein Endothelial Cells

FAS	FAS Cell Surface Death Receptor
γ H2AX	Histone 2A variant
NF- κ B	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
I κ B	Inhibitor of κ B
uPAR	urokinase plasminogen activator receptor

Chapter 1: Introduction

1.1 Cancer

Cancer is a multifactorial disease that is characterized by an abnormal growth of cells and the migration of those cells from their original site to other sites in the body (Alberts *et al.*, 2002). Cancer is a major health problem worldwide. It is considered to be the second leading cause of death in high-income countries (cardiovascular diseases is the first) and the third leading cause of death in middle- and low-income countries (after cardiovascular diseases and infectious and parasitic diseases) (American Cancer Society, 2012b). Statistics from the International Agency for Research on Cancer (IARC), indicated that 14.1 million cases were diagnosed with cancer in 2012 worldwide. While total cancer deaths reached 8.2 million in the same year (International Agency for Research on Cancer, 2012). Terrifyingly the global burden is expected to grow to 21.7 million new cancer cases and 13 million cancer deaths by 2030 (American Cancer Society, 2012a). In UAE 1729 cancer incidences were reported in 2012 (Health Authority, 2012).

There are over 100 types of cancer that have been classified based on their tissue origin. Carcinomas are types of cancer that originate from epithelial cells. Cancer that affects the cells of the lymph system is known as lymphoma. Sarcomas are cancers that occur in mesoderm such as bone and muscles, while adenocarcinomas are cancers that derived from glandular tissue such as breast (Alberts *et al.*, 2002).

Cancer can be caused by several factors that can be classified into internal and external factors. Internal factors include genomic instability and mutations that could occur during DNA replication in addition to different genomic factors. On the other

hand, external factors include environmental causes such as ultraviolet, diet and smoking (American Cancer Society, 2012b).

Basic hallmarks of cancer were described in a considerable scientific work by Hanahan and Weinberg. These hallmarks include abnormal induction of proliferative signals, over expression of oncogene, cells survive rather than performing apoptosis, unlimited cell division, angiogenesis (formation of new blood vessels) and tumor metastasis (migration of the cells from their original site) (Hanahan & Weinberg, 2011). Additional hallmarks are indicated in Figure 1.

Tumors can be classified according to their migration status into benign or malignant. Benigns are primary tumors consist of abnormal cells that accumulate in a dormant state which originate in a specific tissue and do not migrate to different locations (Adams *et al.*, 2010; Teiten, Gaascht *et al.*, 2010). However, malignant tumors are known to migrate from their primary site, through the extracellular matrix (ECM), blood, lymph and the endothelium of the affected tissues to develop secondary tumor (Pecorino, 2012).

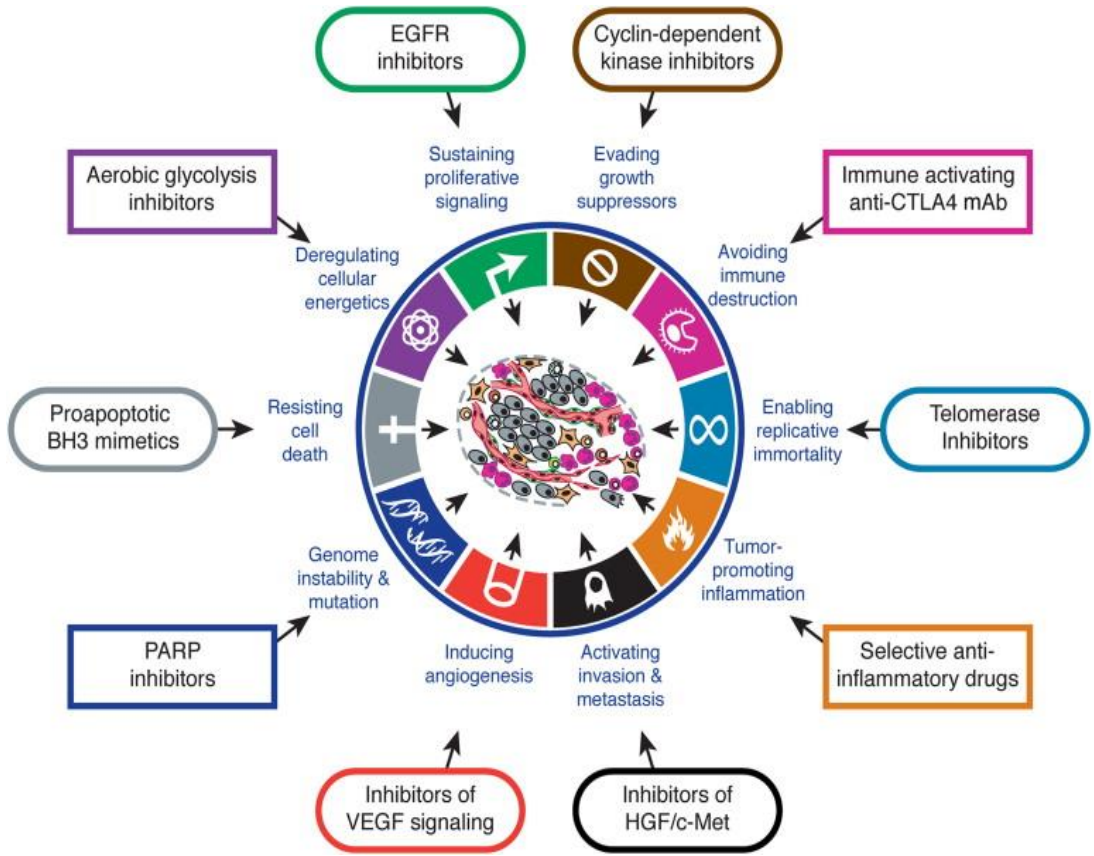


Figure 1: Cancer hallmarks (Hanahan & Weinberg, 2011)

1.2 Colorectal Cancer

1.2.1 Functions of the Colon

Colon also called the large intestine is an organ of the digestive system that function to absorb water and store the waste-products of digestion until the body is ready to empty them out (Elias, 2012). Under normal condition, the colonic epithelium is renewed constantly by crypt proliferative cells which migrate upward along the crypt-villi axis as they terminally differentiate (Lipkin, 1973). The average time for these cells to proliferate is one day, and around 25% of the colonic epithelium is rejuvenated every day. Moreover, the crypt epithelial cells of the entire colon and rectum are replaced every three to four days (Lipkin, 1973).

1.2.2 Understanding Colorectal Cancer

Colorectal cancer is one of the most common cancers worldwide. According to the American Cancer Society, in 2012 colorectal cancer found to be the third most common cancer in men and the second in women. Approximately, 1.4 million cases of colorectal cancer occurred in 2012 worldwide. Around 693,900 deaths from colorectal cancer occurred in 2012 worldwide, which accounts for 8% of all cancer deaths (American Cancer Society, 2012b). As for UAE, colorectal cancer is considered to be the third common cancer, and the fourth leading cause of death among other cancers (Health Authority, 2012).

Colorectal cancer induced from multiple genetic and epigenetic changes leading to the transformation of colon epithelial cells into invasive adenocarcinomas (Ng & Yu, 2015). In addition, genetics has been attributed to colon cancer to either polyposis or

non-polyposis syndromes. Polyposis syndrome is the familial adenomatous polyposis (FAP), which is associated with mutated gene or loss of FAP (also called the adenomatous polyposis coli (APC) gene (Grady, 2003). Non-polyposis colorectal cancer (HNPCC) syndrome is hereditary, which is associated with germ-line mutations in DNA mismatch repair genes (Labianca *et al.*, 2010).

1.3 Apoptosis

1.3.1 Programmed Cell Death or Apoptosis

There are several types of cell death such as autophagy, necrosis and apoptosis. Apoptosis is a highly regulated process of cell death that is characterized by morphological changes such as cell shrinkage, membrane blabbing, chromatin condensation, and nuclear fragmentation (Kerr *et al.*, 1972). The balance between cell growth, differentiation and cell death for maintenance of cellular homeostasis (Rupnarain, Dlamini, Naicker, & Bhoola, 2004).

Apoptosis is an effective therapeutic mechanism in cancer therapy by designing drugs that induce apoptosis in tumor cells without any disturbance to the healthy tissues (Micheau & Tschopp, 2003; Zouet *al.*, 1999). Cells may be induced to undergo apoptosis by extracellular signals “death factors” which is considered as an extrinsic pathway. While the other pathway induced by internal physical or chemicals signals “oxidative stress or DNA damage” called intrinsic pathway (Gupta, 2003).

1.3.1.1 The Extrinsic Pathway

External signals are part of the normal control which insure that individual cells behave for the good of the organism as a whole, through surviving when they are needed and killing themselves when they are not. Several extracellular signals stimulate apoptosis, while other inhibit it (Alberts *et al.*, 2002; Pecorino, 2012).

Some extracellular signals stimulate apoptosis such as death factors. Examples of death factors are Fas ligand or tumor necrosis factor (TNF) and both received through transmembrane death receptors such as Fas receptor or TNF receptor respectively. Fas ligand is bound to the plasma membrane of neighboring cells, while TNF is a soluble factor. Once ligand bind to the death receptor, the receptor undergoes conformational changes in-order to transmit the signal into the cell. These conformational changes lead to the exposure of the death domains which are located on the cytoplasmic tail of the receptors and enable intracellular adaptor proteins to bind through their death domains (Micheau & Tschopp, 2003). Example of intracellular adaptor proteins are Fas-associated death domain protein (FADD) and TNF receptor-associated death domain protein (TRADD) (Backus *et al.*, 2002).

These adaptor proteins found to transduce the death signal from the receptor to caspases. The adapters recruit procaspase-8 molecules through death effector domains (DEDs). Once all the procaspases-8 molecules close to each other, they become activated by self-cleavage. Caspase-8 is well-known to link between the receptor and the apoptotic proteases for that reason it's called as an initiator caspase for the extrinsic cascade. All of them together, the death ligands, receptors, adaptors, and initiators caspases are called death inducing signaling complex (DISC). Caspase activation is initiated by caspase-8 which in turn activate other caspases which called

the executioner caspases (caspase 3, 6 and 7). As a result, cleavage of specific target proteins occur which lead to apoptosis (Pecorino, 2012).

1.3.1.2 The Intrinsic Pathway

Cells can activate their apoptosis program internally, usually in response to injury or stresses (e.g., DNA damage or lack of oxygen or nutrients). The intracellular activation of apoptotic pathway occurs through the intrinsic pathway of apoptosis, which depends on the release of cytochrome c from the mitochondria to the cellular cytosol. Once it is released into the cytosol it binds to procaspases activation adaptor protein called apoptotic protease activating factor-1 (Apaf1) (Adrain & Martin, 2001). That causes the oligomerization of Apaf1 into a wheel like heptamer called apoptosome. Consequently, the apoptosome recruits initiator procaspase proteins (Procaspase-9) that will be activated to form caspase-9 (cleaved form). The activated caspase-9 which will activate downstream executioner procaspases to induce apoptosis (Pecorino, 2012). Figure 2 shows both apoptotic pathways.

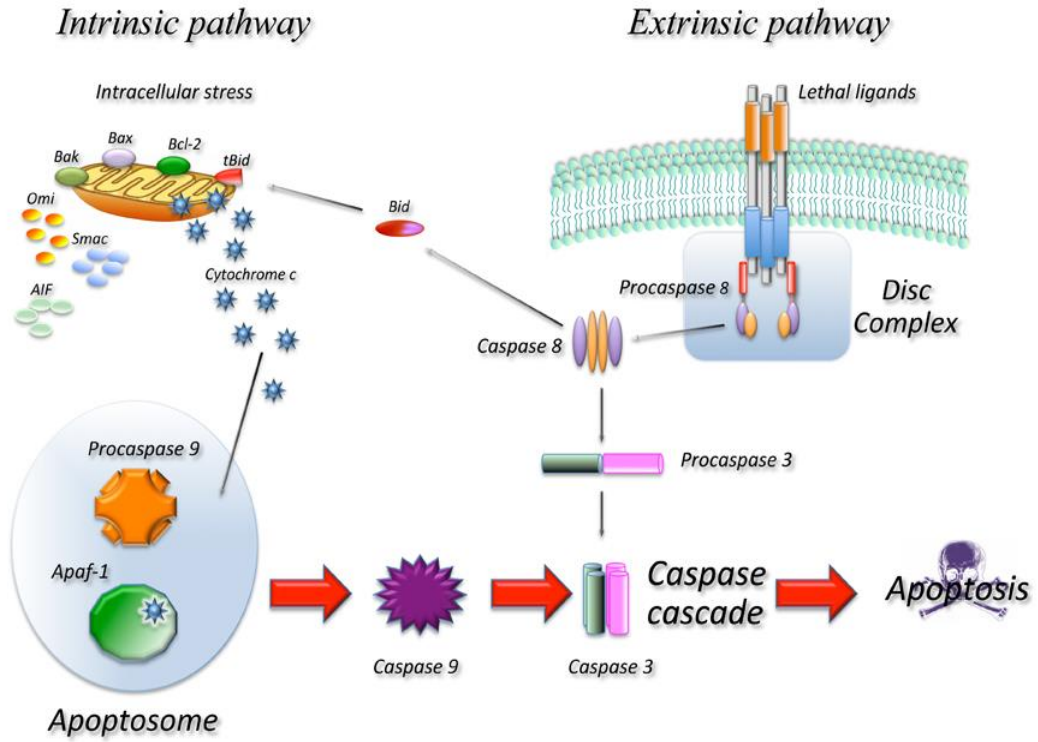


Figure 2: Extrinsic and intrinsic apoptotic pathways (Favaloro et al., 2012)

1.4 Mitotic Cell Cycle

Cells reproduce via duplicating their contents and dividing into two. This cycle of division and duplication known as a cell cycle. These processes are defined into two major phases which are S phase (DNA synthesis and chromosome duplication) and M phase (chromosome segregation and cell division). The M phase includes two major events; nuclear division (mitosis) and cell division (cytokinesis) (Alberts *et al.*, 2002).

Most cell cycles contain additional phases that are called gap phases. These gap phases provide additional time for chromosomes duplication and segregation that are needed for cell growth generally. Additionally, these gaps serve as transition regulators in the cell cycle progression. The first gap phase, G1, found before S phase, while G2 occurs before M phase (Morgan, 2007).

The central components of the cell-cycle control system that include check points which regulate the transition from one phase to another are family of enzymes called cyclin-dependent kinases (Cdks). Cdks belong to serine/threonine protein kinases which are activated at specific points during cell cycle. They get activated via binding to regulatory proteins called cyclins. Different cyclin-Cdks complexes are present at specific stage in the cell cycle and are important to regulate irreversible phase transition. Any defects in cell cycle regulation can result in developmental abnormalities and cancerous growth (Keaton, 2007).

The p16 (from INK family) and p21 (from Cip/Kip family) are proteins inhibitors that involved in regulating cyclin-Cdks activity. The p16 family includes p16, p15, p18 and p19. While, the p21 family's members are p2, p27 and p57. These inhibitors

found to interact with cyclin-Cdks complexes by blocking the ATP-binding site, therefore disabling their kinase activity which leads to cell cycle arrest (Pecorino, 2012).

1.5 Treatments of Cancer

There are many types of cancer treatments, and these types are depending on the type of cancer and how advanced it is. These treatments include surgery, chemotherapy, radiation therapy, hormonal therapy or combined therapy. However, these kinds of therapies known to have different side-effects. Hence, identification and development of new chemotherapeutic agents from plants “Phytochemicals” have gained significant recognition in the field of cancer therapy and become a major area of experimental cancer research (Lin,*et al.*, 2002). Recently, scientists all over the world are concentrating on the herbal medicines to fight against cancer. By understanding the complex synergistic interaction of various constituents of anticancer herbs, new novel herbal anticancer agents can be discovered and designed to attack the cancerous cells without affecting normal cells of the body (Pratheeshkumar, 2012).

1.5.1 Phytochemicals

Plants have been shown to be an excellent source of new drugs, including anticancer agents. There are convincing evidences from experimental studies that highlighted the importance of naturally occurring compounds derived from plants in reducing the risk of several cancers and inhibit the development and spread of tumors. With the

development of molecular and biochemical isolation and purification techniques, many anticancer agents derived from plants have been identified and developed. During the last 20 years, more than 25% of drugs that are used were directly derived from plants, while the other 25% were chemically altered natural products (Cragg & Newman, 2000) . Different examples of anticancer drugs derived from plants are currently in use in clinical fields. These include vinca alkaloids vinblastine and vincristine that were isolated from *Catharanroseus*, terpene paclitaxel from *Taxusbrevifolia* Nutt., and the DNA topoisomerase I inhibitor camptothecin from *Camptothecaacuminata* (Kaczirek et al., 2004). Moreover, there are other types of promising bioactive compounds derived from plants, are currently in clinical trials or preclinical trials or undergoing further investigation. Examples include flavopiridol, roscovitine, combretastatin A-4, betulinic acid and silvestrol. Different studies mentioned the importance of consuming fruits and vegetables, as a source of phytochemicals, in reducing the incidence of cancer. These includes vitamins (carotenoids) and food polyphenols, flavonoids, phytoalexins, phenolic acids indoles and sulfur rich compounds (Vági *et al.*, 2005). The advantage of using such compounds for cancer treatment is their non-toxic nature. An ideal phytochemical is one that has anti-tumor properties with minimal or no toxicity and has a defined mechanism of action.

1.5.1.1 *Origanum majorana*

One of these plant used for their availability of phytochemicals is *Origanum majorana* which belongs Lamiaceae family. Commonly known as marjoram. A large number of known species of the genus *Origanum* are used worldwide as spices and

has a long history of both culinary and medicinal use. *O. majorana* is used as a home cure for chest infection, stomach disorders, cough, nervous disorders, sore throat, rheumatic pain, cardiovascular diseases, and skin care (Al-Harbi, 2011; Vági *et al.*, 2005). Several studies used *marjoram* species both *in vitro* and *in vivo* approaches. These studies indicated that *O. majorana* is very rich in phenolic compounds. The high phenolics content in *Origanum* has a capacity to scavenge free radicals and is shown to be associated with strong antioxidant activity (Miron, Plaza, Bahrim, Ibáñez, & Herrero, 2011). *O. marjoram* was shown to contain phenolic terpenoids (thymol and carvacrol), tannins, flavonoids (diosmetin, luteolin, and apigenin), phenolic glycosides (arbutin, methyl arbutin, vitexin, orientin, and thymonin), hydroquinone, and triterpenoids (ursolic acid and oleanolic acid) (Tsimogiannis, Stavrakaki, & Oreopoulou, 2006). In addition, *O. majorana* has been reported to exhibit a significant anti-microbial activity (Leeja & Thoppil, 2007). Several studies have also revealed that ethanolic, aqueous extracts and essential oil of *O. majorana* could protect against kidney and liver damage and genotoxicity induced by lead acetate (I. El-Ashmawy, 2014; I. M. El-Ashmawy, El-Nahas, & Salama, 2005; I. M. El-Ashmawy, Saleh, & Salama, 2007). *O. majorana* has also been shown to inhibit platelet adhesion aggregation and secretion (Yazdanparast & Shahriyary, 2008). A study by Al Harbi shows that extract of *O. majorana* reduced the side effects of cyclophosphamide, an established anticancer drug, without altering its cytotoxicity (Al-Harbi, 2011).

1.5.1.2 *Origanum majorana* and Cancer

In previous studies, it has been shown that *Origanum majorana* extract (OME) was able to inhibit the proliferation of the MDA-MB-231 breast cancer cell lines in time and concentration-dependent manner. By using different concentrations, OME elicits different effects on the MDA-MB 231 cells (I. M. El-Ashmawy *et al.*, 2007). Low concentration induced an accumulation of apoptotic –resistant population of cells arrested in mitosis and overexpressing the cyclin-dependent kinase inhibitor, p21 and the inhibitor of apoptosis, survivin. On the other hand, higher concentration of OME triggered a massive apoptosis through the extrinsic pathway, including the activation of tumor necrosis factor- α (TNF- α), caspase 8, caspase 3, and cleavage of PARP, downregulation of survivin and depletion of the mutant p53 in MDA-MB-231 cell (Al Dhaheri, Eid, *et al.*, 2013a). Moreover, it was demonstrated that non-cytotoxic concentrations of *O. majorana* significantly inhibited the migration and invasion of the MDA-MB-231 cells as shown by wound-healing and matrigel invasion assay. Also it has been showed that *O. majorana* induced homotypic aggregation of MDA-MB-231 associated with an upregulation of E-cadherin protein and promoter activity. Furthermore, *O. majorana* decreased the adhesion of MDA-MB-231 to HUVECs and inhibited transendothelial migration of MDA-MB-231 through TNF- α -activated HUVECs. Gelatin zymography assay showed that *O. majorana* suppressed the activities of matrix metalloproteinase-2 and -9 (MMP-2 and MMP-9). ELISA, RT-PCR and Western blot results revealed that *O. majorana* decreased the expression of MMP-2, MMP-9, urokinase plasminogen activator receptor (uPAR), ICAM-1 and VEGF. Further investigation revealed that *O. majorana* suppressed the phosphorylation of I κ B, down-regulated the nuclear level of NF κ B and reduced Nitric Oxide (NO) production in MDA-MB-231 cells. Most importantly, we also

showed that *Origanum majorana* promoted tumor growth inhibition and blocked metastasis *in vivo* using chick embryo tumor growth assay (Al Dhaheri, Attoub, *et al.*, 2013) .

Chapter 2: Materials and Methods

2.1 Preparation of *O. majorana* Extracts

Dried leaves of the plant (5.0 gm) were ground to powder by using mortar and pestle. Then, the fine samples were embedded in 100ml of 70% ethanol and kept in dark at 4°C. This combination was refrigerated without stirring. After for 72 hours, the mixture was filtered and evaporated at room temperature. The green residue was vacuumed for 2–3 hours. Finally, the extract mass was recorded.

2.2 Cell Culture

Human cancer cell lines HT-29, were maintained in DMEM cell growth medium completed with 10% fetal bovine serum and 100U/ml penicillin/streptomycin and L-Glutamine. Cells were maintained in a humidified incubator at 37°C in a 5% CO₂atmosphere.

2.3 Cell Viability

Cancer cells were seeded at a density of 6,000 cells / well in 96-well plates. After 24 h, cells were treated for various time (24, 48 or 72h) with increasing concentrations of the *O. majorana* extract (0, 75, 150, 300, 450, 600µg/ml). Experiments were carried out in triplicate and repeated 5 times. Control cells were treated with vehicle (ethanol). The effect of drugs on cell viability was determined using cytotoxicity kit (ABCAM) according to the manufacturer instructions and the results were measured

using microplate reader. The data presented as proportional viability (%) by comparing the treated group with the untreated cells, the viability of which is assumed to be 100%.

2.4 Western Blot

Western blot technique was used to detect the expression levels of several proteins involved in cell death, DNA damage as well as cell cycle regulators. Proteins were extracted from treated and untreated cells. Using RIPA lysis buffer (25mM Tris•HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). The lysates were centrifuged at 13000 rpm for 30 min. and the supernatants were collected and protein concentration measurements were performed. Aliquots of the lysates were separated via SDS–PAGE, and then it was transferred onto a nitrocellulose membrane. After blocking with 5% non-fat dried milk containing phosphate-buffered saline (PBS) and 0.1% Tween-20 for one hour, the membranes were incubated overnight with primary antibodies, followed by an additional incubation with secondary antibodies. A variety of human antibodies were used at different dilutions according to the manufacturer instruction as primary antibodies, and secondary antibodies such as horseradish peroxidase-conjugated goat anti-rabbit or mouse at a 1:5000 dilution. The membranes were then exposed to X-ray film.

2.5 Statistical Analysis

Results were expressed as means \pm S.E.M. of the number of experiments. A Student's t-test for paired or unpaired values was performed and a p value of <0.01 was considered statistically significant.

Chapter 3: Results

3.1 *O. Majorana* Extract Inhibits the Viability of the HT-29 Colon Cancer Cells

We started our study by testing the effect of *Origanum majorana* extract (OME) on colon cancer cells by measuring the viability of these cells using different concentrations of the extract (0, 75, 150, 300, 450 and 600 $\mu\text{g/mL}$) on the cultured HT-29 colon cancer cell line for different incubation periods (Figure 3). Our results which have been compared to a vehicle containing control have shown that the exposure of the HT-29 to OME decreased cellular viability in a concentration- and a time-dependent manner. In the control culture of HT-29 cells which was treated with ethanol only, cancer cells proliferated in a constant rate. The IC₅₀ (producing half-maximal inhibition) was approximately 450 $\mu\text{g/mL}$ at 24 h, 250 $\mu\text{g/mL}$ at 48 h, and 300 $\mu\text{g/mL}$ at 72h treatment. Obviously, there is a decline in the number of the viable cells treated with the (OME) depending on the time and concentration.

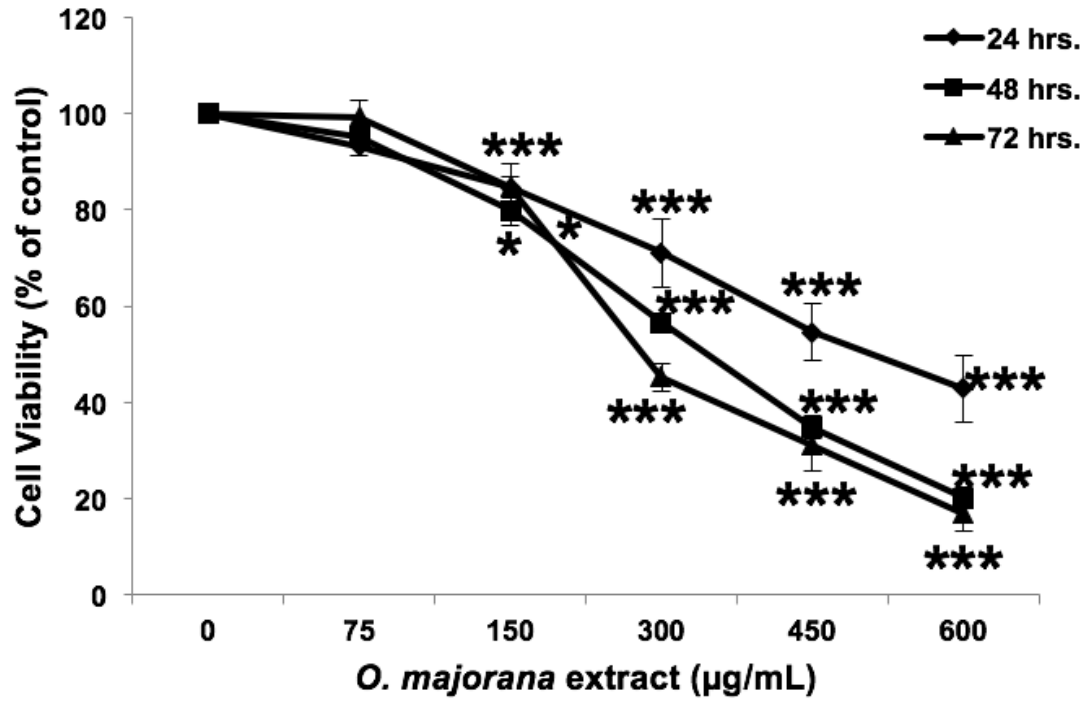


Figure 3: Effect of *O. majorana* on cell viability of colon cancer HT-29. Cell viability in percent after incubation with the indicated concentrations of OME

3.2 *O. majorana* Extract Induces Morphological Changes in HT-29 Colon Cancer Cells.

The observation of the OME-treated HT-29 cells based on light microscopy also showed that the number of treated cancer cells decreased when the concentration of drug increased. Furthermore, under light microscopy observation of HT-29 cells treated with various concentrations of 150, 300, 450 and 600 µg/mL OME. Cells underwent morphological changes which occurred after 48 hours of treatment such as rounding and shrinkage, which are characteristic of apoptosis as shown in Figure 4.

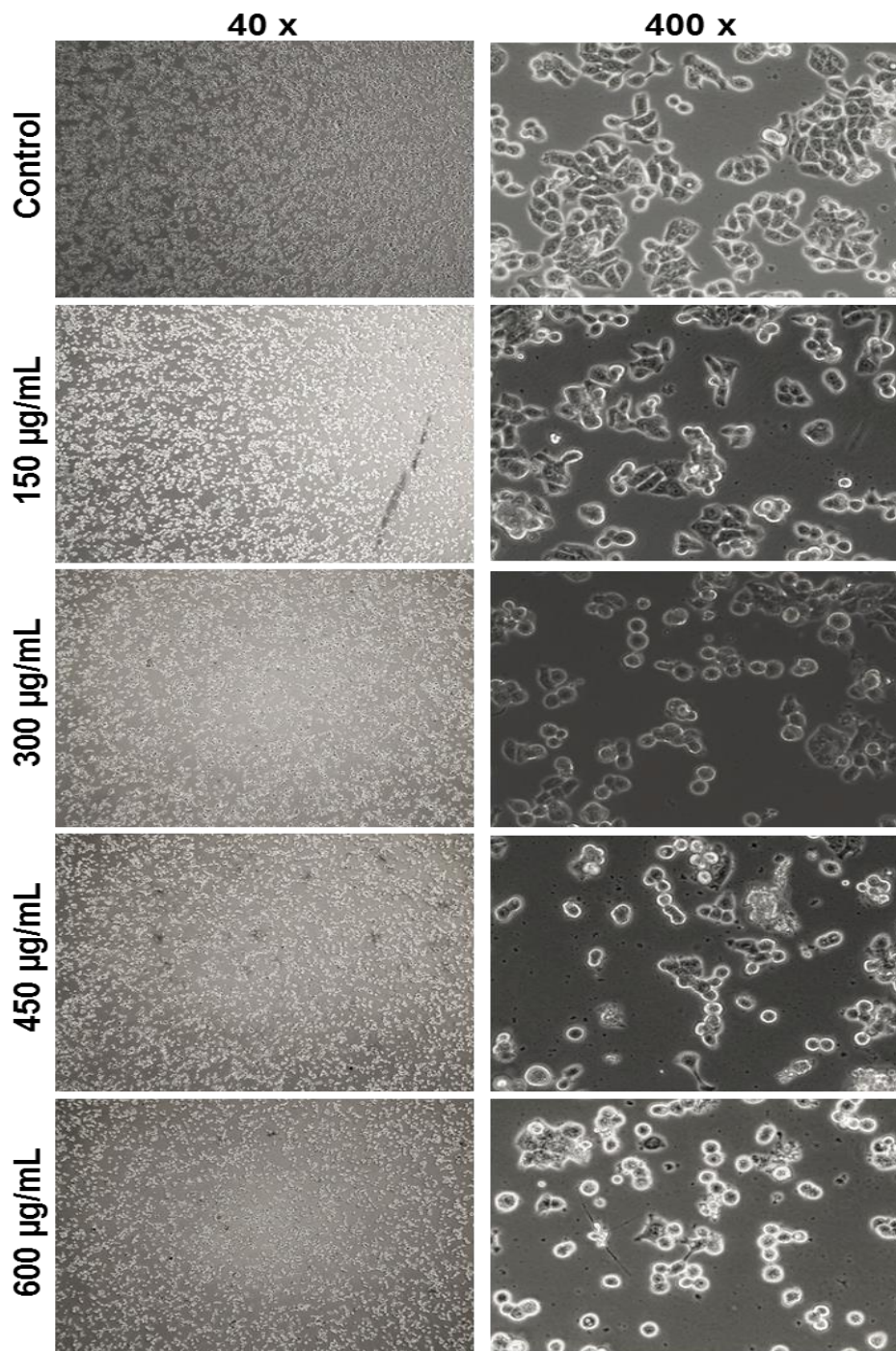


Figure 4: Morphological changes in HT-29 cells treated with OME. Morphological changes observed in the treated HT-29 cells after 48 hours. Images taken at 40x and 400x magnifications. Cells were examined under EVOS XL Core Cell Imaging System (Life Technologies)

3.3 *Origanum majorana* extract induces cell death (apoptosis) in HT-29 colon Cancer Cells through caspase 3 activation

Caspase 3 is considered as one of the most important executioner caspases in apoptosis which causes cell death. As shown in Figure 5, after exposure of HT-29 cells to OME, our experiment revealed that there is a basic level of caspase 3 in the control cells. However, there was an increase in the activecaspase 3 level in the treated cells especially at concentration of (300 µg/ml 450 µg/ml and 600ug/ml) which means there is cell death caused by caspase 3 activation.

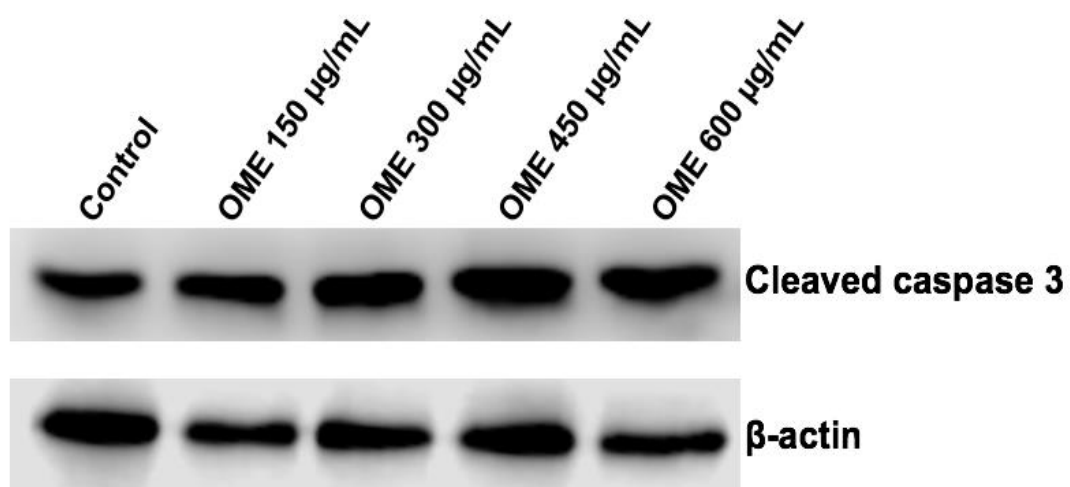


Figure 5 : An increase in the level of caspase 3 in cells treated with OME treated cells. Cells were treated with or without increasing concentrations of OME for 48 hr. Western blot analysis was carried out using anti-cleaved caspase 3

3.4 Significant induction of apoptosis by *O.majorana* extract in HT-29 cells.

To further confirmation the induction of apoptosis using OME on HT-29 cell, we decided to investigate PARP expression in the treated cell. Because PARP is considered as an apoptosis marker, cells were treated with different doses for 48h. As shown in figure 6, a high accumulation of cleaved PARP was detected in concentrations (300, 450 and 600 $\mu\text{g/mL}$ of OME) which confirm the occurrence of apoptosis.

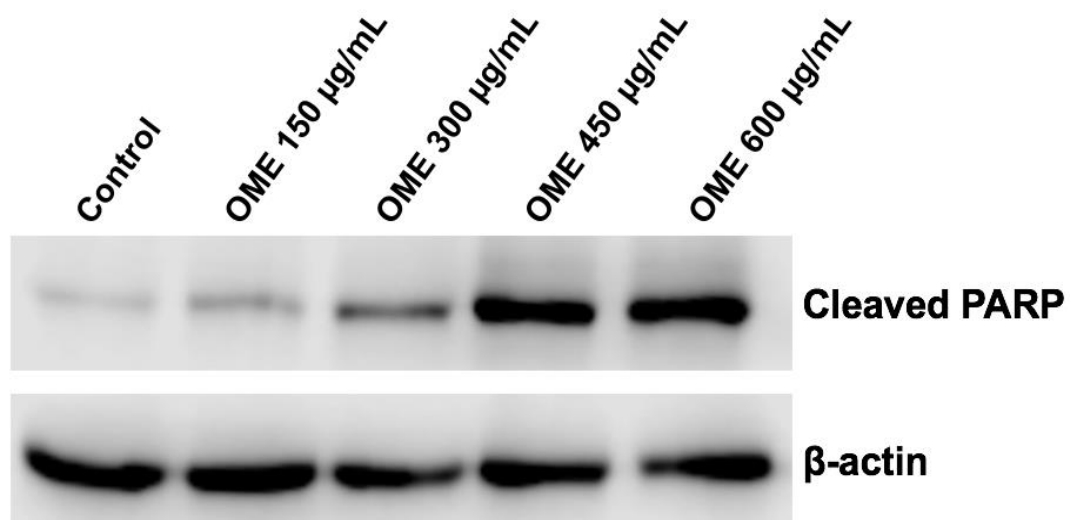


Figure 6: Induction of apoptosis by *O.majorana* extract in HT-29 cells. Cells were treated for 48 hr. with different concentrations of the OME extract and proteins were extracted. Western blot analysis was carried out using anti-cleaved PARP antibodies

3.5 *Origanum majorana* Extract Activates the Extrinsic Pathway for Apoptosis via down regulation of Pro-caspase 8.

After we explored the activity of caspase 3 induced by OME, we decided to determine the cell death pathway if it's extrinsic or intrinsic by examining the activity of the initiator caspase. Therefore, we investigated the activity of pro-caspase 8, the inactive form, in different OME concentrations on HT-29. Interestingly, we found that after 48h of treatment, pro-caspase 8 level decreases in a concentration dependent manner (Figure 7). As a result, that lead to the increase of active form of caspase 8 (cleaved form). Therefore, we concluded that OME induce apoptosis through extrinsic pathway.

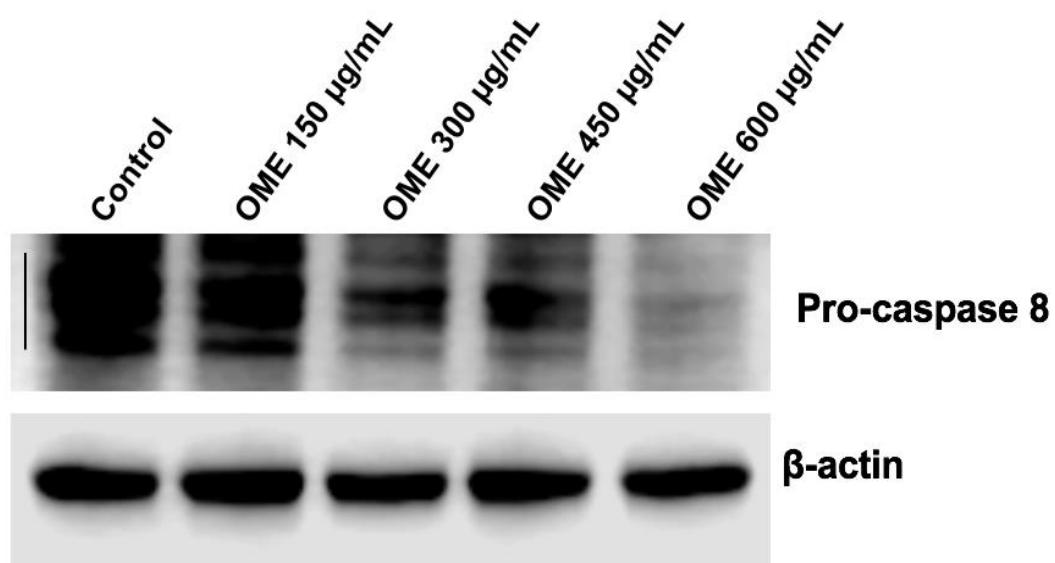


Figure 7: A decrease in the expression of pro-caspase 8 in cells treated with OME treated cells. Cells were treated with or without increasing concentrations of OME. Western blot analysis was carried out using anti-pro-caspase 8.

3.6 *Origanum majorana* Leads to Upregulation of p21 in treated HT-29 cells

By western blot analysis, we observed an up-regulation in the expression of p21 in the treated cells. Actually, the cell cycle (in normal cases) occurs in a checked matter in the presence of regulator proteins, CDK and cyclin. CDK protein becomes active when it binds to cyclin. In cancer cells, there is uncontrolled cell division. Hence, the role of CDK inhibitor protein P21 plays a critical role in inducing cell cycle arrest. P21 binds to CDK and stops its activity by inhibiting cyclin binding. Abbas and Dutta showed that p21 might has a role in promoting apoptosis via both p53-independent and p53-dependent mechanisms below certain cellular stresses and responses. However, the exact role of how p21 promotes apoptosis is not yet clear. Nonetheless could depend on both p53-independent and p53-dependent up-regulation of the proapoptotic protein BAX, effects on DNA repair or activation of members of the TNF family of death receptors (Gartel, 2005). Different studies showed a pro-apoptotic role for p21, it was revealed only that apoptosis coexisted with induction of p21 without defining whether p21 is needed for the induction of apoptosis. Therefore, a careful analysis is required to explore the exact role of p21 under these conditions. Upon applying different concentrations of OME, low concentration of OME (150 $\mu\text{g/mL}$ of OME) did not show any induction of p21 compared to higher concentrations. Upregulation of p21 protein started with higher dose of OME (Figure 8). Based on that, we can demonstrate that p21 expression contributes to the cell cycle arrest and also to apoptosis (Abbas & Dutta, 2009).

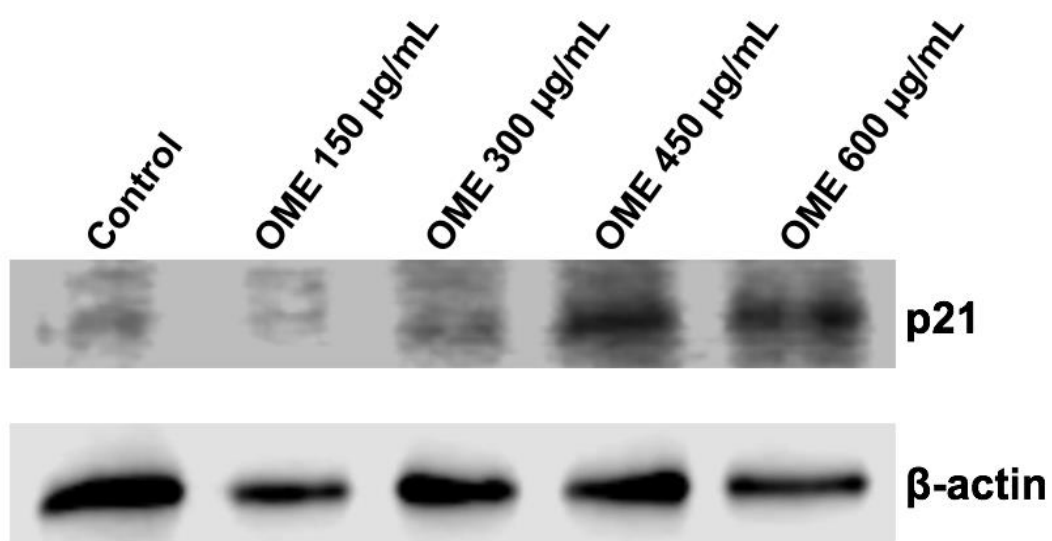


Figure 8: Expression levels of p21 in *O.majorana*-treated HT-29 cells. Cells were treated with or without increasing concentrations of OME. Western blot analysis was carried out using anti-p21 antibody

3.7 Detection of γ H2AX, a Marker of DNA fragmentation, in Treated HT-29 Cells with *O. majorana* Extract

Finally, we wanted to investigate whether OME caused DNA damage in HT-29 cells. HT-29 cells were treated with either ethanol (control) or various concentrations of OME (150, 300, 450 and 600 mg/mL). After 48 h, the level of γ H2AX was measured to indicate the DNA damage. A Western blotting analysis revealed a concentration-dependent increase in the levels of γ H2AX in response to OME treatment (Figure 9), this can be explained by the accumulation of double strand DNA breaks in these cells.

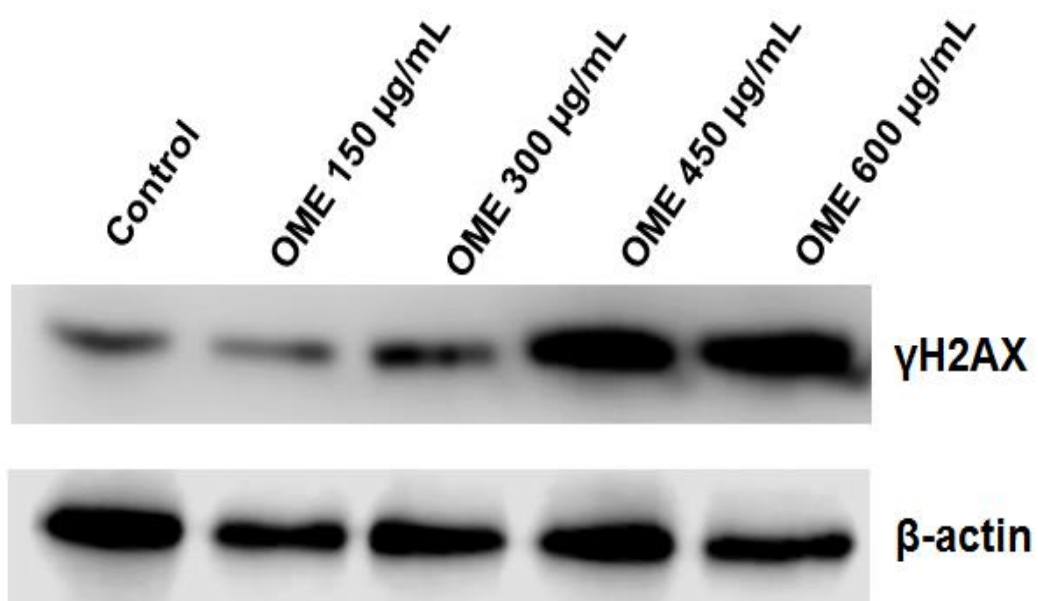


Figure 9: *O. majorana* extract induces a dose-dependent activation of γ H2AX, a marker of DNA double-strands breaks in HT-29 colon cancer cells. Western blot analysis of γ H2AX in HT-29 cells exposed for 48h with the indicated concentrations of OME or equal volume of vehicle (ethanol) as control

Chapter 4: Discussion

Nowadays, finding a balance in cell proliferation expected to be a master key in homeostatic maintenance by inhibiting uncontrolled cells proliferation. Through arresting the cell cycle and induction of apoptosis in progressed cancer cells. An increasing number of evidences focused on the importance of phytochemicals and their effect in the metastasis of different cancers including colon cancer. At this study, we have illustrated that the *Origanum majorana* extract inhibited the proliferation of the colon cancer cell line HT-29. We have investigated a cell death mechanism which inhibit the cell cancer growth.

Firstly, we studied cell viability of treated cells with *O. majorana* extract. We have shown a significant cytotoxic effect of the OME on the HT-29 which was in time and dose manners. Morphological changes were detected on the treated cells which revealed that colon cancer cells went through cell death. Consequently, we tried to investigate the mechanism that caused the cell death.

Our data based on experimental molecular studies demonstrated that *O. Majorana* extract induced a cell cycle arrest linked with a high expression of P21. OME triggered the apoptotic cell death by activation of caspase 8 as we showed that by detecting the level of caspase 8 as a marker of extrinsic pathway. Furthermore, we revealed a DNA damage by a high expression of γ H2AX.

Apoptosis is a very important cell death process that kill cancer cells in two different pathways, either extrinsic pathway or intrinsic pathway which can be characterized by the caspases that are stimulated and activated in the cells. In general, the extrinsic pathway triggered by binding of the ligands with their receptors such as TNF- α and

Fas (Pecorino, 2012). The death receptors ligation with their ligands activate caspase 8 and this event consequently activate caspase 3 which lead to PARP cleavage and ultimately apoptosis (Gupta, 2003). In our study, we found that *O.majorana* extract inhibited the colon cancer cells growth by activation of caspase 8. In our experiments, the level of procaspase 8 was very high at the control then it started to decrease with higher concentrations of OME, that means procaspase-8 has been cleaved into its active form which is caspase 8.

Actually, caspase 3 is activated by both pathways of apoptosis, extrinsic and intrinsic and it leads to an effective destruction of the cell followed by a death. Apoptotic cell death is caspase mediated through the cleavage of multiple proteins crucial in cellular functioning (Fischer, Jänicke, & Schulze-Osthoff, 2003). For further conformation of apoptosis, cleavage of PARP should be detected (Kaufmann, Desnoyers, Ottaviano, Davidson, & Poirier, 1993; Tewari *et al.*, 1995). PARP is known to have an essentialrole in DNA damage detection and repair. Therefore cells with damaged DNA have amplified PARP (Herceg & Wang, 1999; Lemaire, Andréau, Souvannavong, & Adam, 1998).

We wanted to highlight the cleavage of PARP in our treated cells. PARP inhibition is found to be related to many cellular alterations. However, PARP fragments detected by western blot is recognized as biomarkers for specific protease activity in prograded cell death. As shown in our results, PARP cleavage was increase dramatically with dose dependent manner.

On the other hand, DNA damage is estimated as one of the molecular signals associated with cell cycle arrest and apoptosis. So, we measured the level of γ H2AX to assess the DNA damage. Successfully, we found that γ H2AX accumulated in high

concentrations in OME treated cells. Our result demonstrates that OME caused a double stranded DNA damage in a concentration dependent manner.

Alongside with DNA damage and cell cycle arrest, we liked to explore if DNA damage is leading to a cycle arrest. p21 plays a major role in inhibiting CDK which leads to a growth arrest. Therefore, we studied the expression of the CDK inhibitor, p21, in treated HT-29. Interestingly, an increase expression of P21 was detected in the treated cells. The cycle cell arrest or in other word the growth arrest is correlated with apoptosis in most cases of cell death (Sambucetti *et al.*, 1999).

In previous study, a team of the lab have demonstrated that the *Origanum majorana* extract inhibited the proliferation of the mutant p53 triple negative breast cancer (TNBC) cell line, MDA-MB-231. They induced a cell cycle arrest at G2/M phase and this cell cycle arrest correlates with an overexpression of the CDK inhibitor p21. Growth arrest is successfully induced by OME in our present study with a need of more exploring in investigating the expression of CDK inhibitor. By the previous study data, At high concentrations of OME, apoptosis was induced via activating the cell death extrinsic pathway which was mediated by the activation of TNF- α and activation of caspase 8. They have also shown that apoptosis is also mediated by an increase in DNA damage, revealed by the expression of γ H2AX, (Al Dhaheri, Eid, *et al.*, 2013b). We have shown the same results of the OME effect in activating the extrinsic apoptotic pathway and how this extract induces DNA damage in human colon cancer cells HT-29 cell line. The similarity in our present study results and the previous study data on the effect of OME on different human cancer cells confirms the efficiency of the extract in inhibition the cancer cells growth and more study and careful experiments are needed to be done in the future to explore more pathways and mechanisms induced by this plant extract.

In conclusion, our study demonstrated that *Origanum majorana* extract effects HT-29 in concentration-dependent manner. At high concentrations, OME induced DNA damage indicated by the upregulation of γ H2AX and this DNA damage leads to growth arrest. Moreover, DNA damage also induced an extrinsic apoptosis pathway, mediated by activation of procaspase 8 which is connected to PARP cleavage through caspase3. The suggested apoptosis pathway of OME on HT-29 is shown in the model (Figure 10).

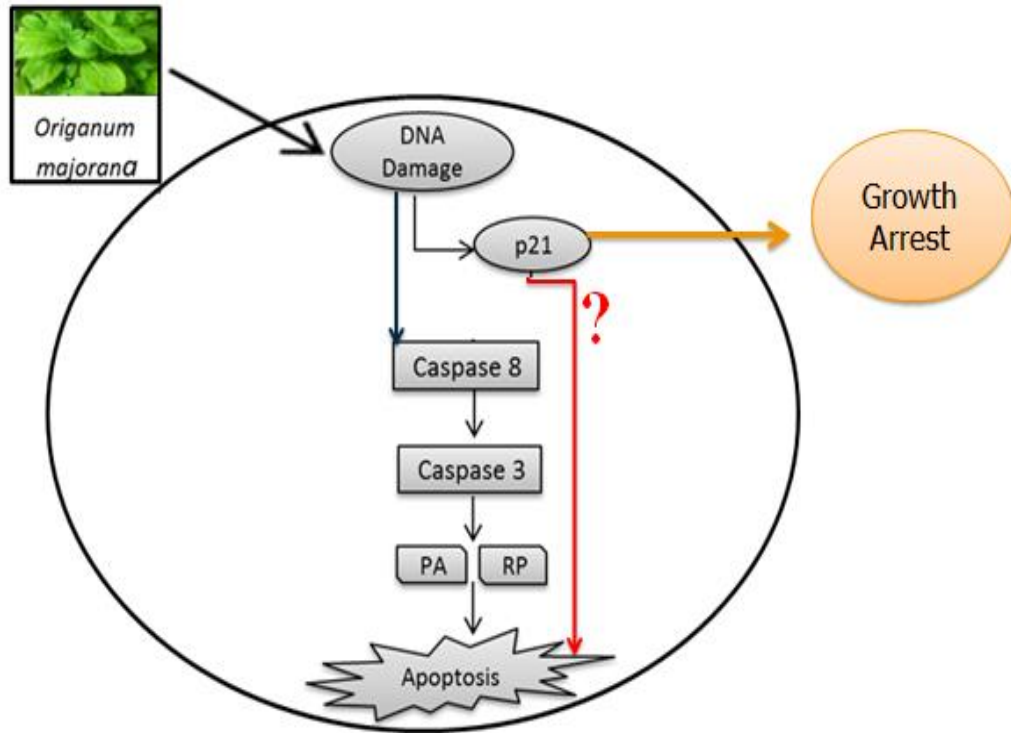


Figure 10: The proposed model shows the effect of Origanum majorana on HT-29 human colon cancer cells

Chapter 5: Conclusion

In conclusion, we have illustrated that the *Origanum majorana* plant extract effect the colon cancer cell line HT-29 successfully by inducing DNA damage and growth arrest. Furthermore, our data have shown the potential of this treatment in activating apoptosis in the colon cancer cells. In future, we are looking for further laboratory research and investigations to study the effect of OME on the HT-29 cells migration to confirm the capacity of OME in inhibiting colon cancer metastasis.

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