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Anti-Colon Cancer Effect of Origanum Majorana Essential Oil.

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ANTI-COLON CANCER EFFECT OF *ORIGANUM MAJORANA* ESSENTIAL OIL

Asma Nasser Hamad Rashed Alrashedi

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 Professor Rabah Iratni

April 2018

Declaration of Original Work

I, Asma Nasser Hamad Rashed Alrashedi, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Anti-colon Cancer Effect of Origanum majorana Essential Oil*", hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Professor 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 Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions.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.

Student's Signature: Asma Alrashedi Date: 15/05/18

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Declaration of Original Work

I, Asma Nasser Hamad Rashed Alrashedi, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Anti-colon Cancer Effect of Origanum majorana Essential Oil*", hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Professor 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 Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions.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

Plants have been shown to be an excellent source of new drugs, including anticancer agents. *Origanum majorana* commonly known as marjoram is a plant that is known to possess different therapeutic values including antioxidant and antimicrobial activities.

Our research team has previously tested the ethanolic extract of *O. majorana* on triple negative breast cancer and published the findings. The ethanolic extract promoted mitotic arrest at G2/M phase, induced apoptosis as well as inhibition of migration and metastasis. The promising potential of the ethanolic extract encouraged us to test the effects of *O. majorana* essential oil on human colon cancer cell lines.

We demonstrated that *O. majorana* essential oil inhibited the proliferation of HT-29 and Caco-2 colon cancer cell lines in a time- and dose- dependent manner. Colony forming assay illustrated that *O. majorana* essential oil reduced the ability of HT-29 to form colonies, and when established colonies were treated with the essential oil, it showed that the treatment was able to reduce colonies' size at low concentrations while at higher concentrations, the oil was able to completely eliminate the already formed colonies. Moreover, the essential oil, induced cell death and minimal cell cycle arrest at G1 phase. Annexin V staining revealed induction of apoptosis in HT-29 cells treated with the essential oil. Western blot assessment further confirmed apoptosis for being the main programmed cell death mechanism triggered by the plant's essential oil. Blotting for survivin, which is a protein that belongs to the inhibitor of apoptosis protein (IAP) family, levels indicate that *O. majorana* essential oil exerts its cytotoxic anti-cancer effect at least partially through the down-regulation of survivin. These preliminary results make *O. majorana* oil a promising alternative candidate against colon cancer.

Keywords: *Origanum majorana*, colon cancer, apoptosis, cell cycle.

Title and Abstract (in Arabic)

أثر زيت البردقوش المضاد لسرطان القولون

الملخص

تستخدم النباتات كمصدر مهم في صناعة الأدوية الجديدة التي تتضمن مضادات السرطان. أحد هذه النباتات هو نبات ال *(Origanum majorana)* و الذي يعرف بنبات البردقوش. أشارت دراسات سابقة على فاعلية نبات البردقوش كعلاج مضاد للأكسدة ومضاد حيوي.

قام فريق بحثنا بدراسة أثر مستخلص نبات البردقوش على سرطان الثدي في بحث تم نشره مسبقاً، وأشارت هذه الدراسة إلى أن مستخلص البردقوش أدى إلى تعليق إنقسام الخلايا السرطانية في مرحلة (G2/M)، وإلى الموت المبرمج للخلايا (apoptosis) بالإضافة إلى أنه منع انتشار هذه الخلايا. وبالتالي فإن هذه النتائج الواعدة لمستخلص نبات البردقوش شجعتنا لدراسة أثرالمستخلص الزيتي لنبات البردقوش على خلايا سرطان القولون. ولقد توصلنا في هذا الدراسة إلى أن زيت البردقوش يمنع نمو وتكاثر الخلايا السرطانية (HT-29) و (Caco-2) بالاعتماد على الوقت والتركيز. كما أنه قلل من قدرة خلايا سرطان القولون على تكوين مستعمرات، بالإضافة إلى أنه أدى إلى تقليل حجم المستعمرات المتكونة مسبقا عند إضافة تركيزات منخفضة من الزيت وجعل المستعمرات المتكونة مسبقاً تتلاشى عند إضافة تركيزات عالية من الزيت. بالإضافة إلى ذلك فإن زيت البردقوش أدى إلى تعليق دورة حياة الخلايا في مرحلة (G1) وإلى الموت المبرمج للخلايا السرطانية (apoptosis). وكان الموت المبرمج هو الطريقة الرئيسية التي تموت فيها هذه الخلايا والتي تم تأكيدها بالقيام بالعديد من التجارب. وأخيراً، فإن رصد مستوى البروتين (survivin)، والذي ينتمي لعائلة البروتينات التي تمنع الموت المبرمج للخلايا، أشار إلى أن آلية عمل زيت البردقوش تؤثر على الخلايا السرطانية على الأقل جزئياً من خلال تقليل مستويات هذا البروتين. كل هذه النتائج تجعل زيت البردقوش بديلاً واعداً كمضاد لسرطان القولون.

مفاهيم البحث الرئيسية: نبات البردقوش، سرطان القولون، دورة حياة الخلية ،الموت المبرمج للخلايا.

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Dedication

To my beloved parents

Table of Contents

List of Figures

List of Abbreviations

CRC	Colorectal Cancer
FBS	Fetal Bovine Serum
OMEO	*Origanum majorana* Essential Oil
PARP	Poly ADP-Ribose Polymerase

Chapter 1: Introduction

1.1 Cancer

Cancer was and still is a major public health problem globally and in the United Arab Emirates. Worldwide, one in every five deaths is cancer-related. An estimate made back in 2012 reported that there were approximately 14.1 million new cases of cancer worldwide, with an estimated 8.2 million deaths linked to cancer in the same year. Moreover, cancer occurrence is predicted to increase by 50% by 2020 to reach 15 million new cases [1]. In the United Arab Emirates, 1768 new cases were diagnosed in the Emirate of Abu Dhabi in 2014, while cancer caused around 427 deaths in 2015. Cancer is the third leading cause of death in the UAE; it accounts for 16% of total deaths. Colon cancer is the second most common type of cancer in both woman and men [2].

1.2 Definition and characteristic

Cancer is a Multifactorial disease which is characterized by uncontrolled cell division, the invasion of those cells and their spreading from the primary site to a new, different location where they establish new colonies of cancerous tumors resembling the ones in the primary site [3]. Cancers can be classified in many different ways depending on the criteria of classification. However, one of the simplest consented upon ways to classify is by their tissue of origin. Carcinoma, which is the most prevalent type of cancer, develops from epithelial cells, while Sarcomas arise from mesoderm cells. Tumors that comprises of epithelial cells with a glandular organization are called adenocarcinoma [3]. Leukemia and lymphoma are derived from white blood cells and precursors [4].

1.3 Hallmarks of cancer

Cancer cells acquire a set of specific hallmarks that can be used to distinguish them from healthy cells. Cancer cells stimulate their growth by responding to self-generated signals, which allows them to maintain proliferative signals and evades inhibitory signals that might otherwise interfere with their growth. Besides, they escape programmed cell death and enable a possibly unlimited replicative ability, inducing angiogenesis by forming new blood vessels to supply and nurture the tumors, migrating from the primary site and metastasize to new distant locations Additionally, cancers cells have been reported to be able to evade immune system destruction [5].

1.4 Causes of cancer

Cancer has been studied extensively for decades now with the hope of finding a definite cause of cancer. Many factors have been recorded to increase the risk of cancer occurrence, some of these factors include genetic predisposition. Mutations located in tumor suppressor genes or oncogenes or a combination of the two can trigger the first onset of a healthy cell's cancerous path. Lifestyle, chemical agents, and viruses are only the standard and most studied factors; many other factors may contribute to the development of cancer such as injury and inflammation [3].

1.5 Colon cancer

Colorectal cancer (CRC) is the third most common type of cancer in incidence and mortality, and it affects both sexes almost equally [6]. CRC used to be rather uncommon back in the 1950s. However, it has seen an increase in incidents in the past six decades that now it became one of the predominant cancers, now it is the third most common type of cancer, and it accounts for approximately 10% of all cancer-related mortalities. Reasons that might explain these alarmingly increased incidents include

an aging population where the primary demographic of patients diagnosed with CRC is at age 50 years and older, poor diet and lifestyle, smoking, low rate of physical activities and finally obesity [7]. CRC is a complex disease; it usually grows in the lining of the colon and the rectum in the form of a polyp, which is a mass bulging in the lumen. Not all the polyps are neoplastic nor do all of them develop into cancer. However, the majority of CRC evolves from adenomatous polyps [6]. During the development of colorectal adenocarcinoma, gastrointestinal epithelial cells acquire a consecutive genetic and epigenetic mutations in oncogenes and tumor suppressor genes; these mutations give the cells proliferative and self-renewal abilities. Therefore, the transitioning epithelium cells become hyper-proliferative which in time develops into a benign adenoma that might evolve into malignant carcinoma that can spread and metastasize forming new tumor colonies in neighboring organs [8].

1.5.1 Mechanisms and pathophysiology of CRC

The risk factors such as environmental and genetic factors that contribute to the development of CRC can do so by promoting the acquisition of cancer hallmarks in colon epithelial cells. One possible way by which these hallmark traits can be acquired is through the sequential and progressive accumulation of genetic mutations and epigenetic alterations where it aids in the activation of oncogenes and inactivation of tumor suppressor genes [9, 10]. The majority of the cells in early neoplastic lesions in the colon have been observed to have lost their genomic and epigenetic stability. Thus, it is highly probable for these instabilities to be a central molecular and pathophysiological event in the initiation and formation of CRC [7]. However, the majority of CRC follows what is known as the ‘classic' model of formation, where the vast majority of malignant tumors arise from polyps, which then if left unchecked

might develop into an early adenoma which is less than 1 cm in size, with tubular histology. Then the adenoma could obtain enough hallmarks to progress to an advanced adenoma which is roughly the same size as the early adenoma, but, with villous histology, before they are finally and fully becoming CRC. This Process is established by the sequential accumulation of mutations and epigenetic alterations, and it takes between 10 to 15 years from the formation of the polyps to the development of CRC. However, in certain circumstances, this process could be more rapid such as if the individual has a genetic predisposition for example Lynch syndrome [11]. Notably, not all polyps develop cancer, only about 10% of all polyps do so [7].

1.5.2 Prevention and treatment

Even though there are no definite prevention steps that an individual with high risk could take, however, there are few precautionary procedures and measures that can lower the risk. Firstly, regular screening for people with higher risk, when screening, the physician is looking for cancer or pre-concourse polyps even when the patient does not exhibit any symptoms. Individuals with no increased risk for CRC are advised to start screening at the age of 50 years, while people with higher risk such as strong familial history will benefit more from screening earlier. Secondly, there are some improvements in lifestyle habits that can lower the risk such as physical activities, diet, body weight, and smoking [7].

1.6 Apoptosis (programmed cell death I)

Apoptosis is a tightly orchestrated multi-step pathway, where cells commit to dying. This mechanism of programmed cell death is extremely crucial during development, however it also important in adult multicellular organisms' homeostasis. Key characteristics of apoptosis include cellular shrinkage and condensation of the

nucleus followed by DNA fragmentation [12]. Cells committing to apoptosis firstly become rounder in shape and detach from neighboring cells which is coupled by plasma membrane blebbing [13]. Phosphatidylserine translocation from the inner to the outer side of the plasma membrane is a fundamental signal of apoptosis which is coined as the 'eat-me' signal which is recognized by phagocytic cells to engulf and consume apoptotic cells and recycle their biomolecules [14]. This process is primarily, however not exclusively, orchestrated by the cysteine proteases family members which are known as caspases [15].

1.6.1 Apoptosis and cancer

The DNA is well protected by the cell, it is the cell's most prized possession, that is why in case of DNA damage, an efficient and immediate response is required. DNA repair systems are both efficient and immensely sophisticated. However, if the repair systems fail to fix the problem that could spills all sorts of trouble to the cell namely genomic instability. In normal physiological circumstances, irreparable DNA damage triggers apoptosis and sets it in motion. Mutations located on apoptosis signaling proteins or on oncogenes ensuing to their activation could result in the evasion of apoptosis, which ultimately might lead to tumorigeneses and cancer development [14].

1.7 Treatment

Many types of cancer treatment are used -independently or combined- depending on the type of cancer and how advanced it is. Surgery is the most common type of therapy for CRC where the tumor alongside with some of the healthy tissues are removed [16]. Radiation therapy exerts its effect by damaging DNA of all cells in the body, while healthy cells can repair the damage, cancer cells will accumulate DNA

damage that would eventually be lethal to them [17]. Chemotherapy targets fast dividing cells aiming at inhibiting mitosis and cellular growth and by that stopping the progression of cancer. Chemotherapy is used to both treat cancer and, in an attempt, to prolong a patient's life [18].

Although great advancements in cancer treatment and control have been achieved, the undesired side effects that are accompanied by such treatments have serious effects on the health of the person. Therefore, alternative therapies that include less toxic and more potent anticancer drug are needed to be developed [17, 19].

1.8 Phytomedicine

For thousands of years, mankind looked to plants for utilizing their medicine. Plants were used starting from the leaves to the roots, and they were extracted as crude extracts such as tinctures, teas, powders and other forms of formulations. The use of plants for medicinal purposes kept evolving throughout history, in the early 19th century active compounds were isolated and purified, beginning with the purification of morphine from opium. Medicinal plants are a rich source of a wide variety of active compounds which can offer a lot of possibilities in the development of drugs [19, 20].

1.8.1 Plants in the management of cancer

Most agents in chemotherapy today are linked to natural compounds, to be more specific about three-quarters of all the chemotherapeutic agents introduced since 1940 are in fact natural compound or are a direct derivative. Irinotecan, etoposide, and paclitaxel are examples of plant-derived compounds used in cancer treatment, and many other compounds are in clinical trial. These drugs exert their effect through targeting many cellular mechanisms such as microtubules interaction, inhibition of

topoisomerases, DNA alkylation, while some interfere with signal transduction of the tumor. Previously in our lab, we have studied the anti-cancer potential of the crude ethanoic extract of *Origanum majorana* on breast cancer, and it showed itself to be a viable candidate [21-24].

1.8.2 *Origanum majorana*

Origanum majorana is an herbaceous plant found in southern Europe and the Mediterranean area. The herb is from the family Lamiaceae, and it is commonly known as marjoram, it can grow up 60 cm. *O. majorana* is widely used as a garnish in food preparation, as well as being a medicinal plant used for different purposes in traditional medicine of different regions. Studies on this plant yielded in the identification of many of its active compounds, notably, marjoram is rich in polyphenols such as flavonoids which are bioactive compounds which, potentially, have beneficial pharmacological activities, in addition, it contains phenolic terpenoids, oxygenated monoterpene, tannins and phenolic glycosides amongst many others. Some of the pharmacological activities that *O. majorana* have been recorded to possess many biological activates such as antioxidant activity, antimicrobial activity and finally, it has anti-cancer and anti-proliferative properties. Our research team has previously tested and published the findings. We have shown, previously in published work, that the ethanolic extract of *O. majorana* has a significant effect on triple negative breast cancer, promoting mitotic arrest at G2/M phase, inducing apoptosis as well as inhibition of migration and metastasis [24-26].

1.8.3 *Origanum majorana* essential oil

Essential oils are complex mixtures made of low molecular weight compounds.

Essential oils are mostly extracted by steam distillation, two of their primary ingredients, terpenoids, and phenylpropanoids provide some of the essential oils aromatic and biological properties characteristic. Essential oils hold a variety of pharmaceutical and biological activities like anti-bacterial, anti-fungal and anti-cancer [27].

O. majorana essential oils is an extract with high medicinal value. Scientific reports show that marjoram oil is generally non-toxic, non-irritant and non-sensitizing. However, it is to be avoided by pregnant women. Some commercial detergents and soaps use *O. majorana* essential oils as an ingredient. Also, it is used as antiseptic, stomachic, and sedative. Marjoram oil is heavily used in aromatherapy and traditional medicine. And since marjoram ethanoic extract showed such high promise and Essential Oils are known for their high biological and pharmacological activities, we wanted to test the anti-colon cancer activity of *O. majorana* Essential Oils and explore some of its underlying mechanism of action [28, 29].

The aim of this study is to investigate the effect of *O. majorana* essential oil on colon cancer cell lines. And to examine the mechanism *O. majorana* plays in inhibiting cancer cells.

Chapter 2: Material and Methods

2.1 *Origanum majorana* essential oil

Origanum majorana essential oil was obtained from PRANARÔM scientific aromatherapy.

2.2 Cell culture, chemicals, and antibodies

Human colon cancer cells HT-29 and Caco-2 were maintained in DMEM supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 U/ml penicillin/streptomycin (Hyclone). Antibodies against caspase-8 and p27 were obtained from Cell Signaling; those raised against Survivin and β-actin were obtained from Santa Cruz Biotechnology. Antibodies raised against Cleaved PARP were obtained from Abcam; those raised against p21 and γH2AX were obtained from Millipore.

2.3 Measurement of cellular viability

HT-29 and Caco-2 cells were seeded in 96-well plates in triplicate at a density of 7,000 cells per well. 24 hours from the seeding, cells were then treated with or without varying dilutions of *Origanum majorana* essential oil OMEO for 24 and 48 h. The cellular viability of HT-29 cells was assessed with the Cell Cytotoxicity Assay Kit (Abcam) conferring to the manufacturer's instructions. The results are illustrative of an average of at least five independent experiments. Data are presented as proportional viability (%) by comparing the treated group with the untreated cells (control), the viability of which is assumed to be 100%. Moreover, cell viability was determined by the Muse™ Cell Analyzer (Millipore) using the Muse Count and Viability Kit (Millipore), where HT-29 cells were plated in 12-well plates (50 × 104

cells/ well). This kit was able to distinguish viable and dead cells based on to their permeability to two DNA binding dyes. Cells were counted on the day of treatment to estimate the approximate number of cells per well. Then following OMEO treatment at specified times, viable cells were counted using Muse™ Cell Analyzer. The effect of OMEO on the viability of the Caco-2 cells were determined using a CellTiter-Glo Luminescent Cell Viability Assay (Promega), according to the manufacturer's instructions.

2.4 Colony formation assay

HT-29 cell-line cells were cultured in 6-well plates at a density of 7000 cells per well and permitted to grow for about ten days to form colonies before OMEO treatment is added in a freshly prepared growth medium, and the colonies were allowed to grow for five additional days. Next, colonies were washed three times with 1X phosphate buffer saline (PBS), then fixed for 15 min with 4% Paraformaldehyde and stained with 0.01% crystal violet for 30 min. Colonies were counted, and their surface area in each well was determined by using the ImageJ software. Moreover, HT-29 cells were allowed to form colonies in the presence of media containing various dilutions of OMEO for 2 weeks and then it was stained as described previously.

2.5 Quantification of apoptosis by Annexin V labeling

Apoptosis was investigated via the Annexin V & Dead Cell kit (Millipore) following the manufacturer's instructions. In brief, HT-29 cells were treated with or without OMEO for 6 h. Both adherent and detached cells were collected and incubated with Annexin V and 7-Aminoactinomycin D (7-AAD), known to be a dead cell marker, for 20 min at room temperature in the dark. The events for live, early and late apoptotic cells were quantified with the Muse™ Cell Analyzer (Millipore).

2.6 Quantification of caspase 3/7 activity

HT-29 cells were seeded at a density of 7,000 cells/well in 96-well plates in triplicate and treated with or without OMEO for a duration of 6 h. Caspase-3/7 activity was studied using a Luminescent Caspase-Glo 3/7 Assay Kit (Promega) following the manufacturer's instructions. Briefly, caspase reagents were added to control and treated cells. The plate was mixed very well on an orbital shaker and incubated for 2.5 h in the dark at room temperature. Then, GloMax Multi-detection System (Promega) was used to measure the luminescent signal.

2.7 Whole cell extract and western blot analysis

HT-29 cells at a density of 6×10^6 were seeded in 150 mm culture dishes and cultured for 24 h prior to treatment. After incubation with OMEO for the specific durations, cells were washed twice with ice-cold PBS, scraped, pelleted and lysed in RIPA buffer (Pierce) complemented with phosphatase inhibitor (Roche) and protease inhibitor cocktail (Roche). After that, cell lysate was incubated for 30 min on ice, then centrifuged at 14,000 rpm at 4 °C for 20 min. BCA protein assay kit (Thermo Scientific) was used to determine the protein concentration of lysates. Cell lysates were then aliquoted in 30 µg and resolved onto 6-15% SDS-PAGE along with Page Ruler Plus Prestained Protein Ladder (Thermo Scientific)

Proteins were transferred onto nitrocellulose membranes (Thermo Scientific) and blocked for 1 hour at room temperature with 5% non-fat dry milk prepared in TBST (TBS and 0.01% Tween 20). Later, nitrocellulose membranes were incubated with specific primary antibodies in blocking buffer overnight at 4 °C. Horseradish peroxidase-conjugated anti-IgG was used as secondary antibody. Immunoreactive bands were detected by ECL chemiluminescent substrate (Thermo Scientific), and

FEMTO and chemiluminescence were detected using the LiCOR C-DiGit blot scanner. Where needed, membranes were stripped in Restore western blot stripping buffer (Thermo Scientific) per the manufacturer's instructions.

2.8 Statistical analysis

Data were described as group mean ± SEM. The data were evaluated via student's t-test. Significance for all statistical comparisons were (*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$).

Chapter 3: Results

3.1 OMEO inhibits cellular viability of HT-29 and Caco-2 human colon cancer cell-lines

First, we examined the anti-cancer activity of OMEO on human colon cancer cells by measuring cellular viability. HT-29 and Caco-2 colon cancer cell lines were treated with various dilutions of OMEO ranging from 1/500 down to 1/10000 and from 1/2000 down to 1/10000 respectively (Fig. 1). Our results show that exposure of HT-29 and Caco-2 cells to OMEO decreased cellular viability in a concentration- and a time-dependent manner. OMEO-treated cells, when observed under light microscopy also revealed that the number of cells is declining when compared with the control. However, it is not explicit whether the decline discerned in the number of cells is reflective of a growth inhibitory effect, or cell death caused by OMEO treatment. Therefore, cellular viability was also monitored using an assay which differentially stains viable and dead cells. We found that depending on the dilution factors the cell growth pattern of HT-29 behaves differently. Firstly, cells treated with 1/10000 which is the highest dilution factor and lowest in concentration tested, showed continued growth throughout the three days period, however, much slower than the control, as illustrated by the reduction of viable cells. Secondly, cells treated with 1/5000, showed a plateau in its growth pattern. However, the third and lowest dilution factor tested 1/2500, was the most effective on cellular viability and caused certain cell death (Fig. 2).

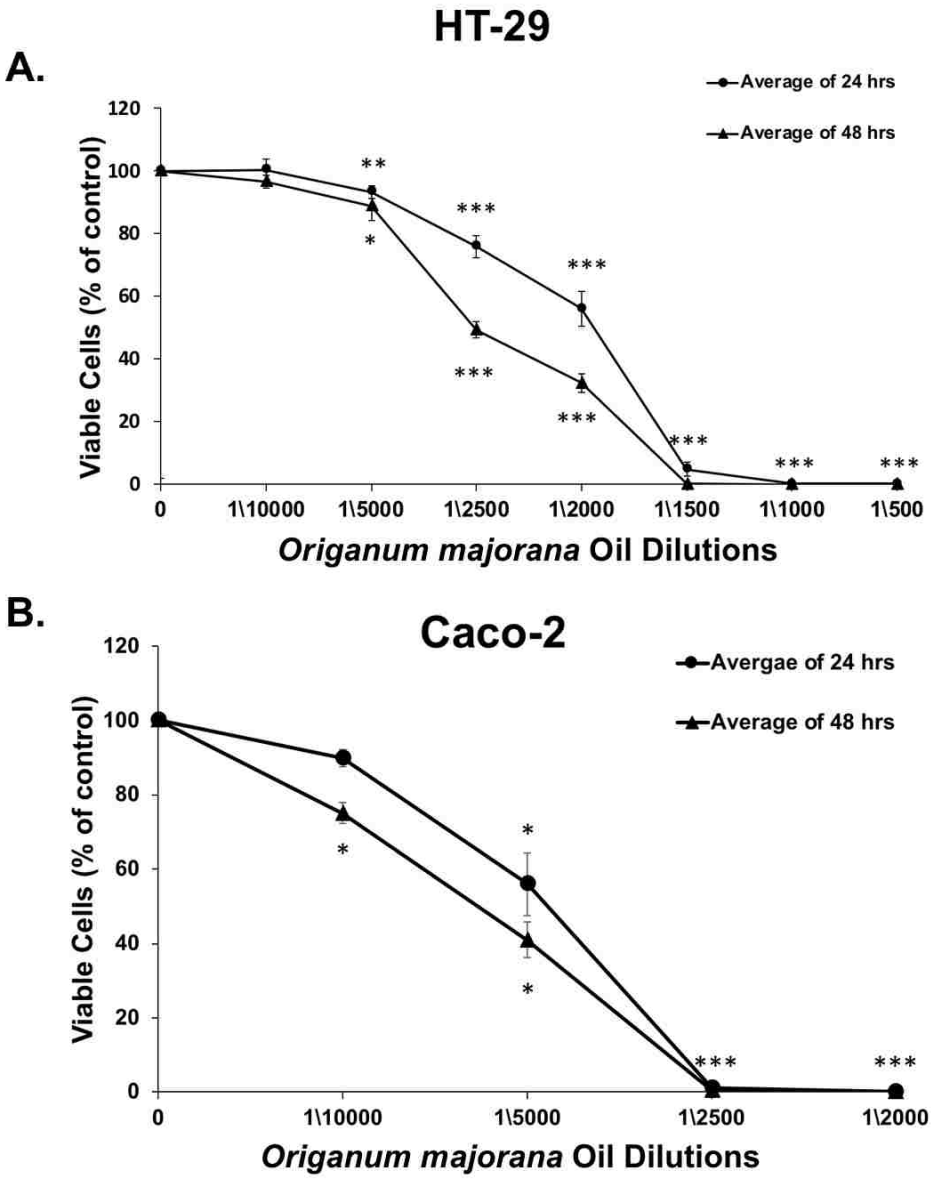

Figure 1: *O. majorana* essential oil inhibits cellular viability. (A) HT-29 and (B) Caco-2 human colon cancer cell lines treated with or without the indicated dilutions of OMEO for 24h and 48h. Data represent the mean of five independent experiments carried out in triplicate. Statistical analysis for cell viability data was performed using student's t test to determine the significance. (*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$).

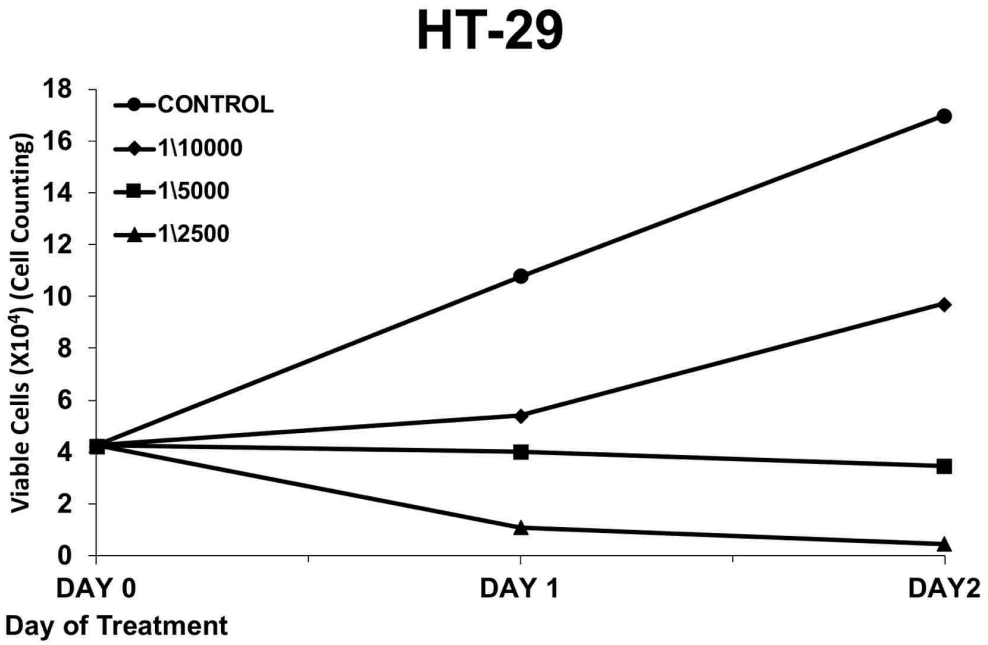

Figure 2: *O. majorana* decreased the number of viable cells of HT-29 cells. HT-29 cells were exposed to OMEO for 24 h and 48 h. Cell viability was monitored using the Muse Cell Analyzer. Data represent the mean ± SEM of three independent experiments.

3.2 OMEO induces morphological changes in HT-29 cells

Light microscopy observation of OMEO-treated HT-29 cells revealed a decrease in the number of cells with decreasing dilutions of OMEO. Furthermore, as shown in Figure 3, HT-29 treated cells, underwent morphological changes characterized by the smaller size and the roundness of the cells which are characteristics of apoptotic cells accompanied with an abundance of de-attached and floating dead cells.

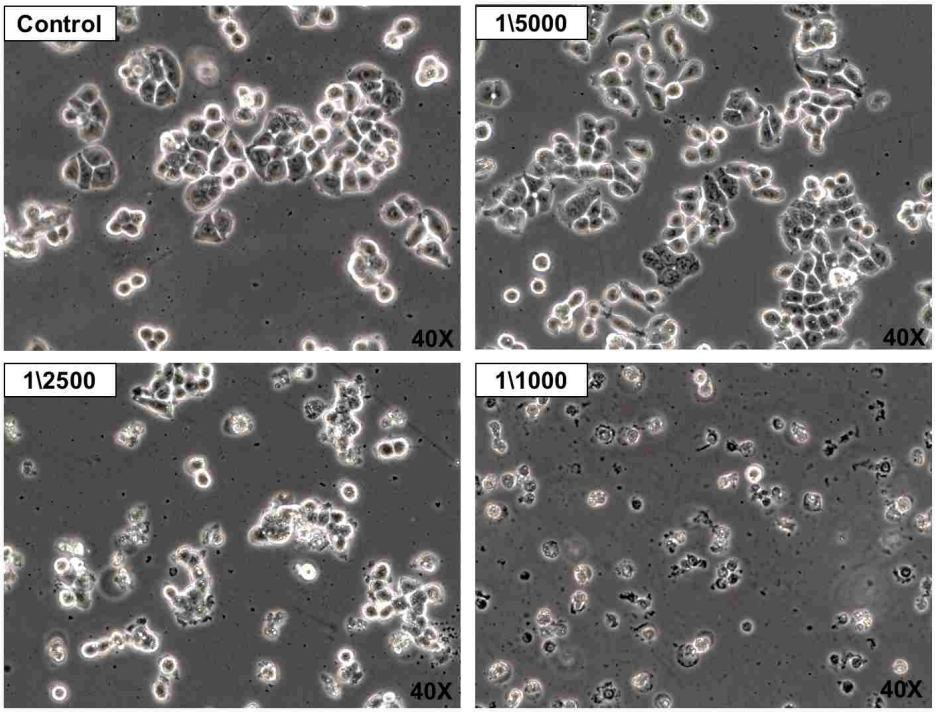

Figure 3: *O. majorana* induces morphological changes in HT-29 cells. Morphological changes observed in HT-29 cells after 24 h treatment with different dilutions of OMEO. Cells were observed under EVOS XL core cell imaging system at 40X magnification.

3.3 OMEO reduces and inhibits HT-29 colony growth

To further confirm the anti-cancer potential of OMEO, we explored the effect of OMEO on HT-29 colonies formed in culture. HT-29 cells were allowed to grow into fully formed colonies on culture plates, which then we treated with three dilutions of OMEO (1/10000, 1/5000, 1/2500) and incubated them with the treatment for three days, after which they were fixed and stained. Figure 4 clearly illustrates the varying effects of the treatment depending on the dilution, where the effect of OMEO is visibly increasing as the concentration is increasing. 1/2500 was the strongest on formed colonies; this dilution was able to eliminate and abolish formed colonies completely.

This result taken together with the previous results further confirm the ability of OMEO to inhibit cellular growth.

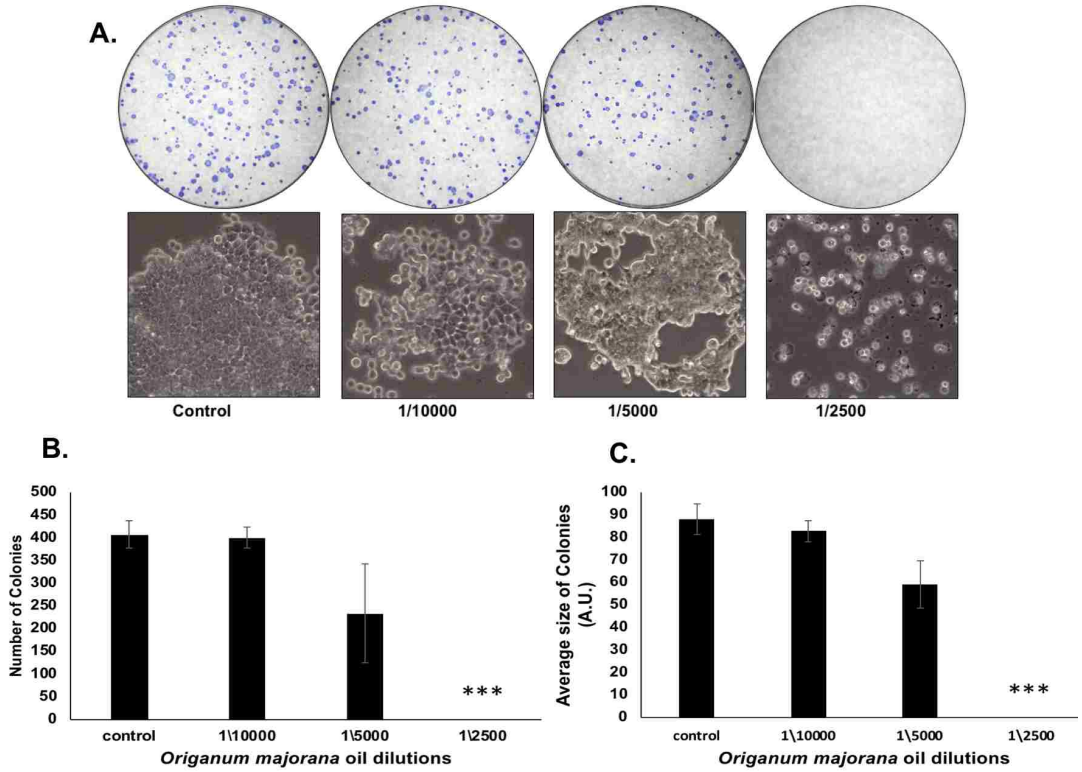

Figure 4: *O. majorana* essential oil reduces and inhibits HT-29 colony growth. HT-29 colonies were first allowed to form in normal media for 2 weeks as described in Materials and methods. Formed colonies were then treated with various dilutions of OMEO and allowed to grow for three more days before staining. Inhibition of colony growth was assessed by measuring the size of the colonies.

3.4 HT-29 cells ability to form colonies is restricted in the presence of OMEO

Unlike the previous colony formation test, in this experiment, we seeded the cells and in the next day treated them and incubated them with the treatment for about ten days. To test whether or not they would retain their ability to form colonies, we allowed the cells to grow and form colonies in the presence of two dilutions (1/10000 and 1/5000). Referring to Figure 5, OMEO reduced the ability of HT-29 cell to form

colonies in 1/10000 dilutions, while it wholly prevented those cells from forming colonies at dilution 1/5000.

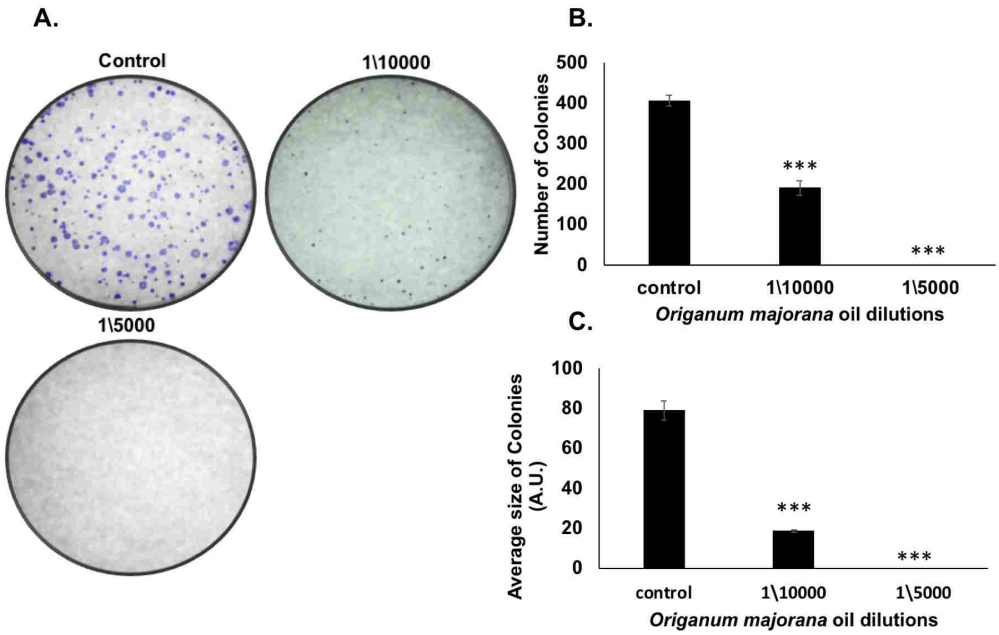

Figure 5: HT-29 cells ability to form colonies is restricted in the presence of *O. majorana* essential oil. HT-29 cells were allowed to form colonies in the presence of media containing various dilutions of OMEO for two weeks and then it was stained. Inhibition of colony formation was assessed by measuring the size and the number of the colonies.

3.5 OMEO induces minimal G1 arrest in colon cancer cells

Giving that at lower concentrations, cells seem to have a slower to a plateau proliferative profile, therefore, we decided to investigate if OMEO exerts an effect on cell cycle progression. Toward this, HT-29 cells were treated with indicated dilutions of OMEO for 24 h; then, they were subjected to cell cycle analysis. We found that treating the cells with OMEO caused minimal cell cycle arrest at G1 phase in HT-29 cells at 1/2500 (Fig 6A-B).

p21 and p27 two CDK inhibitors and markers of growth arrest were examined to inspect the status of cell cycle in OMEO-treated cells further. No significant changes in the levels of p21 nor p27 compared to the control (Fig 6C-D).

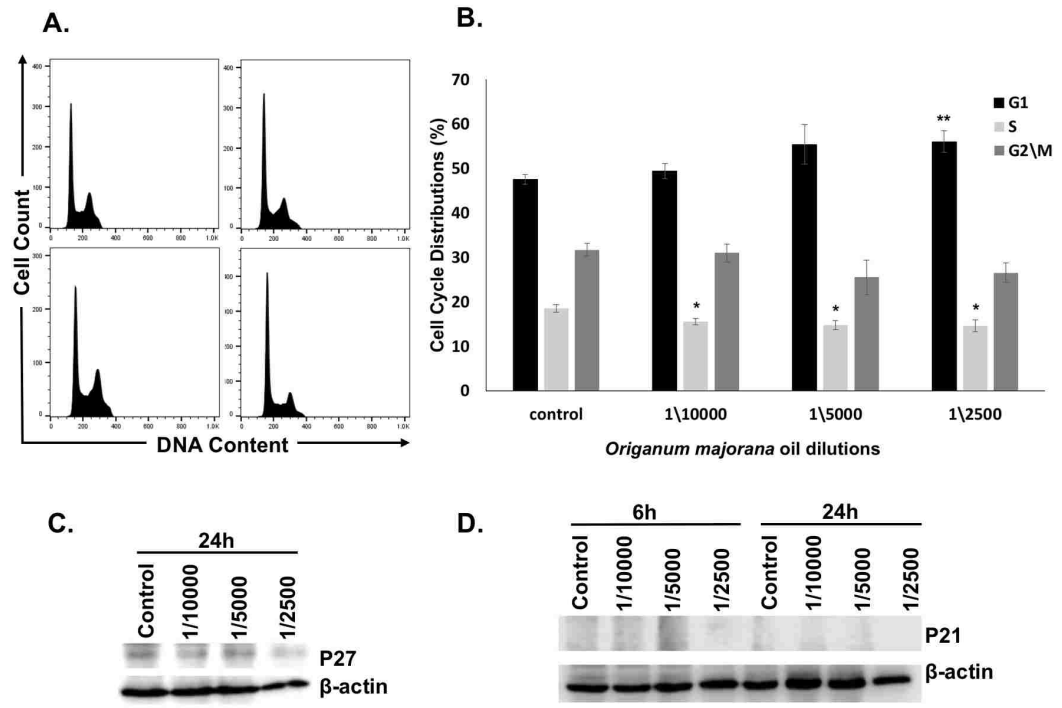

Figure 6: *O. majorana* induces minimal G1 arrest in colon cancer cells. (A-B) Cell cycle distribution analysis of OMEO-induced minimal G1 cell-cycle block. HT-29 cells were first treated with OMEO at the indicated dilutions for 24 h, and then analyzed with Muse Cell Analyzer. Statistical analysis for cell viability data was performed using student's t test to determine the significancy. (*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$). (C-D) Western blot analysis of P21 and P27 in HT-29. Cells were treated with different dilution of OMEO for 6 h.

3.6 Induction of apoptosis in HT-29 colon cancer cells by OMEO

Because both cytotoxicity (Fig. 1) and cell counting assay (Fig. 2), showed that OMEO induced cell death in HT-29 cells, therefore, we sought to detect the onset of apoptosis. Annexin V staining was used to determine the percentage of apoptotic cells induced by OMEO after 6 h treatment. As it is shown in Figure 7A & B, a significant increase in apoptotic cells was detected upon OMEO exposure, cells treated with

1/1000 reached 100% apoptotic cell. Apoptosis was further assessed by PARP cleavage which is a protein marker for apoptosis. PARP cleavage was considerably more pronounced in 1/2500 after 6 h treatment (Fig. 7D). Altogether, Annexin V staining and PARP cleavage data support the conclusion that OMEO induces apoptosis in HT-29 cells.

Activation of the caspase-3/7 is one of the events of the apoptotic pathway. Hence, we assessed the activation of these proteins using a caspase 3/7 activity assay as described in materials and methods section. Cells treated with 1/2500 dilution had an increase in caspase 3/7 activity by 234 folds, while the other two dilutions (1/10000 and 1/5000) had a minimalistic insignificant increase after 6 h (Fig. 7B).

Having shown that OMEO induces the activation of the effector caspases 3/7, we looked at the activity of the initiator caspases of the extrinsic and intrinsic cell death pathway, namely caspase 8 and caspase 9, respectively. Caspase 9 activation detected was not clear in response 1/2500 at different time points (data not shown). On the other hand, however, caspase 8 cleavage and activation was detected in response to 1/2500 at different time points (Fig. 7E). This result suggests that the apoptotic effect of the oil on HT-29 cells is dependent on caspase 8 activity, which associates the extrinsic cell death pathway.

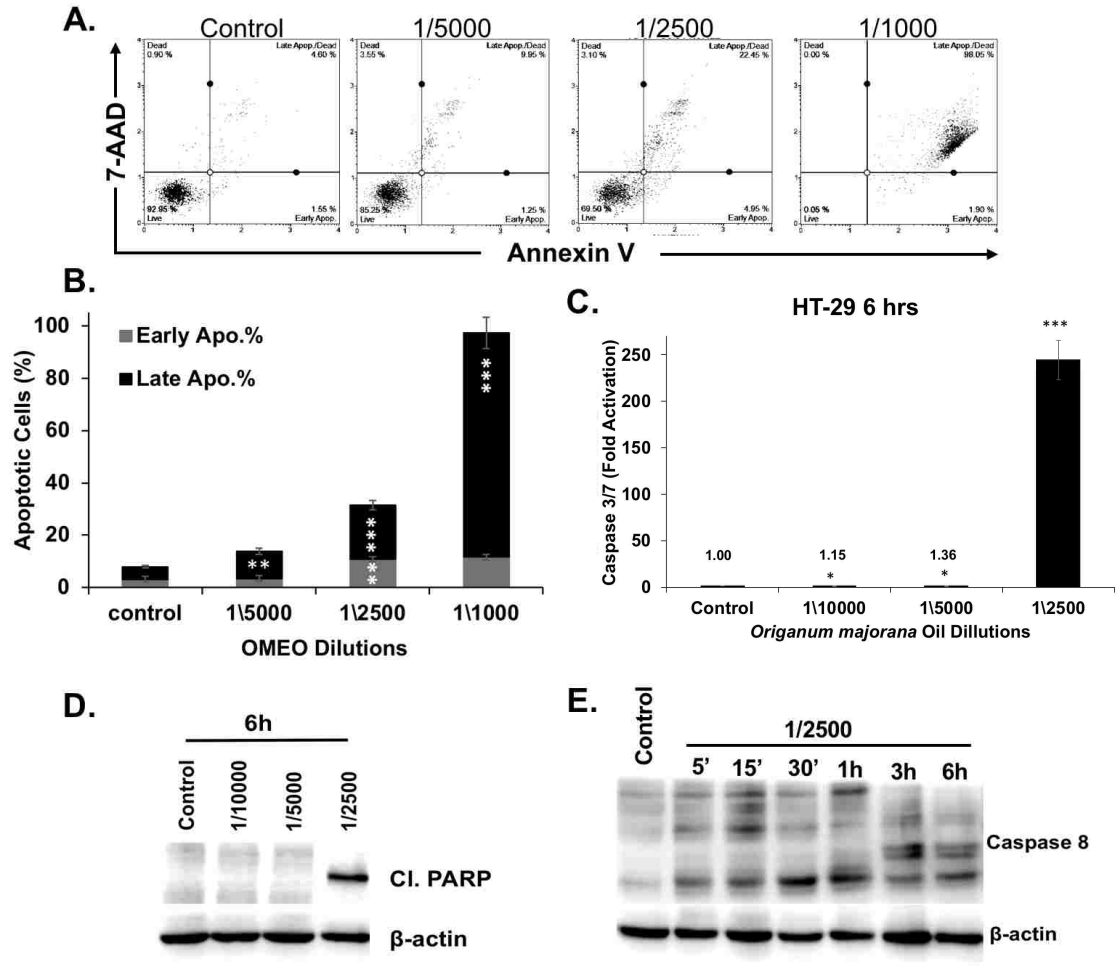

Figure 7: Induction of apoptosis in HT-29 colon cancer cells by *O. majorana*. (A-B) Annexin V binding was carried out using Annexin V & Dead Cell kit. Cells were treated with different dilutions of OMEO for 6 h. Detached and adherent cells were collected and stained with Annexin V and 7-AAD and then the events for early and late apoptotic cells were counted by Muse Cell Analyzer. Data represent the mean ± SEM of at least 3 independent experiments. Statistical analysis for Annexin V data was performed using student's t test to determine the significance. (*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$). (C) Stimulation of caspase 3/7activity in HT-29 cells after exposure to OMEO for 6 h. The relative caspase 3/7 activity was normalized to the number of viable cells per well and is expressed as fold of activation compared to the control cells. (D-E) Western blot analysis of cleaved PARP and cleaved caspase 8 in HT-29. Cells were treated with different dilution of OMEO for 6 h.

3.7 Induction of Survivin in HT-29 cells in response to OMEO

Survivin is one of the proteins that belongs to the inhibitor of apoptosis protein (IAP) family, as it inhibits the caspases and therefore blocks cell death [30]. Conversely, a decrease in survivin levels provokes the cells to undergo apoptosis.

Hence, we examined a possible involvement of survivin in apoptosis triggered by OMEO. Western blotting analysis shows that the expression of survivin in response to various dilutions of OMEO after 6 h treatment sees an initial increase at 1/10000 and 1/5000, while 1/2500 causes this protein to dramatically decrease in its expression levels. Nonetheless, when we analyzed the same dilutions after 24 h, there was a universal gradual decrease in the level of surviving level throughout (Fig. 8A). Since the level of survivin at 1/2500 was very low after 6 h, we wanted to see at which time point does this effect start to take place. Figure 8B demonstrates that this dilution starts to exert its effect as early as 5 min, where the levels of survivin first see an increase at earlier time points and starts to decrease after 1 h.

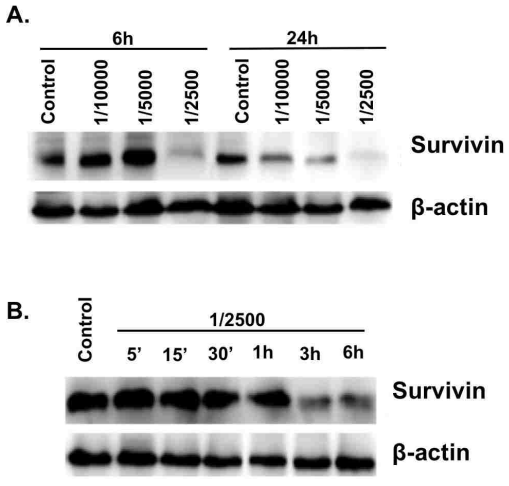

Figure 8: Induction of Survivin in HT-29 cells in response to *O. majorana*. (A) HT-29 cells were treated with different concentrations of OMEO for 6 h and 24 h and survivin level was detected. (B) Time-course measurement of survivin in treated HT-29 cells. Cells were treated with the dilution 1/2500 of OMEO and survivin was examined, at different time-points starting from 5 min up to 6 h.

3.8 Minimal increase in the expression of γH2AX, in OMEO treated HT-29 cells

Finally, we decided to examine the effect of OMEO on γH2AX which is a known marker for DNA double strand breakage and DNA damage. Western blotting analysis revealed a slight increase in the levels of γH2AX in response to OMEO treatment at 1/2500 dilutions (Fig. 9), indicating a minute accumulation of double strand breaks in these cells.

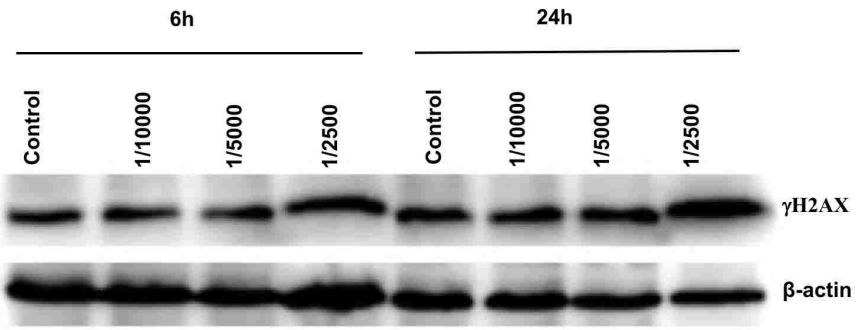

Figure 9: Minimal increase in the expression of γH2AX, in *O. majorana* essential oil-treated HT-29 cells. HT-29 cells were treated with various dilutions of OMEO for 6 h and DNA damage was analyzed, by determining the level of γH2AX accumulation using anti- phospho-H2AX (ser 139) antibody.

Chapter 4: Discussion

An ever-increasing amount of evidence supports the claim that plants are, indeed, an essential player in the search for better cancer treatment or even a cure. Many of these plants or plant-derived drugs are acting through modulating apoptosis. Programmed cell death is the process of which a defective cell commits to dying in an organized well-orchestrated manner. Thus, plants present themselves as candidates for cancer thereby, with great potential and investigating their pharmacological capacity is of impending importance [31].

In this study, we examined the anti-cancer activity of *Origanum majorana* essential oil on human colon cancer cells. Our work demonstrated that OMEO was able to decrease the cellular viability of two colon cancer cell lines (HT-29 and Caco-2) in a time- and a concentration-dependent manner. Also, it induced minimal cell cycle arrest at G1 phase. Furthermore, we found that OMEO caused programmed cell death in HT-29 through the mechanism of apoptosis. Induction of DNA damage was minimal at the highest concentration tested by a slightly increased expression of γH2AX, suggesting that cell death induction was independent of DNA damage.

The apoptotic process of cell death can be triggered by either the extrinsic pathway where signals recognized by death receptors on the surface of the cell set the mechanism in motion inside the cell, or by the intrinsic pathway which is mediated by the mitochondria. The death receptor-mediated apoptosis requires the interaction of ligands such as TNFα and Fas with their receptor which are transmembrane proteins. The activation of the death receptor in turns leads to the activation of the effector caspase 8, which subsequently activates caspase 3 either directly or by a mitochondria-involved pathway [32]. However, activation of the intrinsic pathway is associated with

mitochondrial permeability. Changes in the levels of anti-apoptotic Bcl2 family members and the pro-apoptotic members such as Bax and Bak result in the formation of the apoptosome and activation of caspase 9 which activates caspase 3 [33]. Both, intrinsic and extrinsic apoptotic pathways activate caspase 3 which cleaves poly(ADP-ribose) polymerase (PARP), thus, resulting in apoptotic cell death. In this study, we showed that OMEO induced an intrinsic apoptotic pathway [34]. OMEO induced an increase in the expression of cleaved PARP and the effector caspase 3/7, as well as caspase 8 which is classically associated with the extrinsic pathway of apoptosis.

DNA damage have been associated with cell cycle arrest and apoptosis in many studies, and a variety of anti-cancer drugs have been shown to cause DNA damage [35]. Cancer cells are known to be considerably more susceptible to DNA damage agent than normal cells [36]. DNA damage can be detected by γH2AX, a known marker for double strand breakages. Upon double strands breakage occurrence, H2AX is rapidly phosphorylated at serine 139 to form γH2AX that act as a signal for the recruitments of proteins involved in DNA repair and chromatin remodeling [35, 37]. When the damage is repaired, dephosphorylation of γH2AX occurs and the cell reverts to its normal state [38, 39]. Moreover, studies have shown that γH2AX formation is an early chromatin modification following initiation of DNA fragmentation during apoptosis [40]. When we scored for γH2AX, we detected only a minute increase at the highest concentration tested, which is the same concentration at which we have recorded high levels of apoptotic markers and cell death. Therefore, we speculate that the induction is not cause by OMEO but rather it is a result of late stage apoptosis and that explains the lack of increase in lower concentrations.

Survivin, a pro-growth and one of the inhibitors of apoptosis (IAP) protein family, it exerts its effect by inhibiting caspases and thusly cell death and is involved in the regulation of cell cycle. In most differentiated normal cells Survivin is absent [30]. However, Survivin is reported to be highly upregulated in cancer cells, and that attributes to the poor clinical outcome, not only because of its anti-apoptosis properties but also its linked to resistance to chemotherapy and the aggressiveness of tumors [41]. In our results, we observed an initial increase in the survivin level at earlier time points, which might be explained by the attempt of the cells to survive. However, this rapid increase is followed by a dramatic decrease in all the tested dilutions to varying degree. Therefore, we suspect that OMEO exerts its cytotoxic anti-cancer effect at least partially through the down regulation of survivin.

By comparing these current results with our previous published on *Origanum majorana* ethanoic extract (OME) against breast cancer, we found that OME inhibited the viability of MDA-MB-231 breast cancer cells. The extract induced cell cycle arrest at G2/M phase that was accompanied with upregulation of p21 unlike the oil which induced minimal arrest at G1 phase with no significant changes in the expression of p21 nor p27. Moreover, a differential expression of survivin was observed in cells treated with different concentrations of OME where survivin was induced at low concentrations and then faced reduction at higher concentrations which is in agreement with the current results. Similarly, to *Origanum majorana* essential oil, OME caused a massive induction of apoptosis at higher concentrations through the extrinsic. Finally, OME triggered an upregulation in γH2AX which mediate apoptosis, however, here we have shown that cell death was independent of DNA damage as the expression of γH2AX was minimally changed [24].

In summary, our data suggest that treatment of HT-29 cells with OMEO inhibits cellular viability and induces apoptosis and minimal cell cycle arrest. Moreover, it shows a minimal DNA damage. OMEO seems to exert its effect through the down regulation survivin.

Chapter 5: Conclusion

In conclusion, our study demonstrated, for the first time, the potential role of *Origanum majorana* essential oil, as an anti-colon cancer agent *in vitro*. This study provides preliminary data that proposes *Origanum majorana* essential oil as a valuable source of potentially new natural anti-breast cancer compound(s) that act by triggering apoptosis. Further experiments are needed to study the molecular mechanisms of action and downstream players. In addition to that, exploration of *Origanum majorana* is urged in order to identify the bioactive phytochemical(s) conferring its anti-colon cancer activity.

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Athamneh K, El Hasasna H, Al Samri H, Attoub S, Arafat K, Benhalilou N, Al Rashedi A, Al Dhaheri Y, AbuQamar S, Eid A & Iratni R. *Rhus coriaria* increases protein ubiquitination, proteasomal degradation and triggers non-canonical Beclin-1-independent autophagy and apoptotic cell death in colon cancer cells. Scientific reports. 2017; 7: 11633