SYNTHESIZING OF OLIVE LEAF EXTRACT LOADED CALCIUM ALGINATE CHITOSAN MICROCAPSULES AND INVESTIGATION OF THEIR ANTICANCER ACTIVITY ON CANCER CELLS

A Thesis Submitted to
the Graduate School of Engineering and Sciences of
İzmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in Biotechnology

by

YILDIZ BAL

December 2014

IZMIR

We approve the thesis of **Yıldız BAL**

Examining Committee Members:

_____
Assoc. Prof. Dr. Gülşah ŞANLI
Department of Chemistry, Izmir Institute of Technology

_____
Prof. Dr. Figen KOREL
Department of Food Engineering, Izmir Institute of Technology

_____
Assoc. Prof. Dr. Ali ÇAĞIR
Department of Chemistry, Izmir Institute of Technology

19 December 2014

_____   _____
Assoc. Prof. Dr. Gülşah ŞANLI   **Prof.Dr. Oğuz BAYRAKTAR**
Supervisor, Department of Chemistry   Co-Supervisor, Department
İzmir Institute of Technology   of Chemical Engineering
                                           Ege University

_____   _____
Prof. Dr. Volga BULMUŞ   **Prof. Dr. Bilge KARAÇALI**
Head of Departmant of Biotechnology   Dean of the Graduate School of
and Bioengineering   Engineering and Sciences

ACKNOWLEDGEMENTS

ABSTRACT

SYNTHESIZING OF OLIVE LEAF EXTRACT LOADED CALCIUM ALGINATE CHITOSAN MICROCAPSULES AND INVESTIGATION OF THEIR ANTICANCER ACTIVITY ON CANCER CELLS

As we all know there are too many diseases which can be counted as fatal. But cancer is one of the most terrifying one for human race. Moreover, breast cancer in females and lung cancer in males are the most common cancer types. Although there are so many treatment methods, they have severe side effects. On the other hand, mother earth is so willing to give her hand and open all of the sources she got. *O. europaea* is one of the most important valuable source and Mediterranean area is blessed with that source. Olive and olive products may play an important role in cancer prevention. However, there are some limitations on their direct usage. Since encapsulation technology can seal valuable things into small capsules, it can be used to alleviate these limitations.

In this study, the aim was figure the limitations of olive leaf extract (OLE) out with the encapsulation technology and enhance the effectiveness. Moreover, assess this effectiveness on cancer cell lines. Thus, OLE loaded calcium alginate (OLE-Ca-Alg) capsules produced by ionic-crosslinking. To limit the loss of OLE, capsules were coated with chitosan (CS) and OLE loaded chitosan calcium alginate (OLE-CS-Ca-Alg) were produced–two stage procedure. Optimization studies and characterization of the microcapsules were carried out. To investigate their anticancer effectiveness cytotoxicity, cell cycle and apoptosis analysis were performed for lung and breast cancer cell lines besides cytotoxic effects of the capsules were compared with healthy cell line. Additionally, visual observations were done by optical microscopy.

Consequently, results showed OLE loaded capsules are more cytotoxic than free OLE. These effects were supported with cell cycle, apoptosis analysis and optical microscopy observations. In contrast none of compounds was cytotoxic for healthy cells. Thus, it can be concluded that our results will enhance the drug industry as it is a new approach for anticancer drugs and biocompatible material for biomedical applications.

ÖZET

ZEYTİN YAPRAĞI EKSTRAKTI YÜKLENMİŞ KALSİYUM ALJİNAT KİTOSAN MİKROKAPSÜLLERİN SENTEZLENMESİ VE KANSER HÜCRELERİ ÜZERİNDEKİ ANTİKANSER AKTİVİTELERİNİN İNCELENMESİ

Hepimizin bildiği üzere ölümcül olarak sayılabilecek bir çok hastalık vardır. Ancak kanser insan ırkı için en korkutucularından biridir. Dahası kadınlarda meme kanseri, ve erkeklerde akciğer kanseri en yaygın kanser tipleridir. Bir çok tedavi yöntemi olmasına karşın bu yöntemlerin ciddi yan etkileri vardır. Diğer taraftan Doğa Ana yardım etmekte ve sahip olduğu her şeyi sunmakta cömerttir. *O. europaea* en önemli kaynaklardan biridir, ve Akdeniz coğrafyası bunlarla kutsanmıştır. Zeytin ve zeytin mahsülleri kanserin önlenmesinde önemli rol oynayabilir. Ancak bunların doğrudan kullanımında bazı kısıtlamalar vardır. Enkapsülasyon teknolojisinin değerli şeyleri küçük kapsüllere dönüştürebilmesi bu kısıtlamaları azaltmak için kullanılabilir.

Bu çalışmada hedef enkapsülasyon teknolojisi yardımıyla zeytin yaprağı ekstraktı (OLE) hakkındaki kısıtlamaları saptamak,böylece etkinliğini artırmak, bunların kanser hücre hatlarındaki etkisini belirlemek, ayrıca iyonik çapraz bağlanma yöntemiyle üretilmiş OLE yüklü kalsiyum aljinat (OLE-Ca-Alg) kapsüller üretmekti. OLE kaybını azaltmak için kapsüller kitosan (CS) ile kaplandı ve OLE yüklü kitosan kalsiyum aljinat (OLE-CS-Ca-Alg) üretildi– two-stage yöntemi. Optimizasyon çalışmaları yapıldı. Dahası, mikrokapsüllerin karakterizasyonu sağlandı. Bunların antikanser etkililiği araştırmak adına akciğer ve meme kanserinin sitotoksite, hücre döngüsü ve apoptosis analizleri yapıldı ve kapsüllerin sitotoksik etkileri sağlıklı hücre hatlarıyla karşılaştırıldı. Ek olarak, görsel incelemeler ışık mikroskopu ile yapıldı.

Sonuçlar, OLE yüklü kapsüllerin serbest OLE'ye göre daha sitotoksik olduğunu gösterdi. Bu etkiler hücre döngüsü, apoptosis analizi ve mikroskop gözlemlemeleriyle desteklendi. Bunun aksine, bileşenlerin hiçbirinin sağlıklı hücreler için sitotoksik olmadığı görüldü. Bu yüzden, sonuçlarımız antikanser ilaçlar ve biyomedikal uygulamalardaki biyouyumlu materyallerle ilgili yeni bir yaklaşım olduğu için ilaç endüstrisini geliştireceği sonucuna ulaşılabilir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

1.1. Importance of Olive Leaf Extract

The olive tree (*Olea europaea, Oleaceae*) is a traditional symbol for peace in so many nations. And in additions to its historical mean it has nutritional, medical uses.

Olive cultivation is one of the oldest and main component for the Mediterranean civilizations, and the olive has been always used in traditional remedies in so many countries such as Spain, Italy, France, Greece, Israel, Morocco, Tunisia, and Turkey. (Sibel Karakaya, 2009).

The incidence of cancer in these countries is lower than in the rest of European countries and the United States and the major reason for this, apart from possible genetic factors, could be attributed to the dietary habits. The traditional Mediterranean diet is characterized by high consumption of foods of plant origin (Keys et al. 1981). Especially olive and its products.

Scientific researches revealed that polyphenols are predominant micronutrients of the olive product. (Junkyu Han et al 2009). Therefore, this may be the reason why olive leaves gained much more interest in various fields.

1.1.1. General Properties of Olive Leave Extract

Olive leaves have different chemical composition because of the origins of the tree, storage and climatic conditions. In addition, the structural carbohydrates and nitrogen content in olive leaves depends on factors such as moisture content, contaminations, year and proportion of wood, etc. (Delgado-Pertinez M, Gomez-Cabrera A, Garrido A, 2000).

And there are five groups of phenolic compounds principally present in olive leaves: oleuropeosides (oleuropein and verbascoside); flavones (luteolin-7-glucoside, apigenin-7-glucoside, diosmetin-7- glucoside, luteolin, and diosmetin); flavonols (rutin);

flavan-3-ols (catechin), and substituted phenols (tyrosol, hydroxytyrosol, vanillin, vanillic acid, and caffeic acid). (Benavente-Garcia J, 2000) see in figure 1.1.

Phenolic Compound	Chemical Formula
Oleuropein	
Hydroxytyrosol	
Verbascoside	
Apigenin-7-glucoside	
Luteolin-7-glucoside	

Figure 1.1. Chemical structure of the most abundant phenolic compounds in olive-leaf extract.(Source: Benavente-Garcia et al. 2000).

In vivo and in vitro studies suggest that these bioactive compounds exhibit powerful antioxidant activity (Visioli et al. 2000; Le Tutourand Guedon, 1992).

And it is known that reactive oxygen and nitrogen species are essential to energy supply, detoxification, chemical signaling, and immune function. Moreover they are continuously formed in the human body and are controlled by endogenous enzymes. And when there is an overproduction of these reactive species, an exposure to external oxidant substances, or a failure in the defense mechanisms, damage to valuable biomolecules (DNA, lipids, proteins) may occur. (Sedef N El et al. 2009).

In other word they can damage cellular molecules because they are tend to move so fast and steal an electron from the molecules around them in the cell and oxidize the molecules, it can be said they cause biological rusting. Therefore this damage directly associated with increased risk of cancer and hypothesis of antioxidant claim that antioxidants can prevent this oxidative damage by donating an electron to a free radical and make it stable and numerous lines of evidence demonstrate that antioxidants protect against DNA damage, a major step in oncogenic processes (Hercberg et al., 2006).

In addition, it was found that hydroxytyrosol, oleuropein, caffeic acid, and tyrosol can prevent the generation of reactive oxygen species. In this respect, flavonoids, phenols and oleuropeosides have been shown to possess an important antioxidant activity towards these radicals without evidence of toxicity (Bors, Hellers et al., 1990, Visioli, Bellomo et al., 1998, Visioli, Bellosta et al., 1998).

Benavente-Garcia et al. aimed to identify the main phenolic compounds present in olive-leaf extracts to describe their antioxidant activities. The antioxidant activities of phenolic compounds from olive-leaf extracts are shown in Table 1.1. (Benavente-Garcia et. al. 2000).

Table 1.1. Main phenolic compounds of olive-leaf extracts
(Source: Benavente-Garcia et. 2000)

Phenolic compound	TEAC (mmol/L)
Olive-leaf extract	1.58 ± 0.06
Rutin	2.75 ± 0.05
Catechin	2.28 ± 0.04
Luteolin	2.25 ± 0.11
Hydroxytyrosol	1.57 ± 0.12
Diosmetin	1.42 ± 0.07
Caffeic acid	1.37 ± 0.08
Verbascoside	1.02 ± 0.07
Oleuropein	0.88 ± 0.09
Luteolin-7-glucoside	0.71 ± 0.04
Vanillic acid	0.67 ± 0.09
Diosmetin-7-glucoside	0.64 ± 0.09
Apigenin-7-glucoside	0.42 ± 0.03
Tyrosol	0.35 ± 0.05
Vanillin	0.13 ± 0.01

Whole olive-leaf extract showed a TEAC value of 1.58 mM in this study and this finding suggests that olive phenols may exhibit synergistic behavior due to the capacity of inhibition of free radicals. (Benavente-Garcia et. al. 2000). Researches also showed that oleuropein and its derivates (hydroxytyrosol, tyrosol) are the main phenolic compounds in olive leaves.

Oleuropein is generally the most important phenolic compound in olive cultivars and can reach concentrations of up to 140 mg g^{-1} on a dry matter basis in young olives and 60–90 mg g^{-1} of dry matter in the leaves (Amiot MJ et al. 1986, Guedon D. et al. 1992). According to De la Puerta et al., oleuropein has both the ability to scavenge nitric oxide and to cause an increase in the inducible nitric oxide synthase expression in the cell.

A scavenging effect of oleuropein was demonstrated with respect to hypochlorous acid which is an oxidative substance produced in vivo by neutrophil myeloperoxidase at the site of inflammation and can damage proteins including enzymes (De la Puerta R.et al. 2001, Visioli F et al. 2002, Syed Haris Omar, 2010). Moreover, Hydroxytyrosol, an oleuropein derivative, also improves cardiac and tumoral diseases with effects similar to those of oleuropein (Somova LI et al. 2003).

1.1.2. Effect of OLE on Cancer Treatment

As mentioned before olive leaves have the highest antioxidant and scavenging power among the different parts of the olive tree, therefore olive tree leaves gained so much attention since they have beneficial effects on metabolism when used as a traditional herbal drug. Moreover, there are so many researches which can supply strong proof for polyphenols have cancer-preventive properties. And the most abundant compound in olive leaves is oleuropein, followed by hydroxytyrosol (Sibel Karakaya, 2009).

Coni et al. conducted a study with laboratory rabbits fed special diets that contained oleuropein. The results of the study indicate that the addition of oleuropein increases the ability of low-density lipoproteins to resist oxidation. Hamdi and Castellon showed that oleuropein inhibits growth of LN-18 cells, a poorly differentiated glioblastoma cell line. Menendez et al. showed that oleuropein aglycone is the most potent phenolic compound in decreasing breast cancer cell viability. Subsequently, they also showed that the secoiridoids deacetoxy oleuropein aglycone, ligstroside aglycone, and oleuropein aglycone, induce strong tumoricidal effects, trigger high levels of apoptotic cell death in breast carcinomas (Coni E. et al. 2000, Hamdi HK et al. 2005, Menendez JA et al, 2007).

Recently, Han et al. reported that 200 lg/mL of oleuropein remarkably reduces the viability of MCF-7 cells and decreases the number of MCF-7 cells by inhibiting the rate of cell proliferation and inducing cell apoptosis.

Goulas et al. demonstrated the antiproliferative activity of crude extracts and phytochemicals against cell lines. These extracts inhibit cell proliferation of human breast adenocarcinoma, human urinary bladder carcinoma and bovine brain capillary endothelial (Han J et al., 2009, Goulas V et al., 2009).

Moreover, hydroxytyrosol has been shown to have anti-cancer effect on human colon adenocarcinoma cells and human promyelocytic leukemia cells, have anti-melanogenesis activity (Fabiani et al. 2002, 2006).

In a study Giulia et al. demonstrated that hydroxytyrosol rich extract was previously reported to exert strong inhibitory effects on human colon adenocarcinoma cells proliferation. Such extract targeted the cell cycle progression through the arrest in the G2/M phase. Similarly, pure hydroxytyrosol and olive oil rich hydroxytyrosol extract

induced apoptosis in HL60 cells subsequent to a G0/G1 cell cycle arrest in another trial. (Giulia et al., 2007, Fabiani et al., 2006).

Han J. et al. have showed that oleuropein or hydroxytyrosol decreased cell viability, inhibited cell proliferation, and induced cell apoptosis in MCF-7 cells. Also hydroxytyrosol and oleuropein exhibited a significant block of G1 to S phase transition since the cell number in G0/G1 phase (Han J. et al., 2009).

1.2. General Properties of Cancer Biology

Cancer is one of the most leading cause of death in all over the world. And studies about cancer showed that it effect people of all ages. Over the last 30 years, the most significant development in understanding what cause the cancer was that cancer is a genetic disease at the somatic cell level. And it occurs by the presence of gene products which are derived from mutated genes.

Both uncontrolled growth and spread of cancer occur because of the combined effects of numerous abnormal gene. Most cancer genes are created within somatic cells that then divide and form tumors. Moreover, the nature of cancer is not alike other genetic diseases, because this disease needs mutations which is predominantly arise in somatic cells. Also, a single mutation in a single gene rarely cause cancer, accumulation of mutations in many genes mostly give rise to cancer formation which affect cellular functions, including repair of DNA damage, cell division, apoptosis, cellular differentiation (Concept of Genetic by William S. Klug et al.).

In multicellular organisms cell proliferation is the most essential for all development and reparation of tissue. And it refers the process of cell growth and division. With the data about cancer cells it is well known that all cancer cells lose the control over cell proliferation. And the cellular events include sequences from one cell division to next which means the cell cycle. Figure 1.2.
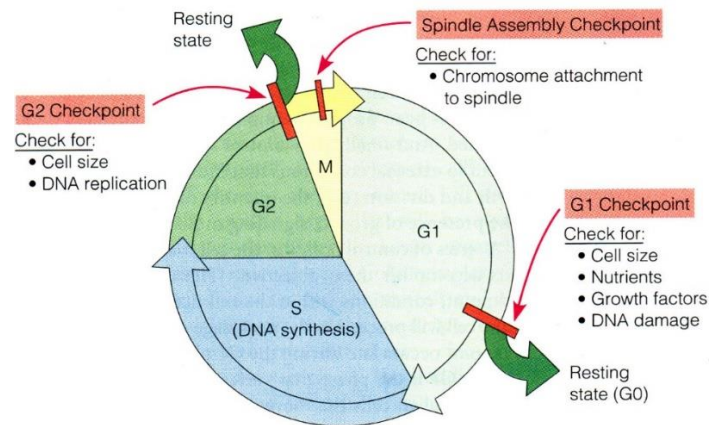
Figure 1.2. Steps and checkpoints of the cell cycle
(Source: Gleesonbiology, 2010).

Normally dividing cells continue through G1, S, G2 and M phases, but if the cell receives signals not to divide, it goes through G0 phase of the cell cycle. In this G0 phase, the cell does not grow but stays metabolically active. However cancer cells are not able to enter G0 and start from the beginning of the cycle over and over again. Since the cell cycle is regulated by genes, its products either promote or suppress the cell division. Mutation or misexpression of those genes cause development of cancer. (Concept of Genetic by William S. Klug et al.). Thus the cell loses control over proliferation and becomes cancerous.

As already described cancer cells are not able to enter G0 and start from the beginning of the cycle over and over again until the condition is corrected. This correction process in the cell reduces the possibility of mutations and chromosomal abnormalities. Although there is a correction system in the cell, sometimes DNA or chromosomal damage could be too severe to repair. Under this circumstance the cell can adapt second defense system and this is called apoptosis or programmed cell death. Apoptosis is a process that cell commits suicide and this system is a genetically controlled process. This process does not only eliminate certain cells that do not contribute to the final adult organism, but also it can prevent cancer formation and its steps are the same in both scenario. During this process, nuclear DNA becomes fragmented, internal cellular structures are disrupted, and the cell dissolves into small spherical structures called as apoptotic bodies. figure 1.3.
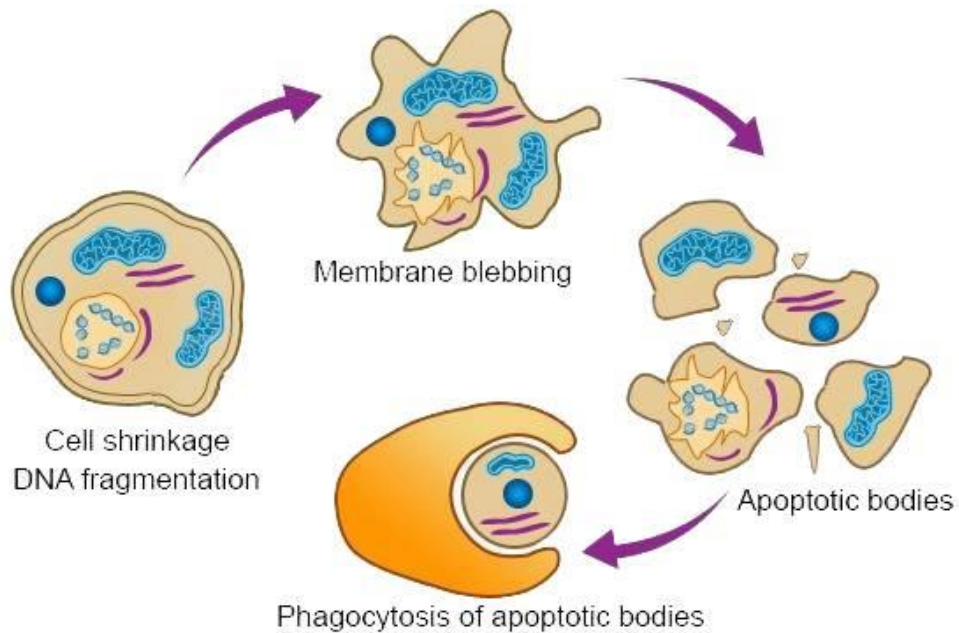
Figure 1.3. Apoptotic pathway of a cell.
(Source: Buzzle, 2014).

In brief, in normal cells, these functions are strictly controlled by genes in right time and place. However in cancer cells, these genes are either mutated or are expressed inappropriately. And this combination of uncontrolled cell proliferation and metastatic spread makes this disease very dangerous. When a cell loses genetic control over cell growth, it may grow into a multicellular mass, in other word create a benign tumor. Such a tumor can be removed by surgery and may not be seriously harmful. However, if cells in the tumor could find a way to enter the bloodstream, invade other tissues, and form secondary tumors, they become malignant which is difficult to treat and may become life threatening (Concept of Genetic by William S. Klug et al).

And studies show, the incidence and mortality rates of this disease are increasing worldwide in both economically developed and developing countries. Breast cancer in females and lung cancer in males are the most common cancer types. Lifestyle-related factors like dietary habits, influence the incidence rate of diseases such as cancer (S. Isık et al., 2012).

1.2.1. Breast Cancer

Breast cancer is the most common cancer and leading cause of cancer deaths among women worldwide. Furthermore, the incidence of breast cancer is increasing all

over the world due to changes in the dietary habits (Jemal et al., 2011, Saad, 2011, Key et al., 2004).

It is known that breast cancer is a heterogeneous disease with distinct clinical behavior and molecular properties. Estrogen receptor (ER) positive and ER negative cancers are the two most distinct subtypes. Since ER plays a central role in the crosstalk between different signaling pathways in breast cancer, the expression of this receptor is important for the behavior of breast cancer cells. ER positive cells exhibit higher proliferative capacity and distinct drug response than ER negative cells. Importantly, ER negative cancers are generally more sensitive to chemotherapy, but associated with poor clinical outcome (Rouzier et al., 2005, Badve et al., 2009, Shen et al., 2012, Andre et al., 2008).

Therefore, genetic, molecular and histological factors alter the mode of treatment of breast cancer. However, breast-conserving surgery followed by whole-breast irradiation, has become the standard of care in the treatment of early-stage breast cancer (Fisher et al., 2002; Clarke et al., 2005).

The implementation of adjuvant therapy either hormonal or chemical has made a major impact on disease-free survival and overall survival in both premenopausal and postmenopausal women with early-stage breast cancer. However, high proportion of treated breast cancer patients suffer from recurrence, and the majority of these patients die because of dissemination of metastatic disease, therefore more efficient and less toxic adjuvant therapeutic strategies are needed (Clarke et al., 2005, Maha H. Elamin et al., 2012).

Moreover, many studies suggest there is a possible correlation between olive products consumption and incidence of breast cancer and hence studies should be continue to reveal whether there is a relation lifestyle and habits could alter the breast cancer risk.

1.2.1.1. MCF-7 Cell Line

MCF-7 (breast cancer cell line) was isolated from a 69-year-old Caucasian female in 1970. It is the best characterized and most widely used of all the human breast cancer cell lines. MCF-7 cell line was discovered by Soule et al. in Michigan Cancer Foundation – 7, and it is named as MCF-7 cell line after this discovery.

This cell line is a perfect model to study the pathway of malignant progression. Because the cell line has retained several ideal characteristics ("MCF-7 Cells, human breast adenocarcinoma cell line, General Information",Altogen, Retrieved on 24 May 2014).

There was no mammary cell line that could live longer than a few months before MCF-7. MCF-7 has multiple characteristics of differentiated mammary epithelium such as the capability of forming domes and the ability to process estradiol via cytoplasmic estrogen receptors. This makes the MCF-7 cell line (Figure 1.4.) an ER positive control cell line. ("MCF-7 Cells, human breast adenocarcinoma cell line, General Information", Altogen , Retrieved on 24 May 2014).
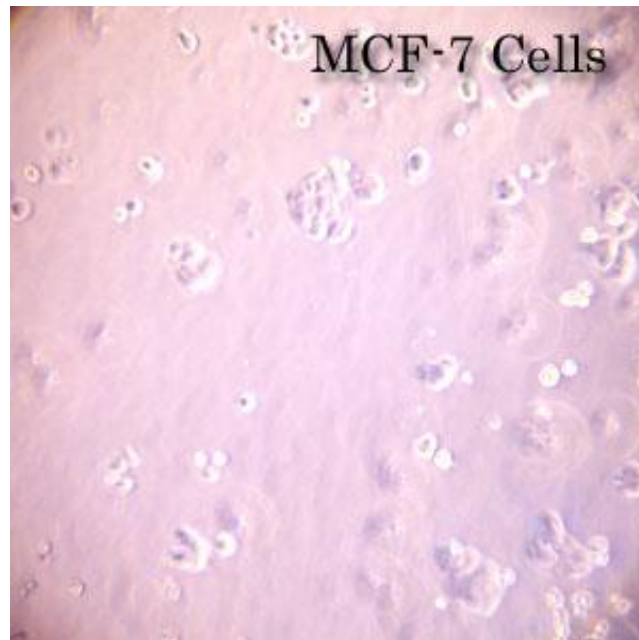
Figure 1.4. Image of MCF-7 Cells.
(Source: Altogen Biosystem, 2014)

1.2.2. Lung Cancer

"The cancer that forms in tissues of the lung" could be the basic definition of this type of cancer. There are two main types of this cancer such as small cell lung cancer and non-small cell lung cancer. And cancer of the lung is the most common type of cancer in the world and it is the leading killer of all cancer patients. (Zhang P. et.al., 2003).

Series of mutations occur and create a lung cancer cell. However, before full cancerous cell formation, cell can be precancerous. Moreover, when a genetically mutated cell divides, it communicate with two new cell and give its abnormal genes, then divide into four cells with DNA errors and this process goes this way. And the cell becomes more mutated with these new mutations therefore, a lung cell may not be effective for doing its job anymore.

In the last 30 years, several advances in lung cancer therapy have emerged with the improvement of immunotherapy, radiotherapy and chemotherapy. The treatment for lung cancer depends on the histologic type, the presence of metastasis and performance status of the patient. The most common treatment approaches include a combination of surgery, radiotherapy and chemotherapy. (Berhoune M et al., 2014, Heidge Fukumas et al., 2014, Chu Q et al., 2014).

Cancers usually present as a heterogeneous population of malignant cells, with some that are drug-sensitive and some that are drug-resistant. Cytotoxic chemotherapy kills drug-sensitive cells, but does not affect drug-resistant cells that are generally in a dormant state. As the tumor begins to grow again, chemotherapy often fails because the remaining tumor cells are primarily drug-resistant. Paclitaxel is one of the most used antineoplastic drug for lung cancer and it blocks the progression of mitosis ultimately leading to cell death by apoptosis (Kreso A et al., 2014, Heidge Fukumasu et al., 2014). And most importantly these kind of treatments have severe side effects, especially for the healty cells. Therefore, more desirable and safety molecules are needed.

Since epidemiological research has provided increasing evidence that dietary habits may play an important role in lung cancer etiology, the potential preventive role of Mediterranean diet in the development of lung cancer is noteworthy (Fortes, C. et al., 2003).

1.2.2.1. A549 Cell Lines

This line was initiated in 1972 by D.J. Giard, et al. through explant culture of lung carcinomatous tissue from a 58-year-old Caucasian male. Further studies by M. Lieber, et al. revealed that A549 cells could synthesize lecithin with a high percentage of desaturated fatty acids utilizing the cytidine diphosphocholine pathway and also the squamous epithelial cells are positive for keratin, as is evidenced by immunoperoxidase

staining. A549 cells (Figure 1.5.) are human alveolar basal epithelial cells. These are squamous in nature and responsible for the diffusion of substances, such as water and electrolytes, across the alveoli of lungs. ("A549 Cell Line- Human Alveolar Adenocarcinoma Cell Line, General Information",Altogen, Retrieved on 24 May 2014).
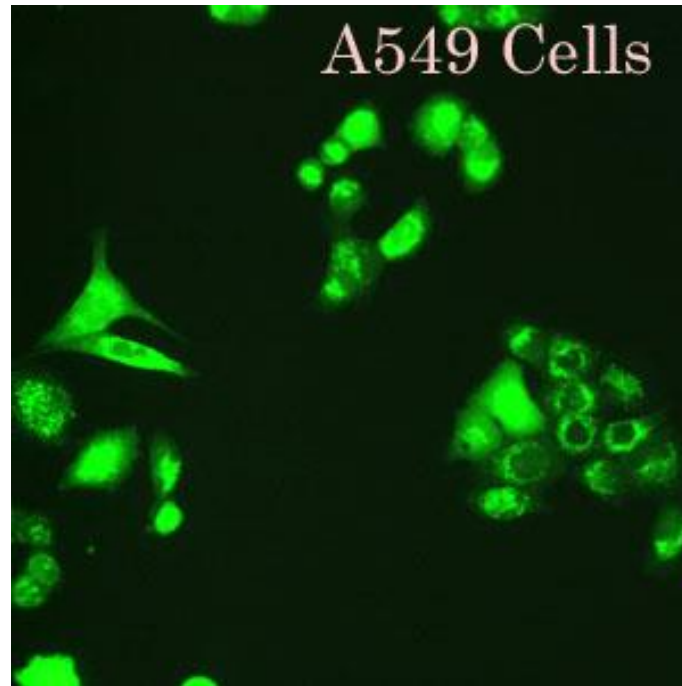
Figure 1.5. Image of A549 Cells
(Source: Altogen, 2014).

1.2.3. BEAS 2B Cell Line

BEAS-2B (human bronchial epithelium) (Figure 1.6.) cells were derived from normal bronchial epithelium obtained from autopsy of non-cancerous individuals. Cells were infected with a replication-defective SV40/adenovirus 12 hybrid and cloned. Squamous differentiation can be observed in response to serum. This ability can be used for screening chemical and biological agents inducing or affecting differentiation and/or carcinogenesis ("General Cell Collection: BEAS-2B", phe-culturecollections, Retrieved on 24 May 2014).

BEAS-2B
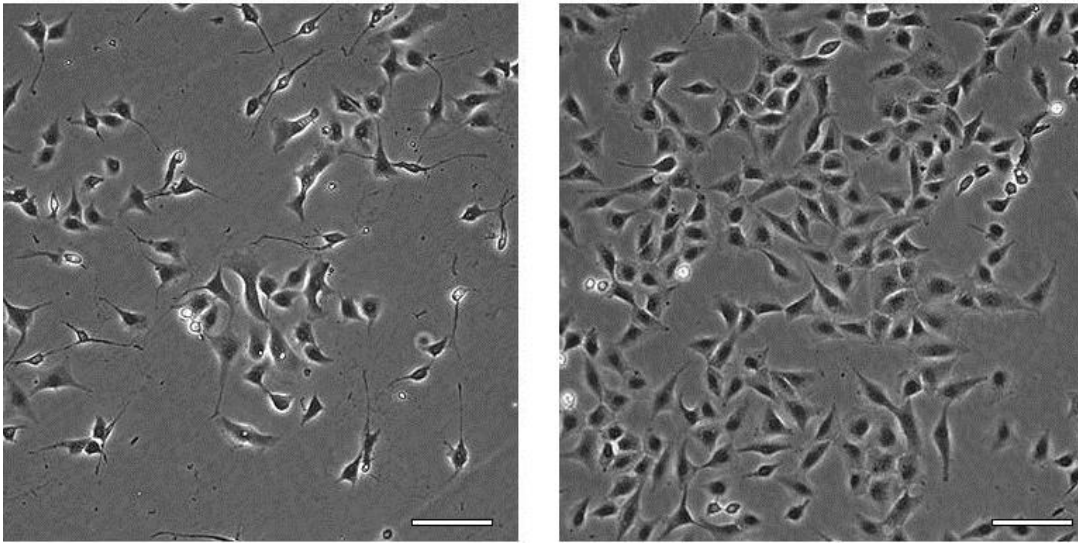
Figure 1.6. Image of Human bronchial epithelium cells
(Source: Altogen, 2014).

1.3. Requirements of Encapsulation Technology

Microencapsulation, developed approximately 60 years ago, is defined as a technology of packaging solids, liquids, or gaseous materials in miniature capsules that can release their contents at controlled rates under specific conditions. The packaged materials can be pure materials or a mixture, which are called coated material, core material, actives, fill, internal phase or payload. On the other hand, the packaging materials are called coating material, wall material, capsule, membrane, carrier or shell, (Desai & Park, 2005, Vilstrup, 2001, Gibbs et al.,1999; Mozafari, 2006).

A 'microcapsule' is defined as a spherical particle with size varying from 50 nm to 2 mm, and it contains a core substance. Microspheres are in a strict sense, spherical empty particles. However, the terms microcapsules and microspheres are often used synonymously. In addition, some related terms are used as well. For example, 'microbeads' and 'beads'. Spheres and spherical particles are also used for a large size and rigid morphology (Majeti N.V. et al. 2000, Yao et al., 1995).

Although their specific shapes depend on the technologies and materials used, there are two major morphologies commonly seen (Figure 1.7.), one is mononuclear capsules, which have a single core enveloped by a shell, while the other is aggregates, which have many cores embedded in a matrix (Schrooyen et al., 2001).
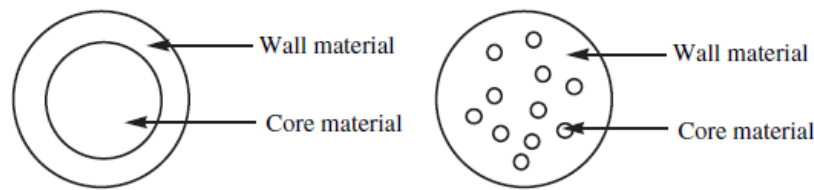
Figure 1.7. Two major forms of encapsulation: on the left, mononuclear capsule and on the right aggregate (Source: Zhongxiang Fang et. al. 2010).

Various techniques are used for encapsulation. But, there are three steps in general:

1) the formation of the wall around the material to be encapsulated;

2) ensuring that undesired leakage does not occur;

3) ensuring that undesired materials are kept out (Gibbs et al., 1999; Mozafari et al., 2008).

Therefore, it is easy to say, the main objective of encapsulation is to protect the core material from undesirable effects of light, moisture, and oxygen. (Shahidi & Han, 1993).

As previously mentioned, the polyphenol have strong antioxidants power. Numerous in vitro studies have demonstrated that polyphenolic compounds can directly scavenge molecular species of active oxygen because polyphenols possess antioxidant properties thus they can donate hydrogen atoms or electrons. Furthermore, polyphenols can chelate metal ions, prevent redox-active transition metals from catalyzing free radical formation. Moreover, the literature shows that polyphenols are able to:

• reduce the inflammation by inhibition of the edema,

• stop the development of tumors,

• present proapoptotic and anti-angiogenic actions,

• modulate the immune system,

• increase the capillary resistance by acting on the constituents of blood vessels

•protect the cardiovascular system (Saija, A. et al., 2003, Leopoldini, M. et.al., 2011, Hanasaki et.al., 1994, Rice-Evans et.al., 1996, Dugas, A.J. et al., 2000, Pietta et al., 2000).

However, there are direct usage limitations of these valuable natural compounds because phenolic compounds suffer during their transit through the stomach since polyphenols undergoes acid hydrolysis in the stomach. Moreover, they oxidize very

quickly, cause dark colored composition and unwanted smell with a considerable loss in activity. Furthermore, many polyphenols have an unpleasant taste which must be masked before their usage as foodstuffs or oral medicines. (Aude Munin et al., 2011).

Since their administration efficiency depends on their bioavailability or only a small proportion of molecules administered orally are absorbed, because of insufficient gastric residence time, low permeability and low solubility, it is a big challenge, keep them stable and valuable. Thus the delivery of these compounds requires product formulators and manufacturers to provide protective mechanisms that can maintain the active molecular form until the time of consumption, and deliver this form to the physiological target within the organism (Chen, Remondetto, & Subirade, 2006).

Encapsulation is an interesting and promising method among the existing stabilization methods. Thus, the utilization of encapsulated polyphenols instead of free forms of the polyphenols can effectively overcome these direct usage limits and also improve its bioavailability and half-life in vivo and in vitro.

Nowadays, various microencapsulation techniques are available that can be classified as follows:

1. Physical methods: spray-drying, fluid bed coating, extrusion-spheronization, centrifugal extrusion, processes using the supercritical fluids,

2. Physicochemical methods: spray-cooling, hot melt coating, ionic gelation, solvent evaporationextraction, simple or complex coacervation,

3. Chemical methods: interfacial polycondensation, in situ polymerization, interfacial polymerization, interfacial cross-linking etc (Aude Munin et. Al., 2011).

Since this technology could protect the encapsulated polyphenols against the harsh environment of the gastrointestinal tract and release them under controlled conditions, drug delivery systems with microcapsules have been so successful for getting a great deal of attention.

Controlled-release technology emerged during the 1980s as a commercially sound methodology. The idea of this technology is the achievement of predictable and reproducible release of an agent into a specific environment over an extended period of time. Moreover, it creates a desired environment with optimal response, minimum side-effects and extended efficacy. Controlled release dosage forms enhance the safety, efficacy and reliability of drug therapy. Thus, microencapsulation is one of the processes designed for the sustained release of drugs, a new dimension into the system. Polymers are potentially useful for this purpose, including synthetic as well as natural degradable

polymers. The release of drugs, which is encapsulated by polymers involves their slow and controllable diffusion through polymeric materials. Production of slow release drugs by the pharmaceutical industry has great deal of interest (K.E. Uhrich et al., 1999, C. Alvarez-Lorenzo et al., 1999, D. Teomim et al., 1999, R.L. Reis et al., 1995, K.C. Gupta et al., 2000, 2000).

Biopolymers are used as encapsulating materials in food and pharmaceutical applications and these polymers are not supposed to affect the activity of encapsulated molecules. Unfortunately, natural extracts systems have much more different behavior than ideal.

Therefore, to obtain an effective protection systems for the natural extracts, selection of encapsulation polymers must be made carefully and among other materials, Alginate and Chitosan have recently been regaining much attention owing to their inherent biocompatibility with the encapsulation of a wide range of drugs and bioactive molecules (F. Shahidi and X. Q. Han, 1993).

1.3.1. Alginate

It is a naturally occurring anionic polymer typically obtained from brown seaweed. Commercially available alginate is typically extracted from *Laminaria hyperborea, Laminaria digitata, Laminaria japonica, Ascophyllum nodosum,* and *Macrocystis pyrifera.* The extract is filtered, and either sodium or calcium chloride is added to the filtrate in order to precipitate alginate. This alginate salt can be transformed into alginic acid by treatment with dilute HCl. After further purification and conversion, water-soluble sodium alginate powder is produced (Smidsrod O et al., 1990, Rinaudo M. et al., 2008).

Matrices formed from alginate have demonstrated longevity within the body and, if highly pure, do not provoke immunogenic responses (Orive, G. et al., 2005). Therefore, its biocompatibility of this polymer make it so popular in this field.

Alginate is a well-known linear copolymer containing blocks of (1,4)-linked β-D-mannuronate (M) and α-L-guluronate (G) residues (Fig. 1.8.). Alginates extracted from different sources differ in M and G contents as well as the length of each block, and more than 200 different alginates are currently being manufactured (Tonnesen HH et al., 2002).
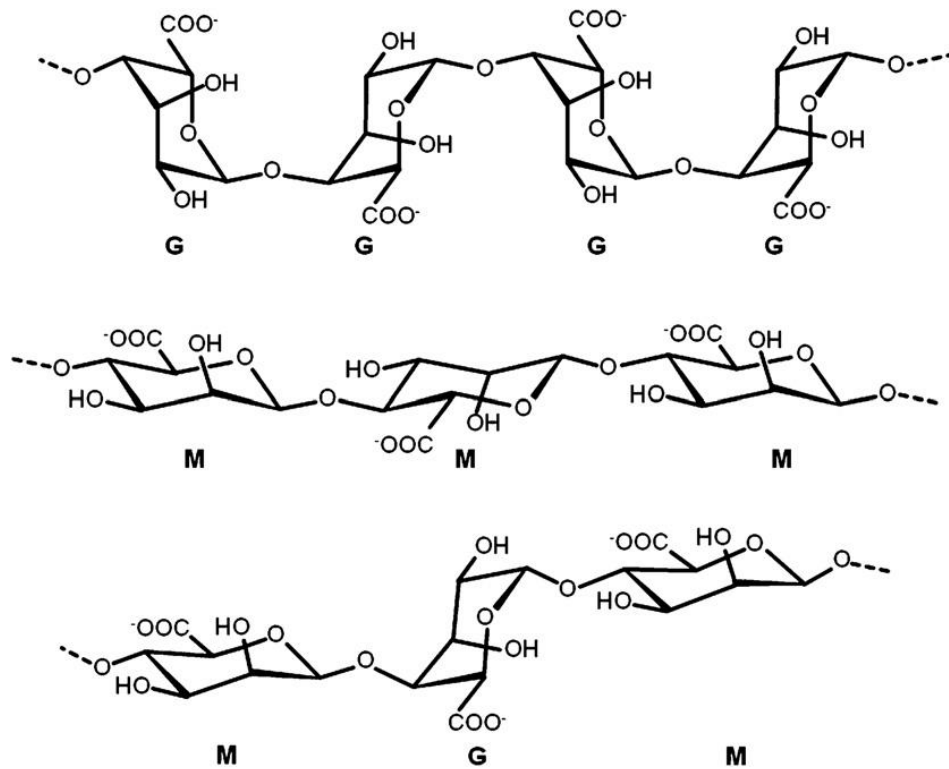
Figure 1.8. Chemical structures of G-block, M-block, and alternating block in alginate
(Source: Kuen Yong Lee et. al. 2011).

Moreover, mild gelation ability of alginate occurred by addition of divalent cations such as Ca^{+2} and it makes the alginate so valuable to investigate. Since only the G-blocks of alginate are believed to participate in intermolecular cross-linking with divalent cations to form hydrogels, Calcium-alginate hydrogels have had widespread application for the protection of encapsulation of molecules for in vitro and in vivo applications.

This cross-linking process will be explained in detail, but basically the gelation and cross-linking of the polymers are achieved by the exchange of sodium ions from the guluronic acids with the divalent cations and the stacks these guluronic groups to form the characteristic egg-box structure shown in figure 1.9.
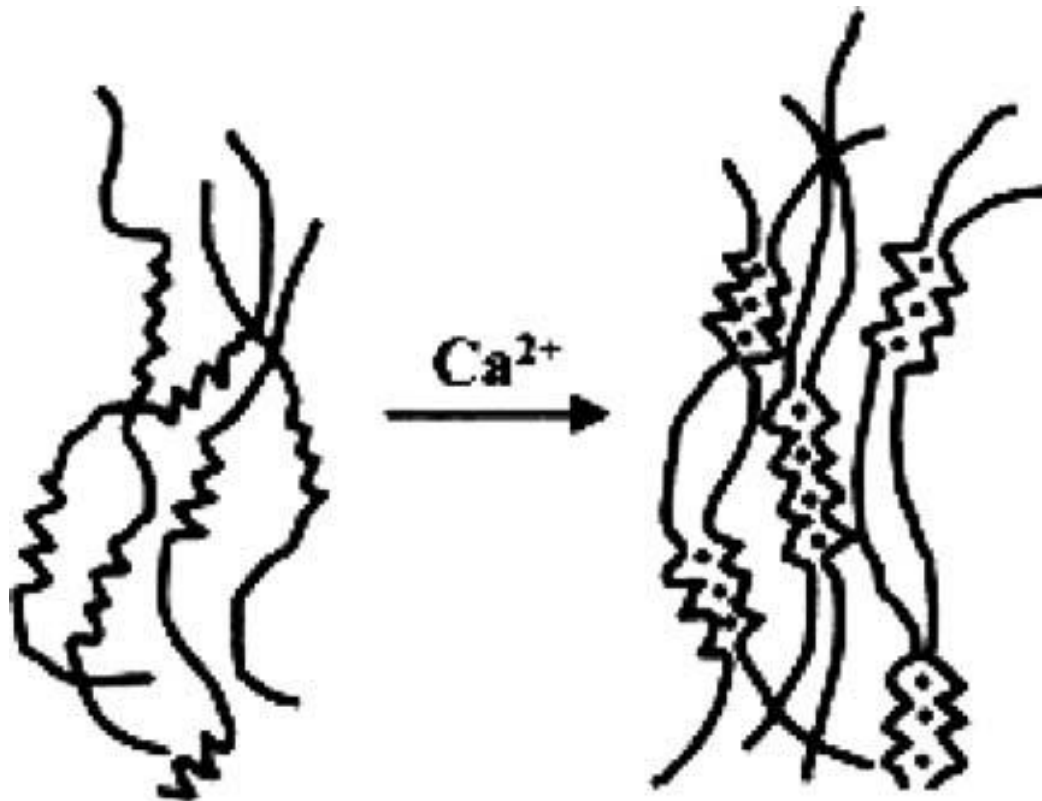
Figure 1.9. Egg-box structure of an alginate gel formed by chelation of Ca^{2+} ions
(Source: Meera George et al., 2006).

Chemical and physical cross-linking of hydrophilic polymers are typical ways to form hydrogels, which can be listed as

- Ionic cross-linking
- Covalent cross-linking
- Thermal gelation
- Cell cross-linking

The use of crosslinking agents to chemically form polymeric hydrogels may lead to toxic side effects or to unwanted reactions with drugs. Therefore, drug encapsulation using alginate is more often carried out physically by dispersion of the alginate/drug solution into a gelation medium in the presence of divalent cations such as Ca^{2+} (Xing L et al.2003, Esposito E. et al., 1996).

1.3.1.1. Ionic cross-linking

Ionic cross-linking is one of the most used method to preaper hydrogels and it includes combining an aqueous alginate solution with ionic cross-linking agents, such as divalent cations. Although, the affinity of alginates towards divalent ions decreases in the following order: Pb > Cu > Cd > Ba > Sr > Ca > Co, Ni, Zn > Mn, Ca^2, is the most commonly used cation (Morch YA et al., 2006).

Guluronate blocks of the alginate can supply a connection with the divalent ions and hence hydrogels can be obtained. The guluronate blocks of one polymer then form junctions with the guluronate blocks of the closest polymer chains, resulting in a gel structure and it is called the egg-box model of cross-linking (Grant GT et al., 1973).

Although Calcium chloride ($CaCl_2$) is one of the most frequently used ionically cross-linking agents, it has high solubility in aqueous solutions thus leading a rapid and poorly controlled gelation. Moreover, the gelation rate is a critical factor in controlling gel uniformity and strength when using divalent cations, and slower gelation produces more uniform structures and greater mechanical integrity (Kuo CK et al., 2001).

To slow and control gelation, a buffer containing phosphate can be used since phosphate groups in the buffer compete with carboxylate groups of alginate in the reaction with calcium ions, and slow down the gelation. The gelation temperature can also influence gelation, at lower temperatures, the reactivity of ionic cross-linkers (e.g., Ca^{2+}) is reduced, and cross-linking becomes slower. The resulting cross-linked network structure has greater order, therefore enhanced mechanical properties (Augst AD et al., 2006)

In addition to these, chemical structure of alginate can affect the mechanical properties of the resultant gels. For example, gels prepared from alginate with a high content of G residues exhibit higher stiffness than those with a low amount of G residues (Drury JL et al., 2004).

Instead of these advantageous properties of the calcium alginate beads, researches have demonstrated that they have a very porous nature and thus a low retention capacity. Additionally, strong ionic interactions between alginate and Ca^{2+} cause minimal swelling at pH 7.4, thus limiting the drug release especially at the intestinal tract. Therefore, to overcome this limitation of calcium-cross-linked alginate microcapsules, a polycationic polymer can be used as a membrane for capsule surface, since a strong electrostatic

interaction can occur between the amine groups of chitosan and carboxyl group of alginate (M. L. Huguet et al., 1996).

1.3.2. Chitosan

As most of the present-day polymers are synthetic materials, their biocompatibility and biodegradability are much more limited than those of natural polymers such as cellulose, chitin, chitosan and their derivatives. However, these naturally abundant materials also exhibit a limitation in their reactivity and processability (W.A. Mass et al., 1998, L. Illum et al., 1998).

In this respect, chitin and chitosan are recommended as suitable functional materials, because their biocompatibility, biodegradability, non-toxicity, adsorption properties, etc. Due to chitin's poor solubility in aqueous solution and organic solvents, it is not practical for bioapplications in contrast chitosan is more suitable as an artificial variant of chitin. Therefore, the chitin is naturally occurring polymer source for the chitosan. And it is easily obtained from crab or shrimp shells and fungal mycelia.

In the first case, chitin production is associated with food industries such as shrimp canning. In the second case, the production of chitosan–glucan complexes is associated with fermentation processes, similar to those for the production of citric acid from *Aspergillus niger, Mucor rouxii,* and *Streptomyces*, which involves alkali treatment yielding chitosan–glucan complexes. The alkali removes the protein and deacetylates chitin simultaneously. Depending on the alkali concentration, some soluble glycans are removed. The processing of crustacean shells mainly involves the removal of proteins and the dissolution of calcium carbonate which is present in crab shells in high concentrations. The resulting chitin is deacetylated in 40% sodium hydroxide at 120 ^{0}C for 1−3 hours. This treatment produces 70% deacetylated chitosan (P. Madhavan, 1992). Therefore, much attention has been paid to chitosan as a potential polysaccharide resource recently (S. Nishimura et al., 1991).

They have been took into consideration because they are made from an abundant renewable resource and also they are very compatible and effective biomaterials that can be used in many applications (S.J. Kim et al., 1994, G. Crini et al., 1997).

The structures of chitin and chitosan are shown in figure. 1.10.
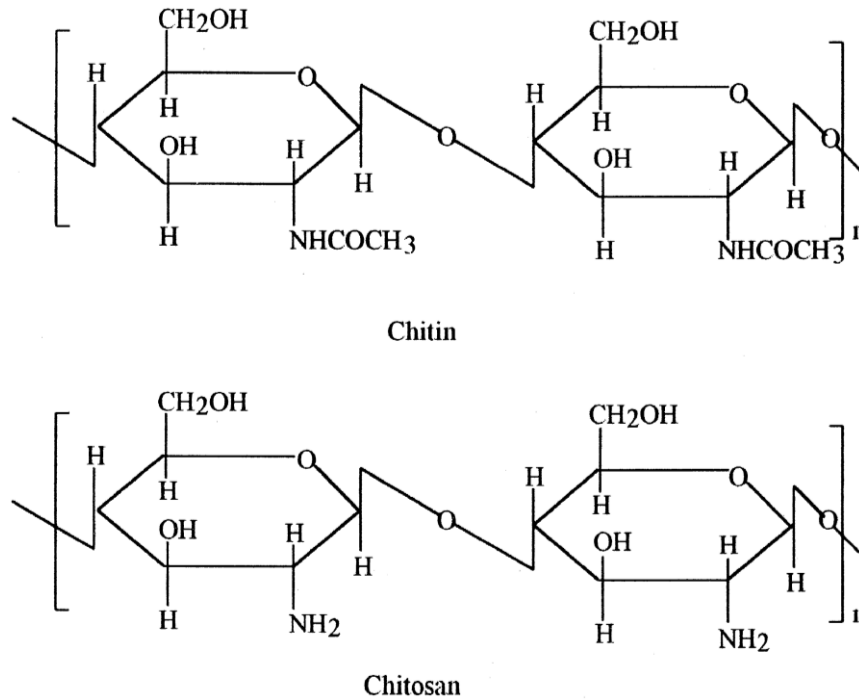
Figure. 1.10. Structures of chitin and chitosan
(Source: Majeti N.V et. al. 2000).

Chitosan is a copolymer composed of N acetyl- d-glucosamine and d-glucosamine units available in different grades depending upon the degree of acetylated moieties. It is a polycationic polymer that has one amino group and two hydroxyl groups in the repeating glucosidic residue. Thus, chitosan is a copolymer consisting of N-acetyl 2-amino-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose, where the two types of repeating units are linked by $(1 \rightarrow 4)$-β-glycosidic bonds (Hoppe-Seiler F., 1994, Agrawal P et al., 2009, Roberts GAF, 1992).

In short, the facile derivatization makes chitosan an ideal candidate for biofabrication. And after refinement, chitosan has a rigid crystalline structure (Yi H. et al., 2005).

As it is indicated at the beginning of this section, the coating for the calcium alginate beads is needed and with the sport of the all information about the chitosan, it could be used as a stabilizing agent, a membrane forming creator to the alginate gel by electrostatic interactions since it is positively charged it can interact with the negatively charged alginate.

With this purpose there are two procedures: in **one-stage procedure**, the alginate solution is dropped directly into a solution of chitosan and capsules with a complex

alginate-chitosan membrane surrounding a liquid alginate core can be obtained. The core can be subsequently gelled either by adding calcium chloride to the chitosan solution or by treating the liquid core capsules with calcium chloride after the membrane formation. The other method is a **two-stage procedure** which includes the formation of calcium alginate beads followed by a membrane forming stage where the beads are immersed in a solution of polycation (Huguet ML et al, 1996).

1.4. Aim of the Study

There are increasing evidence that high consumption of olive and its products may play an important role for prevention of cancer. Dietary agents that present in olive products especially polyphenols are believed to be responsible for these protective effects since they have antioxidant activitiy and hence free radical scavengering effect. These kinds of informations led us to our perspective, therefore the aim of this study is to investigate the anticancer properties of OLE, which is rich in polyphenols, loaded microcapsules on lung and breast cancer. With this purpose, extraction of olive leaves was carried out, in the first place. After characterization trials, OLE was sealed into calcium alginate capsules by ionic-crosslinking. Then capsules were coated with one of the most well-known polycationic polymer, chitosan, by two-stage procedure. Concequently, encapsulation would enhance bioavalibility and bioactivity of OLE.

For this purpose, determination of anticancer effects of OLE loaded capsules and free OLE were determined by cytotoxicity studies for A549 and MCF-7 cancer cells and also healty cells were treated with these compounds. Moreover, cell cycle analysis were carried out for having a deep understanding underlying mechanism of cytotoxicity. Additionally, visual observations of their effectiveness was performed by optical microscopy. Finally, apoptotic pathway of the cells treated with free OLE and OLE loaded capsules was determined.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Olive leaves were obtained Olive Research Institute, Izmir and extraction was performed at laboratory of Oğuz BAYRAKTAR in Chemical Engineering Department, Izmir Institute of Technology.

To produce microcapsules alginate were obtained from AppliChem, calcium chloride from Sigma chitosan from Aldrich.

Chemicals, solutions and some materials which were necessary during molecular biological studies were supplied from Biotechnology and Bioengineering Research and Application Centre, Izmir Institute of Technology.

The chemicals, buffers, solutions used and their compositions are listed in alphabetic order in Appendix A and Appendix B.

2.2. Methods

D. Bayçın et al. (2007) procedure was used for Extraction and characterization of olive leaf extract.

Synthesizing and characterization of Ca-Alg microcapsules and CS coated Ca-Alg microcapsules and olive leaf extract loaded Ca-Alg and CS coated Ca-Alg microcapsules were carried.

Ca-Alg capsules formation is schematically illustrated in figure 2.1. The gelation is achieved by the exchange of carboxyl group of the G blocks of the alginate with the divalent cations, Ca^{+2} ions, and it stuck the G blocks in to characteristic egg box structure.
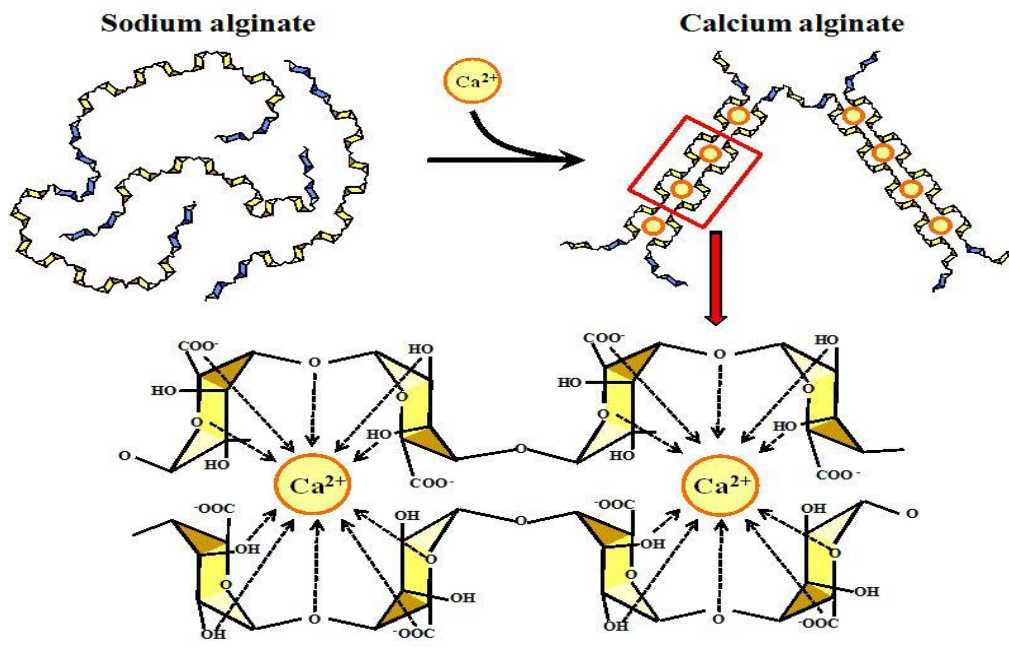
Figure 2.1.   Basically illustration of Ionic cross-linking
(Source: Keita Kashima et. al. 2012).

Since coated beads and microspheres are found to be better oral delivery vehicles, OLE loaded Ca-Alg capsules were coated according to two-stage procedure (Meera George and T. Emilia Abraham, 2009, M.L. Huguet & E. Dellacherie, 1996) as it can be seen in figure 2.2.
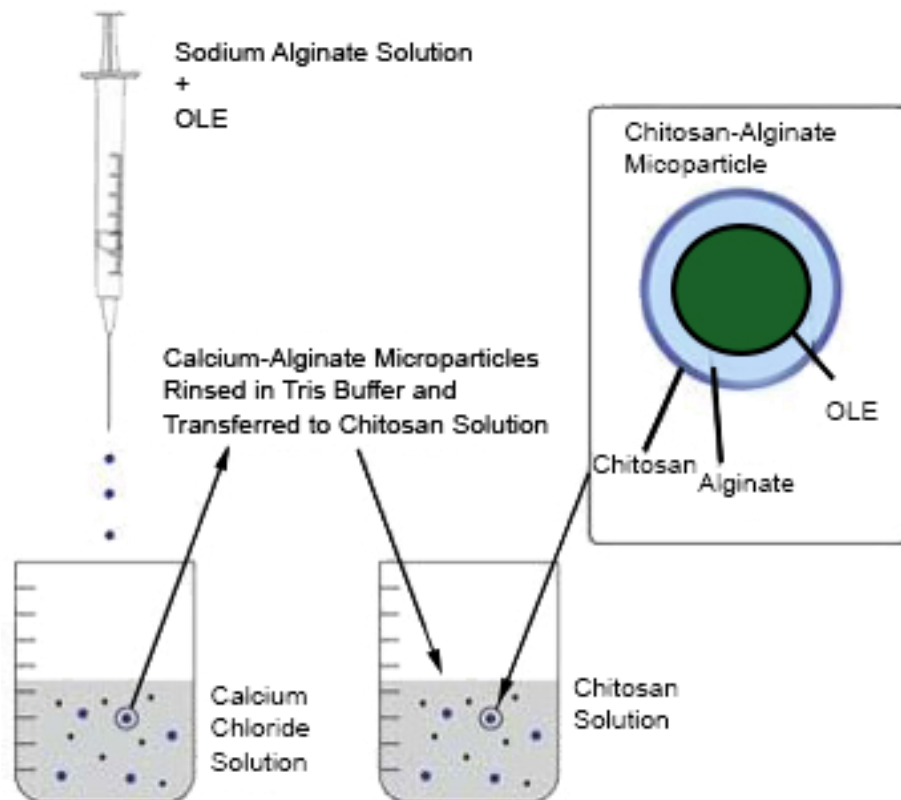
Figure 2.2. Schematic illustration of OLE loaded CS-Ca-Alg capsules

For proliferation of cell lines and in vitro cytotoxicity studies one of the oldest procedure was used (T. Mosmann, 1983). Cell cycle and cell apoptosis studies were carried out according to the Çakmak, Ö.Y (2011) procedures.

2.2.1. Chemical Studies

Chemical studies include extraction of olive leaves, characterization of olive leave extract, synthesizing of microcapsules, synthesizing of OLE loaded microcapsules, optimization of OLE loaded microcapsules and characterization of OLE loaded microcapsules.

2.2.1.1. Extraction of Olive Leaves

Olive leaves were supplied from Olive Research Institute, Izmir. Collected olive leaves were washed with deionized water and then dried at 37 °C for 3 consecutive days. The dried leaves were powdered and extracted in 70% ethanol aqueous solution in an orbital shaker, for 2 h at 25 °C. Liquid phase was drained and separated with vacuum filter. The ethanol was removed by using rotary evaporator at 38 °C, 120 rpm rotation. To remove chlorophyll, solvent-free extract was centrifuged. Then, the solvent-free olive leaf extract was dried using a freeze-dryer system at -52 °C and 0.2 mbar, and it was stored in light-protected glasses until further use. Extraction can be seen in figure 2.3. step by step.

Figure 2.3. The order of the procedure of extraction of olive leave

2.2.1.2. Characterization of Olive Leaf Extract (OLE)

Total phenolic compound content and total antioxidant were determined and total phenolic compounds were analyzed.

2.2.1.2.1. Determination of Total Phenolic Compound Content

To determine total phenolic compound content in olive leaf extract, the Folin-Ciocalteus method was used (Bayçın et al. (2007). Folin reagent was diluted from the stock solution at 1:10 ratio. The extract containing phenolic compounds was diluted with distilled water. Diluted solutions were vortexed and 20 μL sample were plated in 96-well plate. 100 μL of Folin reagent was added into each well. After 2.5 min., 80 μL of 7% Na_2CO_3 was added into each solution and mixed. After 1 h., the intensity of green color of each solution was measured by spectrophotometer (Thermo, Varioskan Flash, U.S.A.) at 725 nm. The procedure is shown in figure 2.4. Analysis was repeated three times for olive leaf extract and the results are expressed as miligrams of gallic acid equavelents per gram of dry olive cake (mg of GAE/g).
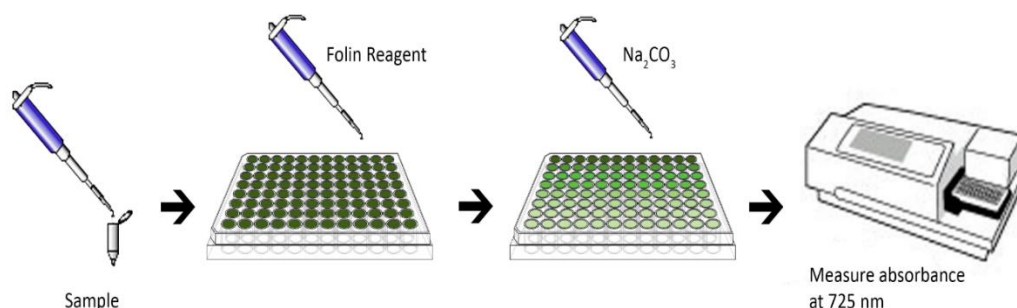
Figure 2.4. Basic illustration of total phenolic compound determination procedure

2.2.1.2.2. Determination of Total Antioxidant Capacity

Trolox Equivalent Antioxidant Capacity (TEAC) assay was performed for obtaining total antioxidant capacity. The method is used due to the ability of antioxidant

molecules to quench the long-lived ABTS+. The addition of antioxidants reduces radical cation to ABTS its neutral form, which can supply determining a decolorization.

To convert the ABTS into its radical cation, $K_2S_2O_8$ (sodium persulfate) was added into solution at 1:1 ratio. This reaction cause a change in color of radical cation solution in to blue-green which can absorbs light at 734 nm, to stand in the dark at room temperature for 12–16 h before use. The reactivity of the various antioxidants tested are compared to that of Trolox, which is a water-soluble analog of vitamin E (Barclay, L. et al., 1985).

At the beginning of the analysis day, an ABTS+ working solution was obtained by the dilution in ethanol of the stock solution to an absorbance of 0.70±0.02 AU at 734 nm, verified by spectrometer (Thermo, Varioskan Flash, U.S.A.), and used as mobile phase in a flow-injection system, according to Pellegrini et al. (2003)

The procedure is shown in figure 2.5 and results were expressed as TEAC in mmol of Trolox per kg (solid foods and oils) or per L (beverages) of sample.
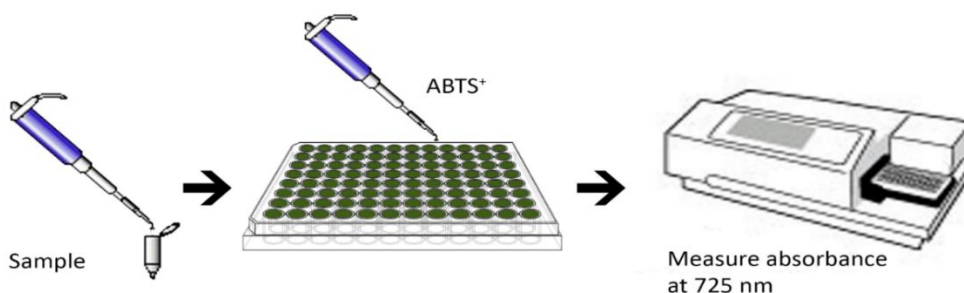
Figure 2.5. Basic illustration of total antioxidant activity determination procedure

2.2.1.2.3. Analysis of Total Phenolic Compounds

To obtain percent amount of phenolic compounds found in olive leaf extract, HPLC analysis was performed. The HPLC equipment used was a Hewlett-Packard Series HP 1100 equipped with a diode array detector. The stationary phase was a C18 LiChrospher 100 analytical column (250 mm×4 mm i.e.) with a particle size of 5 mm thermostated at 30 ºC. The flow rate was 1 mL/min and the absorbance changes were monitored at 280 nm. The mobile phases for chromatographic analysis were: (A) acetic acid/water (2.5:97.5) and (B) acetonitrile. A linear gradient was run from 95% (A) and 5% (B) to 75% (A) and 25% (B) during 20 min; it changed to 50% (A) and (B) in 20 min

(40 min, total time); in 10 min it changed to 20% (A) and 80% (B) (50 min, total time), after reequilibration in 10 min (60 min, total time) to initial composition.

Oleuropein in OLE was identified by comparing its retention times with the corresponding standards. Coumarin was used as an internal standard for the quantification of oleuropein and rutin. Other standards were used only for identification of these compounds in OLE.

2.2.1.3. Synthesizing of Microcapsules

2.2.1.3.1. Synthesizing of Calcium–Alginate (Ca-Alg) Microcapsules

Beads were obtained by mixing sodium alginate (3%) and Tris-HCl buffer solution (0.3M, pH 8.5), then the alginate solution was dropped from a syringe to calcium chloride solution (1.7M). The beads formed were maintained in the gelling bath to harden (60 min). Then, they were filtered through a filter paper and were washed with buffer solution. Ca-Alg microcapsules stabilized in air for 15 minutes.

2.2.1.3.2. Synthesizing of Chitosan coated Calcium – Alginate (CS-Ca-Alg) Microcapsules

Beads were obtained by mixing sodium alginate (3%) and Tris-HCl buffer solution (0.3M, pH 8.5), then the alginate solution was dropped from a syringe to calcium chloride solution (1.7M). The beads formed were maintained in the gelling bath to harden (60 min). Then, they were filtered through a filter paper and were washed with buffer solution. Ca-Alg microcapsules stabilized in air for 15 minutes. To analyze the effect of an additional layer, beads were immersed in the chitosan solution (30 minutes).

2.2.1.4. Synthesizing of OLE Loaded Microcapsules

2.2.1.4.1. Synthesizing of OLE Loaded Ca-Alg Microcapsules

For the preparation of OLE loaded Ca-Alg microcapsules, OLE (1.5%) is dissolved in Alginate solution. Then, the same procedure with the synthesizing of Ca-Alg microcapsules was performed.

2.2.1.4.2. Synthesizing of OLE Loaded CS-Ca-Alg Microcapsules

For the preparation of OLE loaded CS-Ca-Alg microcapsules, OLE (1.5%) is dissolved in Alginate solution. Once homogenized the alginate solution was dropped from a syringe calcium chloride solution (1.7 M). Capsules were maintained in this gelling bath to harden for 60 minutes. Then, they were filtered through a filter paper and were washed with buffer solution. OLE loaded Ca-Alg microcapsules stabilized in air for 15 minutes. Finally, beads were immersed in the chitosan solution (30 minutes).

2.2.1.5. Optimization of OLE Loaded Microcapsules

To have the best loading of OLE, the capsules are prepared under different producing conditions. And optimum conditions were chosen according to the result of loading capacity of OLE and proper shape of the capsules.

The amount of lyophilized extract loaded in capsules was estimated by dissolving a known amount of capsules in sodium citrate (10% w/v) during 20 min for calcium-alginate capsules and 60 min for chitosan coated beads in an Orbital Shaker at 37 °C and 125 rpm. The concentrations of lyophilized OLE loaded in the beads were determined by Folin-Ciocalteau method. A blank of sodium citrate solution was also performed.

The percentage of loading capacity was calculated with the following equation:

$$\%LC = ((A-B)/A) \times 100 \qquad (2.1)$$

Where A is the initial amount of extract dissolved in the alginate solution and B is the amount of extract determined on the solution of sodium citrate.

2.2.1.5.1. Effect of Concentration of Alginate

To understand the effect of alginate concentration on loading capacity and obtaining proper round shaped capsules, four different concentration were chosen and chosen concentrations were 1%, 2%, 3%, 4%. For this purpose, the capsules were prepared with chosen alginate concentrations. Other parameters were kept stable and the same procedure was performed. Then shape of capsules were observed and known amount of capsules were dissolved in sodium citrate solution (10% w/v) during 20 minutes for Ca-Alg capsules and 60 minutes for CS coated capsules and supernatant of the solutions were used for the determination of loading capacity.

2.2.1.5.2. Effect of CaCl$_2$ Amount

To understand the effect of CaCl$_2$ amount on loading capacity and obtaining proper round shaped capsules, five different concentration were chosen and chosen concentrations were 1M, 1.2M, 1.4M, 1.7M and 2M. For this purpose, the capsules were prepared with chosen amount of CaCl$_2$ concentration. Other parameters were kept stable and the same procedure was performed. Then shape of capsules were observed and known amount of capsules were dissolved in sodium citrate solution (10% w/v) during 20 minutes for Ca-Alg capsules and 60 minutes for CS coated capsules and supernatant of the solutions were used for the determination of loading capacity.

2.2.1.5.3. Effect of concentration and pH of Tris- HCl Buffer

To determine the effect of the buffer, it`s concentration and pH were changed one after another. Chosen concentrations were 0.3M, 0.5M, 0.7M, 1M, and chosen pH values were 8, 8.5 and 9. For this purpose, the capsules were prepared with chosen concentration and pH of buffer solution separately. Other parameters were kept stable and the same procedure was performed. Then shape of capsules were observed and known amount of

capsules were dissolved in sodium citrate solution (10% w/v) during 20 minutes for OLE loaded Ca-Alg capsules and 60 minutes for CS coated capsules and supernatant of the solutions were used for the determination of loading capacity.

2.2.1.5.4. Effect of Concentration of Chitosan

To study the effect of chitosan concentration on loading capacity, capsules were prepared with different values and CS solution was adjusted to 0.5%, 1%, 1.5% and 2%. CS coated capsules prepared with a fixed alginate concentration, $CaCl_2$ amount, buffer solution concentration and pH.

Then known amount of capsules were dissolved in sodium citrate solution (10% w/v) during 60 minutes for OLE loaded CS-Ca-Alg and supernatant of the solutions were used for the determination of loading capacity.

2.2.1.5.5. Effect of Concentration of OLE

To study the effect of concentration of OLE on capsule formation and loading capacity, OLE loaded Ca-Alg and CS-Ca-Alg microcapsules were prepared with fixed values. And lyophilized OLE with the amount of 1.5%, 2% and 3% was added and dissolved in alginate solution, and capsules were prepared with the same procedure and fixed parameters as explained before. Then shape of capsules were observed and known amount of capsules were dissolved in sodium citrate solution (10% w/v) during 20 minutes for OLE loaded Ca-Alg capsules and 60 minutes for CS coated capsules and supernatant of the solutions were used for the determination of loading capacity.

2.2.1.6. Characterization of OLE Loaded Microcapsules

2.2.1.6.1. Determination of Loading Capacity

The loading capacity of OLE loaded Ca-Alg and CS-Ca-Alg capsules were examined with Folin-Ciocalteu method by spectrometric analysis with Thermo, Varioskan Flash, U.S.A. For doing it, known amount of capsules were dissolved in

sodium citrate solution (10% w/v) during 60 minutes for OLE loaded CS-Ca-Alg and 20 minutes for OLE loaded Ca-Alg by an Orbital Shaker at 37 °C and 125 rpm and supernatant of the solution was used for the determination of loading capacity. The percentage of loading capacity was calculated with the following equation:

$$\%LC= ((A-B)/A) \times 100 \qquad (2.2)$$

Where A is the initial amount of extract dissolved in the alginate solution and B is the amount of extract determined on the solution of sodium citrate.

2.2.1.6.2. Fourier transform infrared spectroscopy (FTIR)

To study interactions of OLE and used polymers in the experiment, FTIR spectroscopy was used. For this purpose, OLE loaded CS-Ca-Alg, empty capsules were studied. And FT-IR was carried out according to the Miracle Zn-Se ATR method on a Spectrum-100 FT-IR Spectrometer (Perkin Elmer) in the range of 650-4000 cm.

2.2.1.6.3. Morphological Analysis

To have an understanding of the geometry of the OLE-loaded CS-Ca-Alg capsules, optical microscopy studies have carried out (OLYMPUS-CKX41).

In addition the external morphology of OLE loaded microcapsules was analyzed by scanning electron microscope. Quanta 250 SEM, with the ESEM mode, the moist capsule samples were been able to study. The pictures of microcapsules were taken by random scanning under same pressure.

2.2.2. Molecular Biological Studies

In order to understand whether OLE polyphenols have cytotoxic effects on different cancer cell lines and to study whether encapsulation enhance this effectiveness of OLE, molecular biological studies were carried out. In this context, cytotoxicity, cell cycle and apoptosis analysis were carried out.

2.2.2.1. Proliferation of Cancer and Healthy Cell Lines

MCF-7 breast cell line, BEAS 2B and A-549 cells line were obtained from Biotechnology and Bioengineering Research and Application Centre, IZTECH.

As cell lines were stored at -80°C, cells (2 mL) were removed from frozen storage and quickly thawed in a water bath at 37°C so as to acquire the highest percentage of viable cells. When the ice crystals melted, the content was immediately transferred into a sterile filtered tissue culture flask ($25cm^2$) containing 5-6 mL of Roswell Park Memorial Institute-1640 (RPMI-1640) growth medium containing 10% fetal bovine serum (FBS) and 1% gentamicine sulfate and incubated overnight in a humidified atmosphere containing 5% CO_2 at 37°C. After incubation, cells were passaged and the cells were refreshed twice a week.

In order to passage these cells, cells were harvested by trypsinization and washed with RPMI-1640 (10% FBS and 1% Gentamicine sulfate) medium and then cell suspension was taken from tissue culture flask ($75cm^2$ or $150cm^2$) into a sterile falcon tube (50 mL) and then centrifuged at 800 rpm for 5 minutes at room temperature. After centrifugation, the supernatant was removed from the tube and 2 mL RPMI-1640 was added to falcon tube and then pipetting was held for homogenizing the pellet. After homogenized, it was transferred (1mL) into a sterile $75cm^2$ or $150cm^2$ filtered tissue culture flask and growth medium was added 14mL for $75cm^2$ flask, 24mL for $150cm^2$ flask. Then it was incubated in humidified incubator 5% CO_2 at 37°C.

2.2.2.2. In Vitro Cytotoxicity Study

The MTT assay measures the cell metabolic activity whereby the mitochondrial dehydrogenase enzyme of viable cells reduces the yellow tetrazolium salt, 3-[4,5-dimethylthiazol-2-yl]-3,5- diphenyl tetrazolium bromide dye (MTT), to a purple formazan crystals (Nasti et al., 2009) and this reduction only occurs if mitochondrial reductase enzymes are active, thus conversion is directly related to the number of viable cells.

Therefore the MTT assay was used to assess cell viability and the absorbance of this solution can be quantified by spectrophotometer. Additionally, MTT procedure is seen in figure 2.6. with most important details.
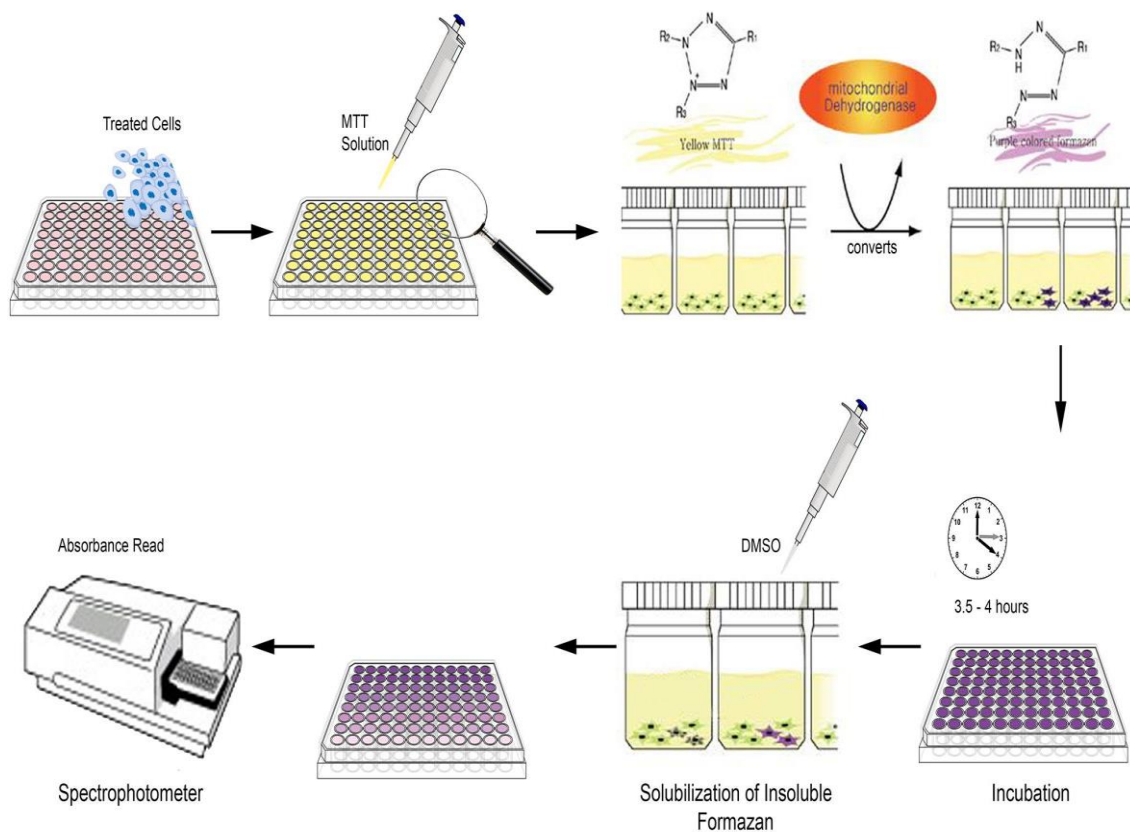
Figure 2.6. Basic illustration of MTT procedure

To asses cell viability in presence of various concentration of the OLE loaded CS-Ca-Alg and Ca-Alg microcapsules, OLE alone, and other polymers, this assay was performed. To investigate the cytotoxic effects of OLE loaded CS-Ca-Alg and Ca-Alg microcapsules, free OLE and other polymers used against A549, MCF-7 and BEAS 2B cells, and 95 μg/mL of cells were seeded in 96-well plates at a density of 1 x 104 cells/mL and incubated for 24h. After 24 h, calculated amount of OLE loaded CS-Ca-Alg and Ca-Alg microcapsules were dissolved in dimethyl sulfoxide (DMSO) and diluted at appropriate concentrations with the culture medium.

Calculated amount of OLE and other polymers only were dissolved in culture medium. Finally, 5 μL of these compounds were added into each cell with the final concentrations were 1.0, 5.0, 10.0, 50.0, 100.0, 500.0 and 1000.0 μg/mL for A549 and 200.0, 300.0, 400.0, 600.0, 800.0, 900.0 and 1000.0 μg/mL for both MCF-7 and BEAS 2B cell lines.

Untreated cells were used as a control groups and cells were incubated further 72 hours in CO_2 incubator at 37. After the incubation, the medium was removed and cells washed with phosphate-buffered saline (PBS). %10 MTT solution (5.0mg/ml in PBS) was prepared with RPMI respectively and after removing the growth medium from plates,

35

100 µL MTT solution was added to each well. After adding MTT solution, plates were incubated at 37 °C for 4 h in dark and then plates were centrifuged at 1800 rpm for 10 minutes at room temperature to avoid accidental removal of formazan crystals. After removing MTT, 100 µL DMSO was added to each well to dissolve the formazan crystals and then 96-well plates placed in a shaker for 15 min. Finally, the absorbance was determined using plate reader at a wavelength of 540 nm.

Cell viability was expressed as follows:

$$\text{Cell viability (\%)} = ((A_T/A_0))*100 \qquad (2.3)$$

Where A_0 is the control absorbance and A_T the absorbance of the treated cells. The concentration inhibiting cell viability by 50% (IC_{50}) was obtained by interpolation of the cell viability curves. Three independent assays were repeated (n=3).

2.2.2.3. Cell Cycle Analysis by Flow Cytometry

For examination to further investigate the mechanism underlying the anti-proliferative activity by analyzing the cell cycle distribution of the arrested cells using flow cytometry.

In order to determine the effects of the OLE and OLE-CS-Ca-Alg on MCF-7 and A549 cell cycle, propidium iodide (PI) staining were carried out which can supply fluorescent staining.

A549 and MCF-7 cells were cultured for the analysis and seeded onto 6-well plates in 1, 98 mL growth medium at 1×10^5 cells/well and incubated overnight for allowing the cells to attach. After incubation, 20 µL free OLE (dissolved in RPMI-1640 medium) and OLE-CS-Ca-Alg (dissolved in DMSO) were added to obtain final concentration of 50, 100, 500 and 1000 µg/mL in both A549 and MCF-7 lines. Then, these treated cells and untreated cells used as a control were continually fostered in the medium at 37°C with 5% CO_2 for 72h. After 72h, the cells were washed with PBS and harvested by trypsinization.

Then, cells were transferred to a falcon tube and then centrifuged at 1200×g for 10.0 min. After centrifugation, supernatant were removed and the cell pellet was solved in 1mL cold PBS and then 4mL PBS was added on ice. The cell suspension was

centrifuged again and the pellet resuspended in 1mL PBS. Since cells must be permeable for the measurements, they were fixed by adding 4mL -20 °C ethanol slowly during vortexing and kept on ice. Fixed cells were kept at -20 °C until the analysis day.

As the cells need to be died with PI before analysis with flow cytometer, the fixed cells were centrifuged at 1200xg for 10 min at 4 °C. After removing of supernatants, cell pellets were dissolved in 1 mL 4 °C PBS and added 4 mL 4 °C PBS and centrifuged again at the same temperature.

After centrifugation the cell pellets were dissolved in 200 µl 0,1 % Triton X-100 in PBS. 20µL RNase A (200 µg/ml) was added to cell suspension and cells were incubated in 37°C in 5% CO_2 for 30 min. After 30 minutes 20 µl PI (1mg/mL) was added and incubated at room temperature for 15 min.

The cell cycle distribution could be determined by flow cytometer (FACSCANTO, BD), and data were analyzed by ModFit software and it were collected at least 10,000 events for each sample.

2.2.2.4. Apoptosis Analysis by Flow Cytometry

The apoptotic mode of cell death can be described as a process which plays an important role in the improvement and homeostasis of multicellular organism and in the regulation and maintenance of the cell populations in tissues upon pathological and physiological conditions (Hengartner, 2000; Jacobson et al., 1997; Leist & Jaattela, 2001; Meier et al.,2000). Also it is called programmed cell death or cell suicide (Vaux, 1999).

Thus it is important to examine the mechanism underlying of the anti-proliferative activity by analysis the cell death. Since Annexin-V is a phospholipid-binding protein with a high affinity for phosphatidylserine (PS), which is normally located on the cytoplasmic face of the plasma membrane but in apoptotic cells it is changed. Thus, it can be used as a marker for apoptotic cells.

To investigate the apoptotic effect of OLE-CS-Ca-Alg on A549 and MCF-7 cells, the compound was tested by using Annexin V- FITC Detection Kit. $1x10^5$ cells/well were seeded in a 6-well plate in 1, 98 mL growth medium and incubated at 37°C in 5% CO_2 for 24h. After incubation, 20 µL OLE-CS-Ca-Alg was added to the concentration of drug ranging from 50, 100, 500 and 1000 µg/mL in both A549 and MCF-7 lines.

Then, these treated cells were continually fostered in the medium at 37 °C with 5% CO_2 for 48h. Untreated cells were used as a control groups. After incubation, the cells were taken to a falcon tube and centrifuged at 800 rpm for 5 minutes. When centrifugation finished, the pellet was dissolved in 5 mL of PBS and centrifuged again. After that, the pellet was resuspended in 200 μL of binding buffer. 2 μL of annexin V-FITC and PI were added.

The stained cells were incubated for 15 minutes at room temperature. And finally, the mixture was determined by flow cytometer (FACSCANTO, BD).

2.2.2.5. Imaging of Optical Microscopy

For visual observation of the effects of the microcapsules and the extract, optical microscopy studies were carried out. For this purpose A549 and MCF-7 cells were seeded onto 96-well plates. After waiting overnight, OLE-CS-Ca-Alg, OLE-Ca-Alg microcapsules and free OLE were added at cytotoxic concentration and incubated for 72h. And also some cells were not treated as control groups. After incubation, cells were imaged by using optical microscopy (OLYMPUS-CKX41).

CHAPTER 3

RESULTS AND DISCUSSIONS

3.1. Chemical Studies

3.1.1. Characterization of Olive Leaf Extract

3.1.1.1. Determination of Total Phenolic Compound Content

Total phenolic compound assay was carried out by Folin-Ciocalteu method due to this reagent is so reactive towards the alkaline solution of polyphenols. Total phenol content of OLE results which are determined by Folin-Ciocalteu assay, were expressed as mg gallic acid equivalent per g of extract (mg GAEq./g).

Total phenol content calculations of OLE were carried out by the gallic acid calibration curve shown in Appendix. C.1. the concentration of total phenolic compounds is 0.26 mg/mL according to the absorbance of OLE at 725nm in UV-Visible Spectrophotometer. Total phenolic compound content of OLE was calculated as 260 mg GAEq/g extract.

In a study, the total polyphenol content of olive tree leaves were determined to be 2,058 mg GAE (gallic acid equivalent) per 100 g (Makris D. et.al., 2007). Since origin of branches on the tree, storage conditions, climatic conditions and moisture content can affect the chemical composition of olive leaves, our result was acceptable.

3.1.1.2. Determination of Total Antioxidant Capacity

Total antioxidant capacity assay depends on ABTS method since antioxidants of olive leaf have scavenging activity hence it is able to inhibit the ABTS radical cations. This inhibition can be observed as a decrease in the absorbance values at 734 nm in UV-Visible Spectrophotometer. The percentage inhibition of absorbance at different concentrations were calculated to be represented in Appendix C.2. The TEAC values of

olive leaves were calculated by comparing the slope of these curves with the slope of Trolox dose-response curve given in Appendix C.2.

Antioxidant capacity was determined as 2.18 mmol of TEAC /gr olive leaf extract. Benavente-Garcia et al. (2000) reported the antioxidant activity of phenolic compounds from olive leaves; Olive-leaf extract 1.5 +/- 0.06 TEAC (mmol/L). And this proves that our result was consistent with the literature.

3.1.1.3. Analysis of Total Phenolic Compounds

Structural analysis were carried out by HPLC. When olive leaves extracted sample was injected to HPLC and the chromatogram (Figure 3.1.) was obtained. And it shows the retention time of polyphenols during the elution and demonstrates that other polyphenols are not as much as oleuropein which is one of the most effective antioxidant. The amount of oleuropein was calculated using the calibration curves (Appendix C.3.).

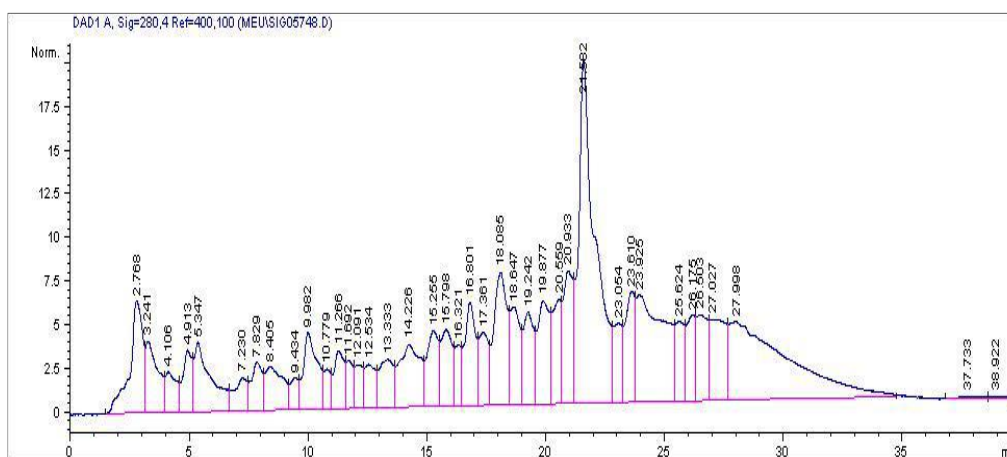Figure 3.1. HPLC chromatogram of olive leaf extract.

Figure 3.1. shows that the retention time of oleuropein was 21.592 min. Elution time of polyphenols is directly proportional to their polarity in reversed phase columns.

The area of oleuropein pick was obtained as 944.4 from the chromatogram analysis. The amount of oleuropein was 0.23 μg/mL and the percentages of oleuropein in olive leaf extract were 2.3 %.

3.1.2. Synthesizing of Microcapsules

Natural polyphenols are valuable compounds which possess scavenging activities towards radical oxygen species. These abilities make polyphenols interesting for the treatment of various diseases especially cancer. Unfortunately, it is well known that there are some direct usage limitations such as a lack in long-term stability, a low water solubility, unpleasant smell and bitter taste, to overcome these limitations, delivery systems have been developed, and encapsulation would appear to be a promising approach among them.

These hydrogels prove that they have a wide applicability as biomaterials. They have been used as delivery vehicles for drugs and bioactive compounds. However, it is well known that calcium alginate beads are very porous and have low retention capacity. Therefore, protecting these encapsulated molecules and deliver them is one of the most challenging thing. For supplying a solution to this difficulty, chitosan maybe used. There are some studies which indicates cross-linked alginate has more capacity to retain the encapsulated molecules and mixing of alginate with other polymers such as chitosan have been found to solve the problem of leaching.

Chitosan has the ability of controlling the release of encapsulated compound thanks to its hydrophilic nature and easy solubility in acidic medium and biodegradability, pH sensitiveness, mucoadhesiveness, etc. Therefore, these properties of chitosan enable them to be considered a better choice for protection and delivery of the encapsulated molecules.

3.1.2.1. Optimization of OLE Loaded Microcapsules

Once OLE was encapsulated, loading capacity of it in the capsules is one of the most important property for using them in an effective way. Thus optimization trials were made to obtain the best loading capacity and also to obtain acceptable round-shape.

3.1.2.1.1. Effect of Concentration of Alginate

To understand the effect of alginate concentration on loading capacity and obtaining proper round shaped capsules, four different concentration were chosen and chosen concentrations were 1%, 2%, 3%, 4% and other parameters are fixed ($CaCl_2$ amount 1.2 M, OLE concentration 1.5%, pH of 8.5). Results are presented in figure 3.2.
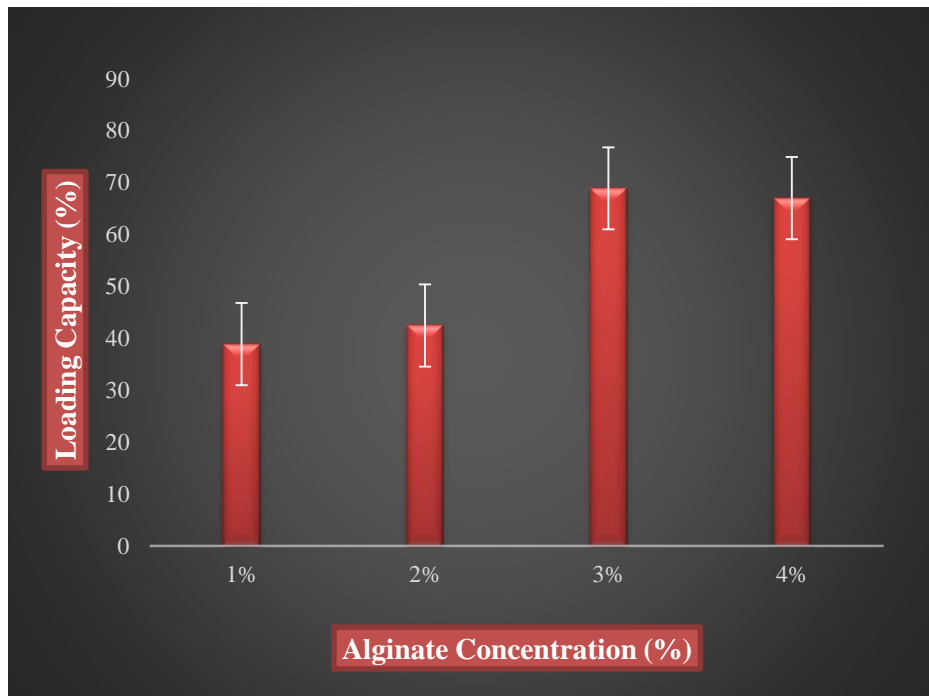
Figure 3.2. The effect of increasing Alginate concentration on loading capacity of OLE.

Figure 3.2. indicates that the addition of the 3% concentration of alginate resulted in a significant increase in the amount of OLE retained (68%), but higher alginate concentrations did not further increase protection of OLE. A relation may be made with our results and G.W. Vandenberg and his colleges' work which indicates protein retention increases significantly with alginate concentration (G.W. Vandenberg et al., 2001).
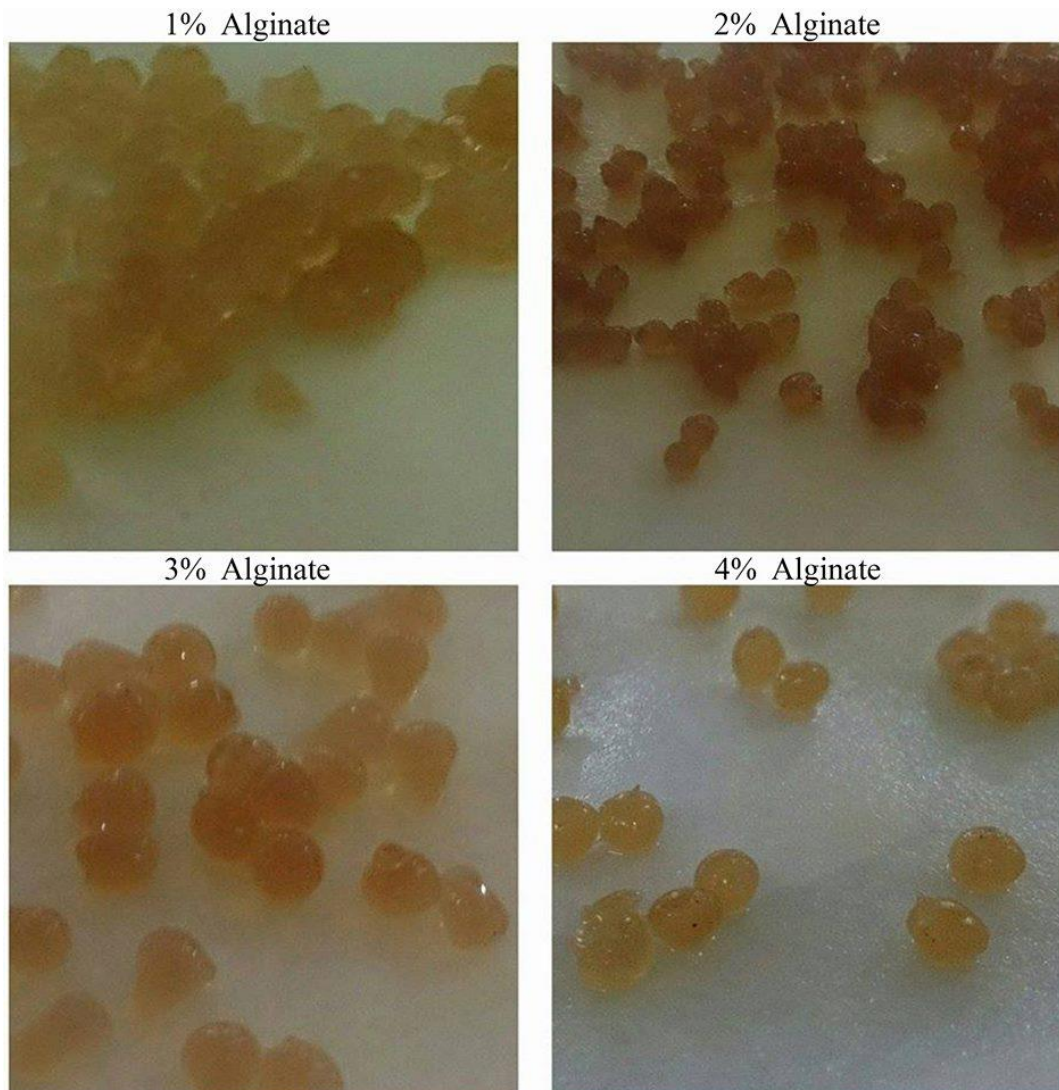
Figure 3.3. Effect of Alginate concentration on the formation of capsules.

In addition increasing alginate concentration effect the capsule formation in positive manner as seen in the figure 3.3. Our results fit with the study of Catherine K.et al., 2000, which shows a higher polymer concentration likely resulted in higher viscosity (increased hindrance and friction among polymer chains), leading to slower rearrangement of the polymer chain conformations to form the effective crosslinks. And also Martinsen et al. suggested that the effect of increasing alginate concentration amplifies the alginate gradient that is established in externally-gelled microcapsules.

The results clearly suggested that increasing concentration has an impact on the formation of capsules and loading capacity of OLE in increasing manner. However the concentration above 3% has no effect, thus this concentration (3%) of alginate has chosen as an optimum value.

3.1.2.1.2. Effect of CaCl$_2$ Amount

To understand the effect of CaCl$_2$ amount on loading capacity and obtaining proper round shaped capsules, five different concentration were chosen and chosen concentrations were 1M, 1.2M, 1.4M, 1.7M and 2M. And the other parameters are fixed as alginate 3%, OLE concentration 1.5%, pH of 8.5. Results of loading capacity and physical impacts are presented in figure 3.4. and figure 3.6., respectively.
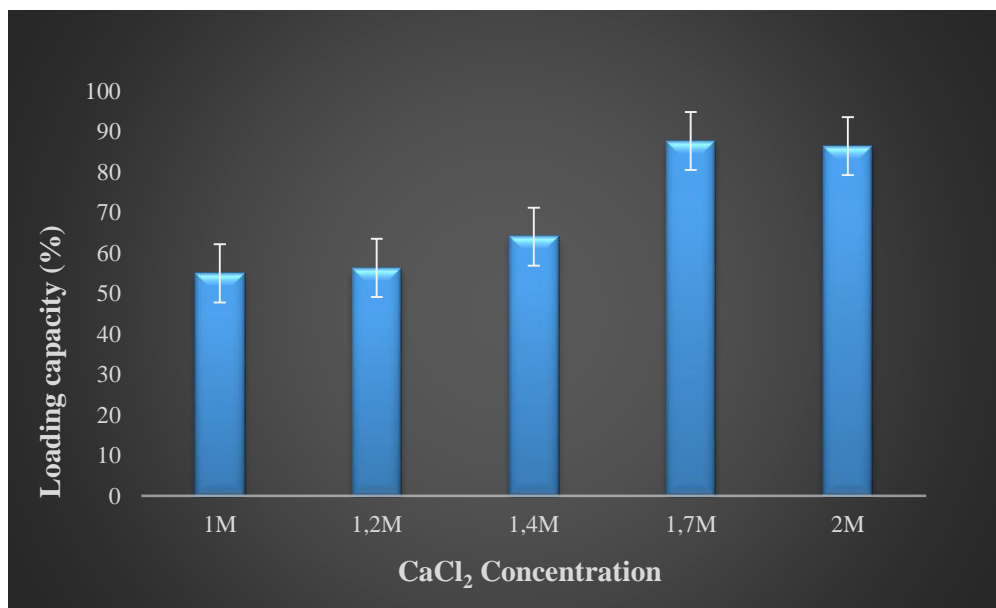
Figure 3.4. The effect of increasing CaCl$_2$ concentration on loading capacity of OLE in Ca-Alg Capsules.

It was assumed that an increased calcium content enhanced molecular interactions of an alginate gel, shortened the average distance between ionic crosslinks, and resulted in greater shrinkage. Thus increasing CaCl$_2$ concentration increases the loading capacity. And as the figure indicates, CaCl$_2$ concentration effects the loading capacity in increasing manner, however further increase was not observed.

Additionally, further studies also showed that CaCl$_2$ concentration effects the loading capacity of OLE in CS coated capsules and results are shown in figure 3.5.
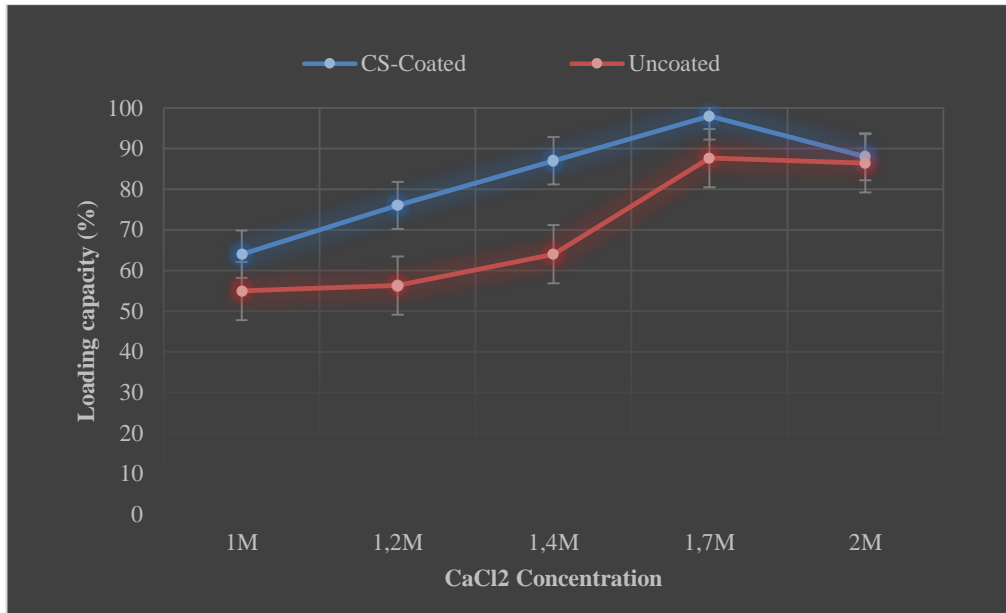
Figure 3.5. The effect of increasing $CaCl_2$ concentration on loading capacity of OLE in Ca-Alg Capsules and CS coated capsules.

These results shows there is an increase in loading capacity of OLE with increasing $CaCl_2$ concentration. Therefore, it can be assumed that the presence of $CaCl_2$ leads more CS coating over the microcapsules. Catherine K. et al., 2001, also showed that, at higher calcium contents the overall contribution of the effective ionic interactions became greater and leading to better mechanical properties of the alginate gels. The study of Olav Gasser et al., 1998, supports this idea as they indicated that Chitosan binds faster and to a higher extent with increasing concentrations of calcium chloride. Thus, it seems that the calcium ions increase the binding of chitosan.

In addition, effects of $CaCl_2$ concentration on the formation of capsules are shown in figure 3.6.
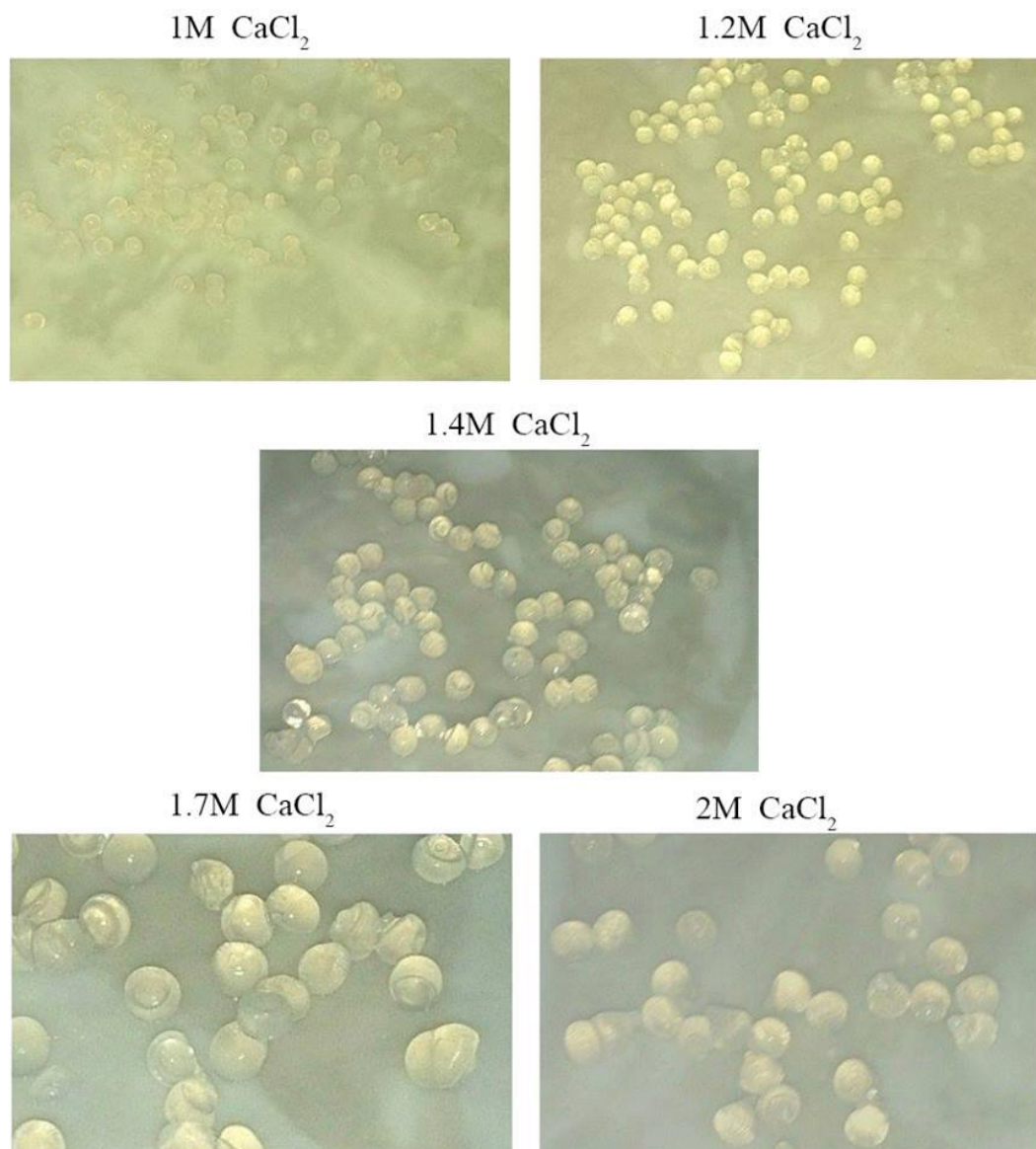
Figure 3.6. The effect of increasing $CaCl_2$ concentration on capsule formation.

It is clearly seen that $CaCl_2$ concentration does not affect formation of the capsules so much, well-shaped capsules were produced with all concentration chosen. Thus, optimal values are chosen according to loading capacity of OLE.

Consequently, the results clearly suggested that increasing concentration has no significant impact on the formation of capsules but on the loading capacity of OLE, it was great in increasing manner. However the concentration above 1.7M has no effect, thus this concentration of $CaCl_2$ has chosen as an optimum value.

3.1.2.1.3. Effect of concentration and pH of Tris- HCl Buffer

To determine the effect of the buffer, it`s concentration and pH values were changed separately. Chosen concentrations were 0.3M, 0.5M, 0.7M, 1M, and chosen pH values were 8, 8.5 and 9. For this purpose, the capsules were prepared with chosen concentration and pH of buffer solution separately. Other parameters (3% alginate, 1.7 M $CaCl_2$) were kept stable and the same procedure was performed. And results of Tris-HCl buffer concentrations are showed in figure 3.7.
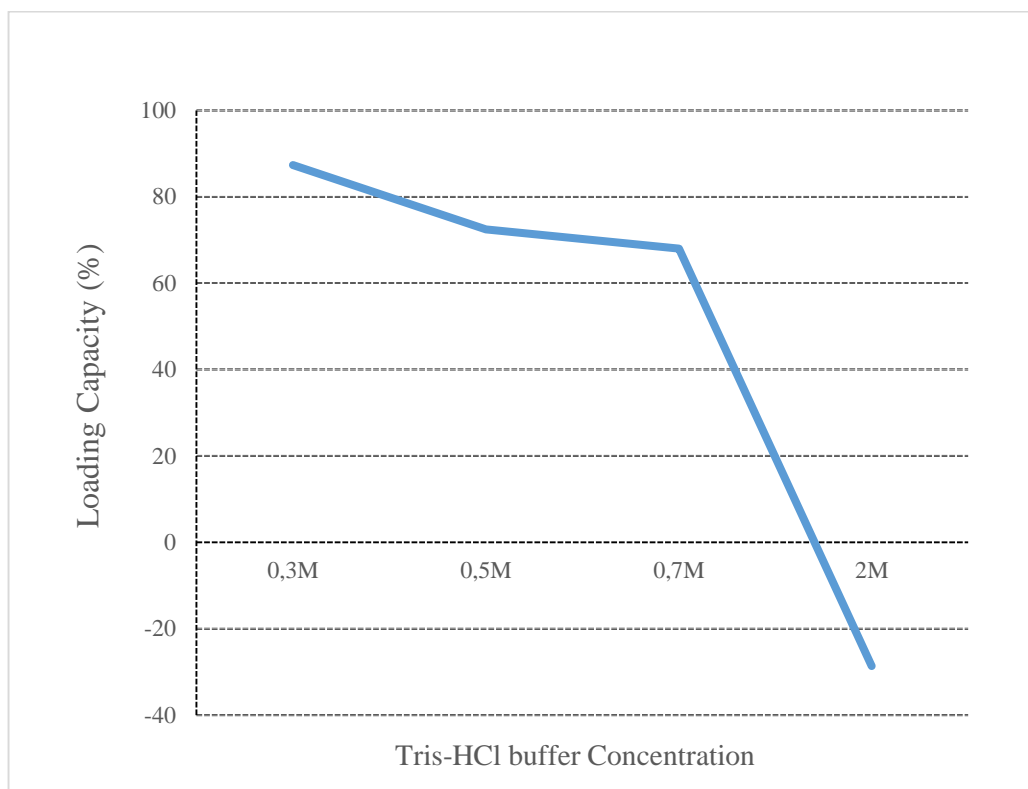
Figure 3.7. The effect of increasing Tris-HCl buffer concentration on loading capacity of OLE.

According to the results, there is a decrease in loading capacity of OLE from 0.3M up to 2M. And the most effective value of the buffer is 0.3M.
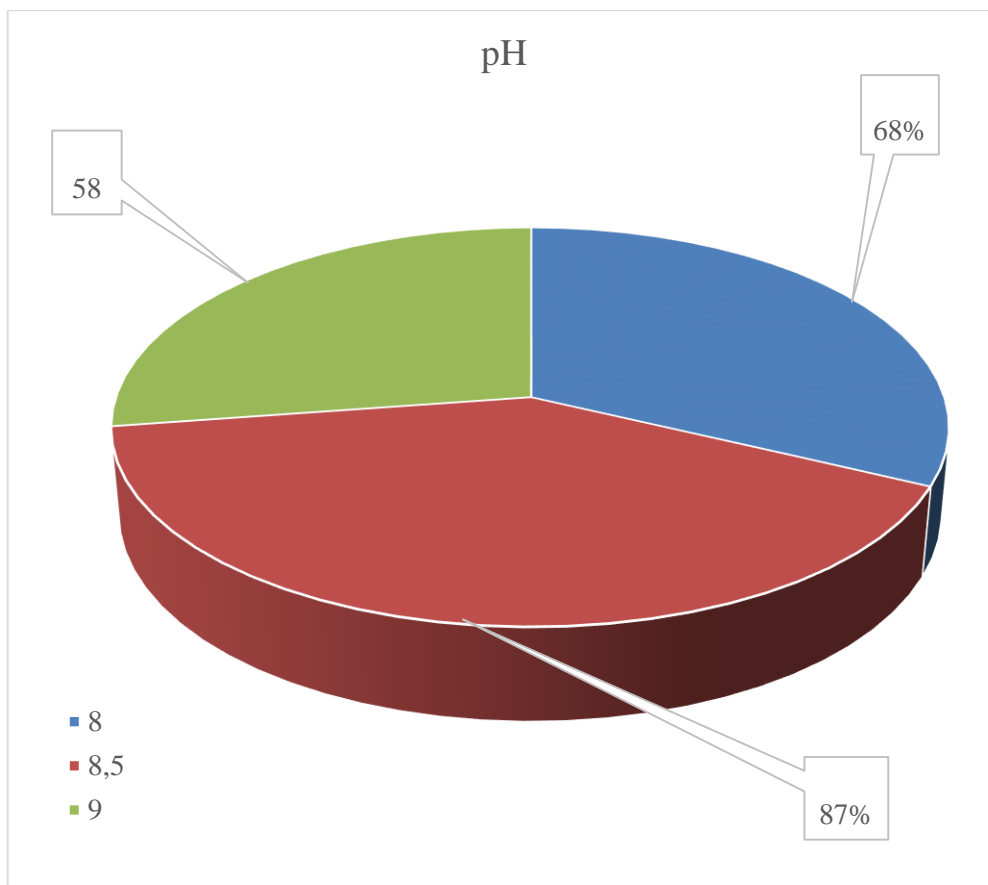
Figure 3.8. The effect of pH of Tris-HCl buffer on loading capacity of OLE.

Additionally trials with these pH values shows (Figure 3.8.) that when it is 8.5, loading capacity was the most highest and hence it could be because under basic conditions negatively charged O^- of alginate attack to metoxy groups of OLE. Moreover, a change is made in the aqueous phase (pH) can induce the formation of microcapsules and it could be proven with the figure 3.9.
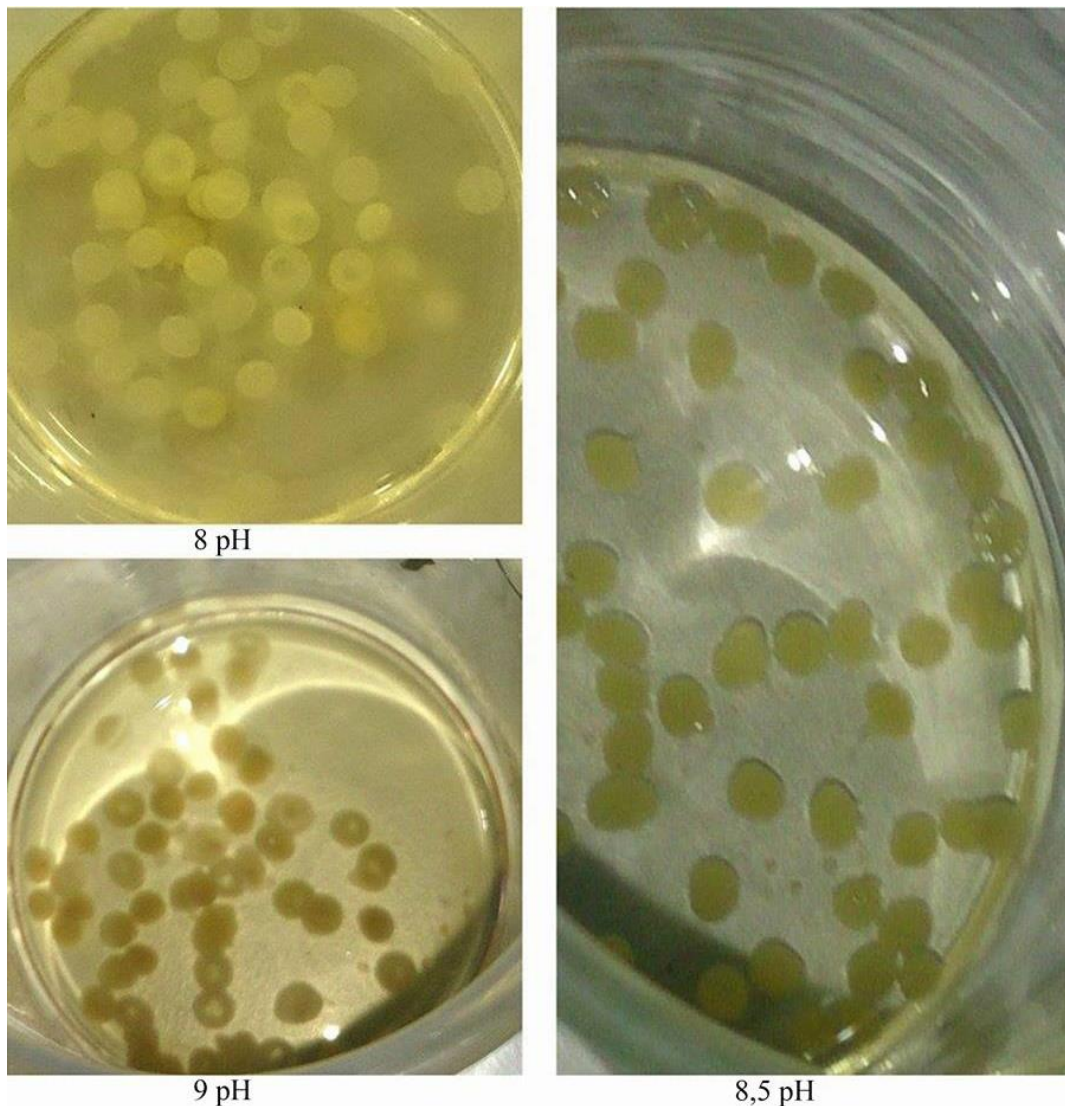
Figure 3.9. The effect of pH of Tris-HCl buffer on capsule formation.

As it clearly seen in the figure 3.9., the capsules formed at 8 and 9 pH values, were like red blood cell and improper but at the 8.5 pH value, capsules were completely round-shaped. Therefore, chosen optimal value of pH was 8.5 when both loading capacity and capsule formation results were taken into consideration.

3.1.2.1.4. Effect of Concentration of Chitosan

To study the effect of CS concentration on loading capacity, capsules were prepared with different values. And CS solution was adjusted to 0.5%, 1%, 1.5% and 2%. And CS coated capsules prepared with a fixed alginate concentration, $CaCl_2$ amount, buffer solution concentration and pH. And results are showed in figure 3.10.
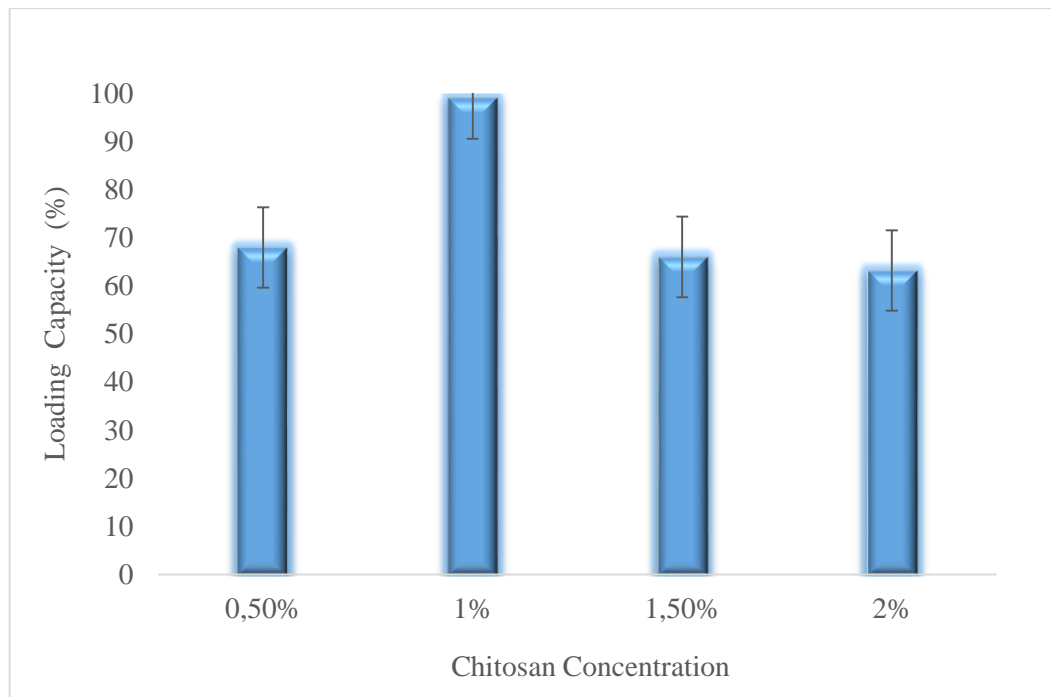
Figure 3.10. The effect of increasing CS concentration on loading capacity of OLE.

In our study chitosan was used as a coating material to reduce microcapsule swelling, improve loading capacity of OLE. And these results show that chitosan do its job, as it has impact on OLE loading into capsules since the enhanced OLE loading percentage in the presence of chitosan could be attributed to the formation of a strong complex membrane that would stabilize and strengthen the ion gel network and limit the loss of OLE by diffusion during microcapsule formation.

Moreover, increasing CS concentration effect the loading capacity of OLE in increasing manner but there is not a further increase above 1% of CS for OLE loading into capsules. And the optimal concentration of chitosan for microencapsulation of OLE was found to be 1%. These results fits with the study of Xiao-Yu Li et al., 2009, and they revealed that the addition of the lowest concentration of chitosan (0.05%; w/v) resulted in a significant increase in the amount of IgY activity retained (67.1%), but higher chitosan concentrations did not further increase protection.

3.1.2.1.5. Effect of Concentration of OLE

To study the effect of concentration of OLE on capsule formation and loading capacity, OLE loaded Ca-Alg and CS-Ca-Alg microcapsules were prepared with a fixed values. And chosen OLE concentrations to assess are 1.5%, 2% and 3%. Results of loading capacity are showed in figure 3.11.
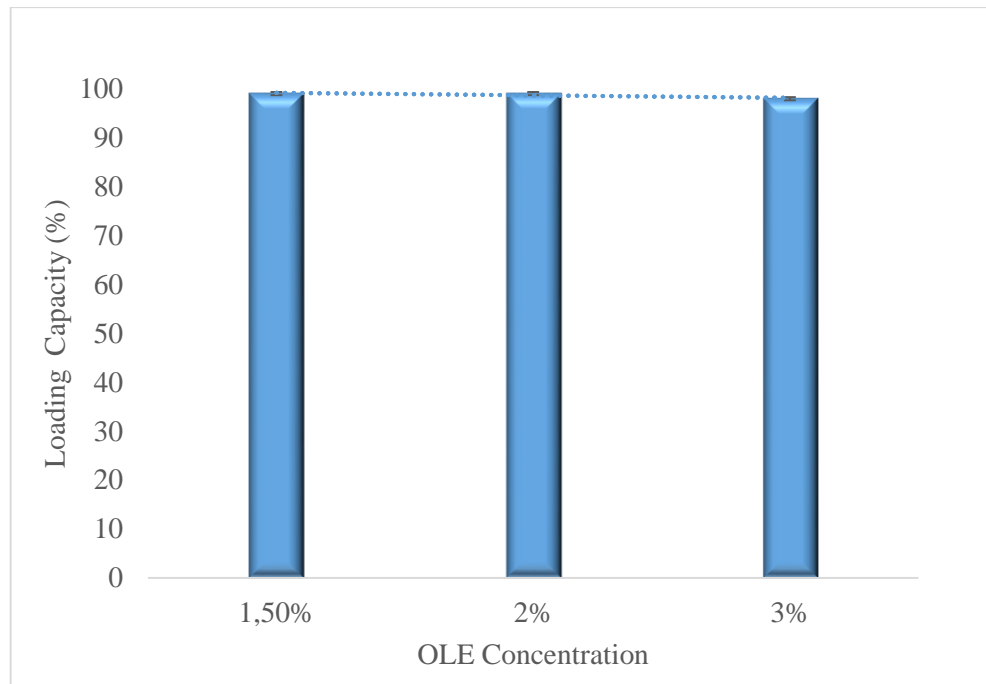
Figure 3.11. The effect of increasing OLE concentration on loading capacity.

The capacity of OLE into alginate solution is only increased up to 3%. Below this concentration, there was no significant changing in loading capacity of OLE into capsules as seen in figure 3.11. It can be concluded from this result that there is no inverse proportion between the increasing concentrations of OLE and loading capacity of OLE. However, increasing concentration of OLE misleads the formation of capsules. Because of this reason 1.5% is chosen as optimal value according to these results. (Figure 3.12).
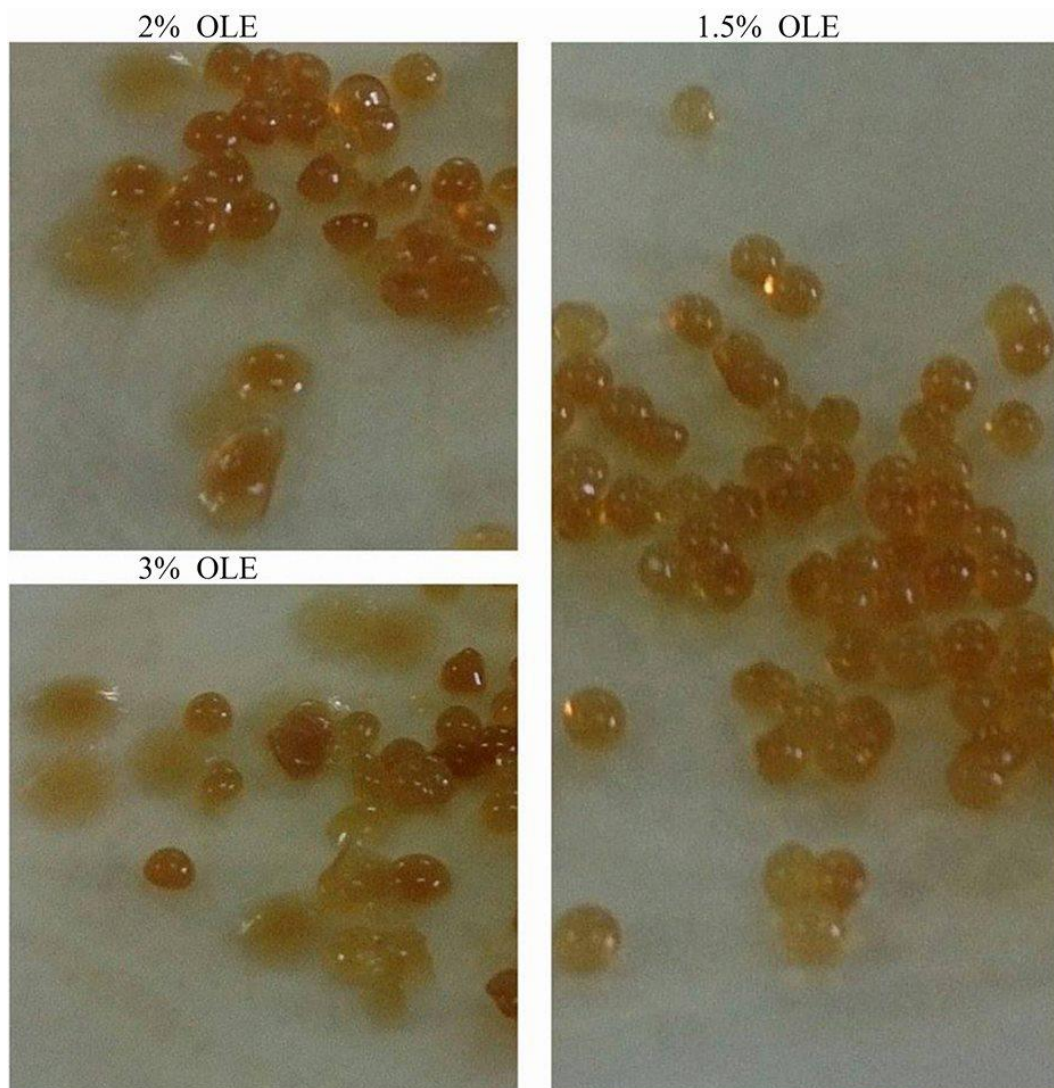
Figure 3.12. The effect of increasing OLE concentration on capsule formation.

3.1.2.2. Characterization of OLE Loaded Microcapsules

3.1.2.2.1. Determination of Loading Capacity

Chitosan was applied after the formation of capsules to increase the OLE loading in the capsules, since chitosan has been shown to possess mucoadhesive properties due to molecular attractive forces formed by electrostatic interaction between positively charged chitosan and negatively charged alginate.

The loading capacity of OLE loaded Ca-Alg and CS-Ca-Alg capsules were examined with Folin-Ciocalteu method by spectrometric analysis For doing it, known amount of capsules were dissolved in sodium citrate solution (10% w/v) during 60

minutes for OLE loaded CS-Ca-Alg and 20 minutes for OLE loaded Ca-Alg microcapsules by an Orbital Shaker at 37 °C and 125 rpm and supernatant of the solution was used for the determination of loading capacity. The percentage of loading capacity was calculated with the following equation:

$$\%LC= ((A-B)/A) \times 100 \tag{3.1}$$

Where A is the initial amount of extract dissolved in the alginate solution and B is the amount of extract determined on the solution of sodium citrate.

After calculations, results represented in figure 3.13. and it demonstrates the effect of chitosan presences on the percentage of OLE loading.
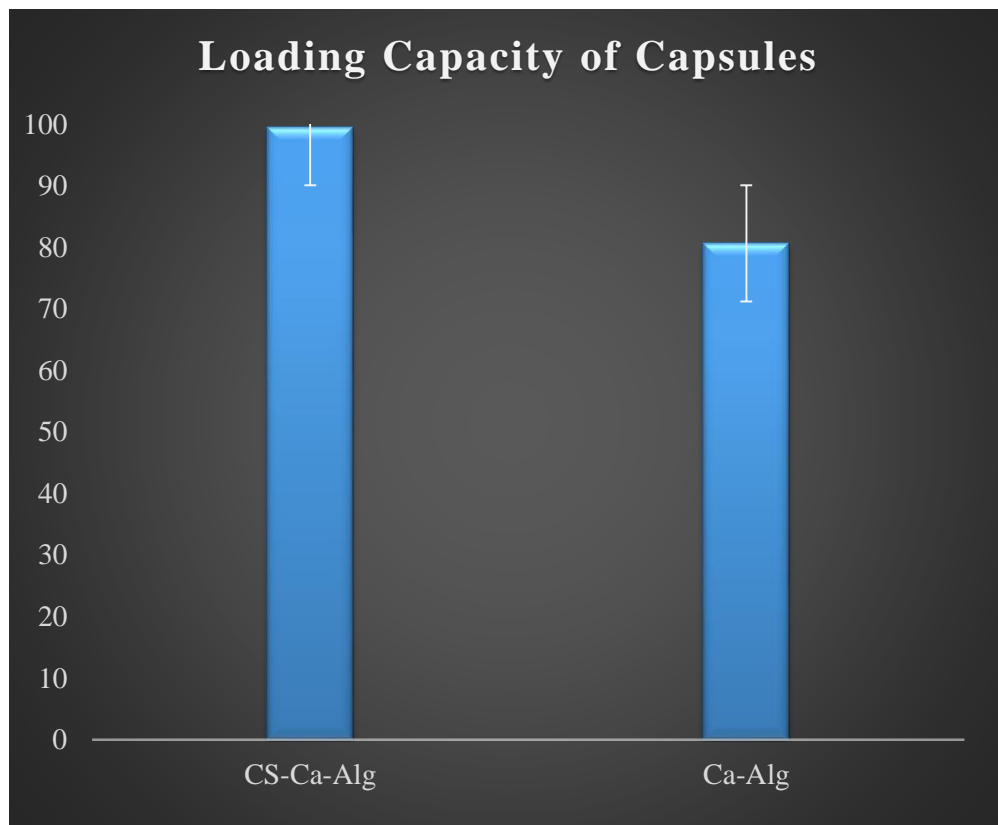
Figure 3.13. The effect of presence of Chitosan on loading capacity.

According to these results, it could be estimated the presence of CS is effective on loading capacity of OLE in the capsules. In addition, chitosan increased the percentage of OLE loading from 80% without chitosan, to an average of 99% with chitosan.

Therefore, when it is compared the CS-Ca-Alg microcapsules appear to be a more effective vehicle for the oral delivery of OLE than Ca- alginate microcapsules.

3.1.2.2.2. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectroscopy was used to study OLE – polymer interaction. For this purpose OLE loaded and empty capsules were studied. The FTIR spectra of empty and OLE loaded CS-Ca-Alg microcapsules are shown in Fig. 3.14.
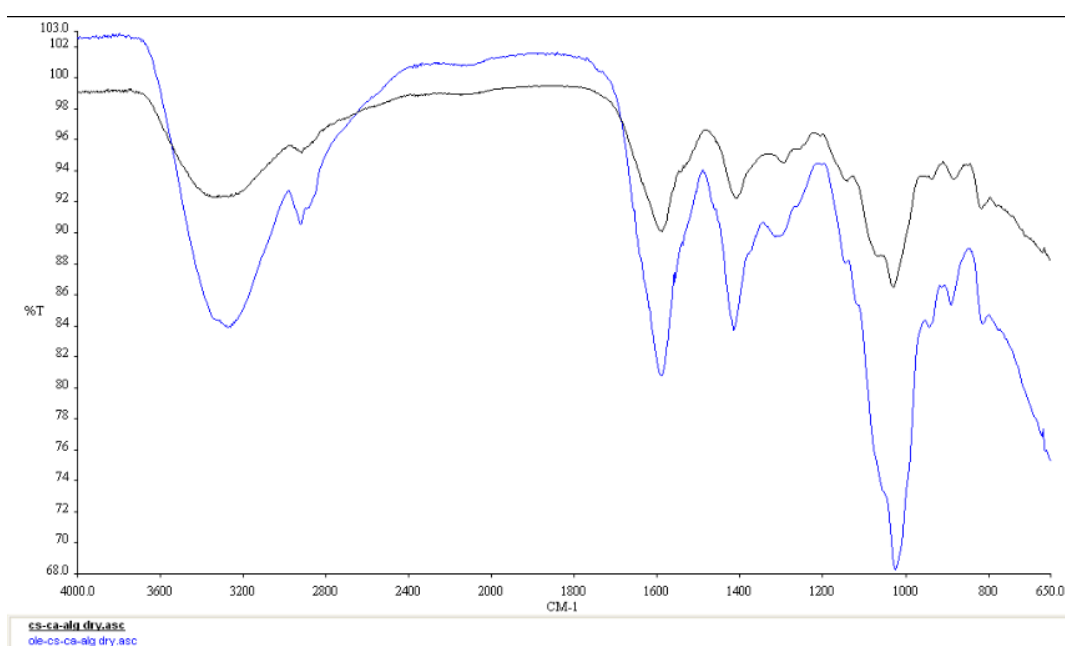
Figure 3.14. The FTIR spectra of empty and OLE loaded chitosan-calcium alginate microcapsules are shown in.

The peaks at 2800-3200 cm^{-1}, 1400- 1200 cm^{-1} and 1000- 800 cm^{-1} are sharper for OLE-loaded chitosan calcium alginate microcapsules when compared to the same peaks for the empty chitosan calcium alginate capsules.

Moreover, the peaks ranging between 2800– 3000 cm^{-1} can be ascribed to the stretching of the –NH$_2$ group with strong overlapping hydroxyl peak between 3000– 3600 cm^{-1}. In addition OLE-loaded CS-Ca-Alg microcapsules show a small peak between 2800-3200 cm^{-1} and 1650 – 1700 cm^{-1} which was not observed in the empty capsules.

These minimal interactions between chitosan and OLE support that the encapsulation of polyphenolic compounds such as OLE in chitosan-alginate based systems.

54

3.1.2.2.3. Morphological Analysis

To study external morphology of capsules Optical microscopy studies carried out for both empty and OLE-loaded CS-Ca-Alg capsules and these observations were showed in figure 3.15.
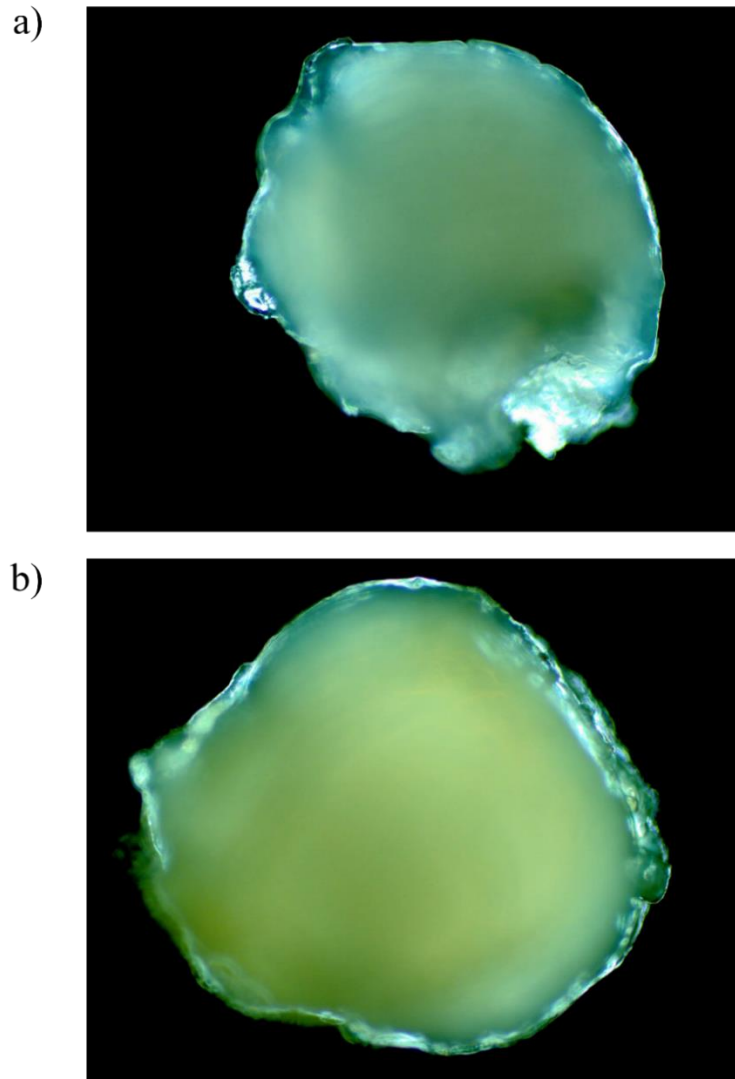
Figure 3.15. Optical microscopy images of capsules a) CS-Ca-Alg b) OLE- CS-Ca-Alg.

As it can be seen these two different capsules are round-shaped. But there are some aggregations on both empty and OLE-loaded capsules. The same observation was recorded by Shantha L. Kosaraju et al., 2006, as they indicated, optical microscopy of both the placebo and OLE-loaded particles revealed the spherical geometry. There were a few aggregates formed in both the placebo and OLE loaded microsphere preparations.

To provide a better understanding of morphological characteristics of both CS-Ca-Alg and OLE loaded CS-Ca-Alg Environmental Scanning Electron Microscopy studies provided.

In this respect, the morphological characteristics of OLE loaded and empty microcapsules were examined by scanning electron microscope. Quanta 250 SEM, with the ESEM mode the moist capsule samples were been able to study.

Microcapsules loaded with OLE, revealed a smooth surface with regard to the blank microcapsules as it is illustrated in figure 3.16. and figure 3.17. at different magnification.
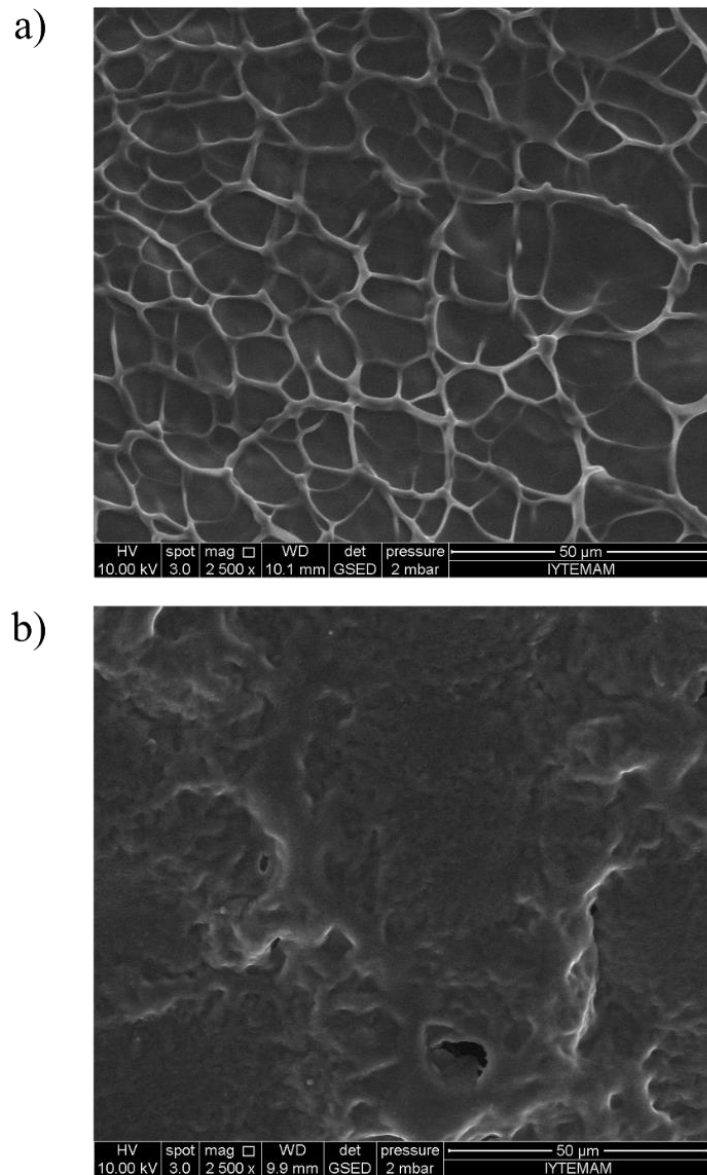
Figure 3.16. ESEM image of capsules a) CS-Ca-Alg b) OLE-CS-Ca-Alg at 50µm magnification.
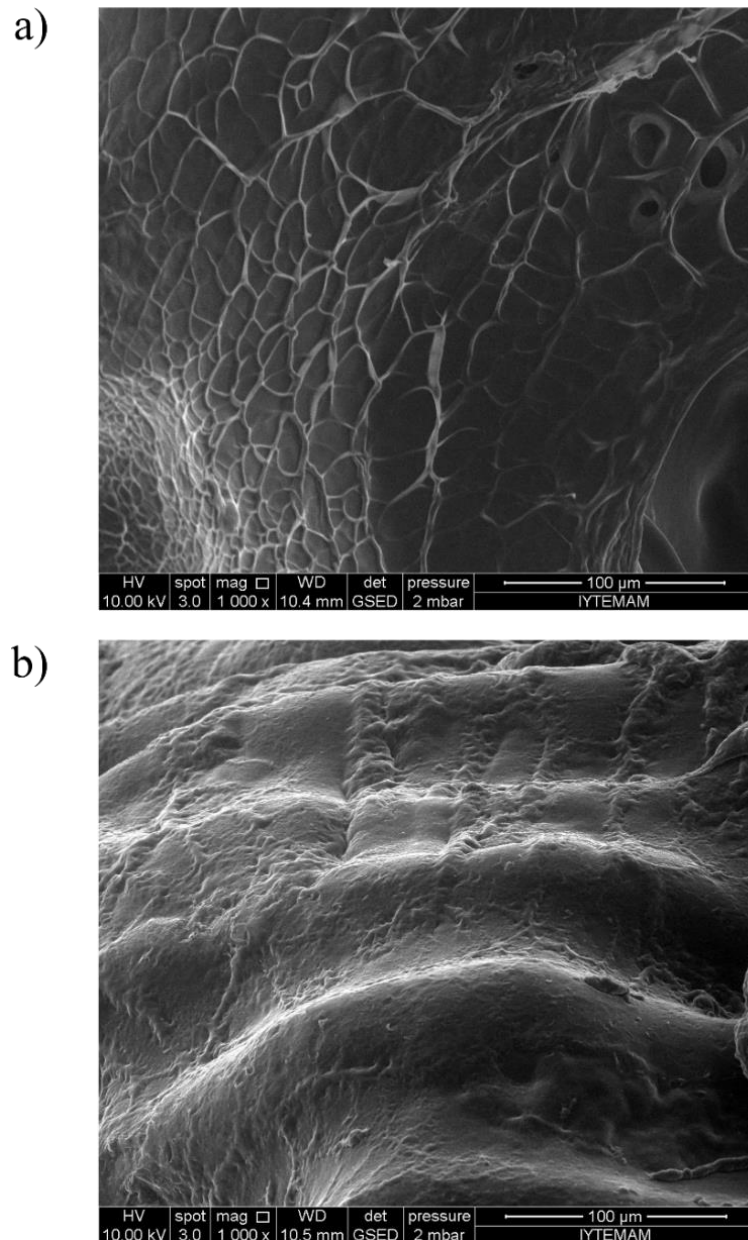
Figure 3.17. ESEM image of capsules a) CS-Ca-Alg b) OLE-CS-Ca-Alg 100µm.
magnification

Empty microcapsules have rough surface in spider web shape and holes on it. These differences may be attributed to influence of structural interactions between polyphenols which is present in this extract and the matrix polymers. The same observation was recorded by Shantha L. Kosaraju et al., 2006, when olive leaf extract in chitosan microspheres were prepared. The SEM of spray-dried placebo chitosan microspheres appeared to have a different morphology when compared to the OLE entrapped chitosan microspheres. The placebo microspheres appeared to have many

wrinkles on the surface and there were gaps between the wrinkles. The OLE-loaded microspheres had a smooth surface.

3.1.2.3. Molecular Biological Studies

In order to understand whether OLE polyphenols have cytotoxic effects on different cancer cell lines and to study whether encapsulation enhance this effectiveness of OLE, molecular biological studies were carried out. In this context, cytotoxicity, cell cycle and apoptosis analysis were carried out. And their results are given regularly.

3.1.2.3.1. In Vitro Cytotoxicity Study

To examine whether the compounds were cytotoxic on cell lines, MTT assay was carried out. Since this assay is a colorimetric assay is to determine cell growth and the viability in response to external factors. Additionally it mechanism goes as, cellular oxidoreductase enzyme may reflect the number of viable cells and by this enzyme, a yellow tetrozolium dye (MTT), is reduced to insoluble purple formazan crystals, then solubilization solution DMSO is added to dissolve formazan into colored solution and this colored solution can be quantified by spectrophotometry.

3.1.2.3.1.1. Cytotoxicity Study on A549 Cell Lines

The cytotoxicity of various concentrations of the OLE-CS-Ca-Alg, OLE-Ca-Alg, CS-Ca-Alg, OLE, Alginate and CS were measured using the MTT assay and results are represented as Log[C]-Cell Viability graph.

In order to understand whether OLE, Alginate and CS are effective when they are used alone, the MTT results of them are compared and represented in figure 3.18. with different concentrations such as 1.0, 5.0, 10.0, 50.0, 100.0, 500.0 and 1000.0 µg/mL.
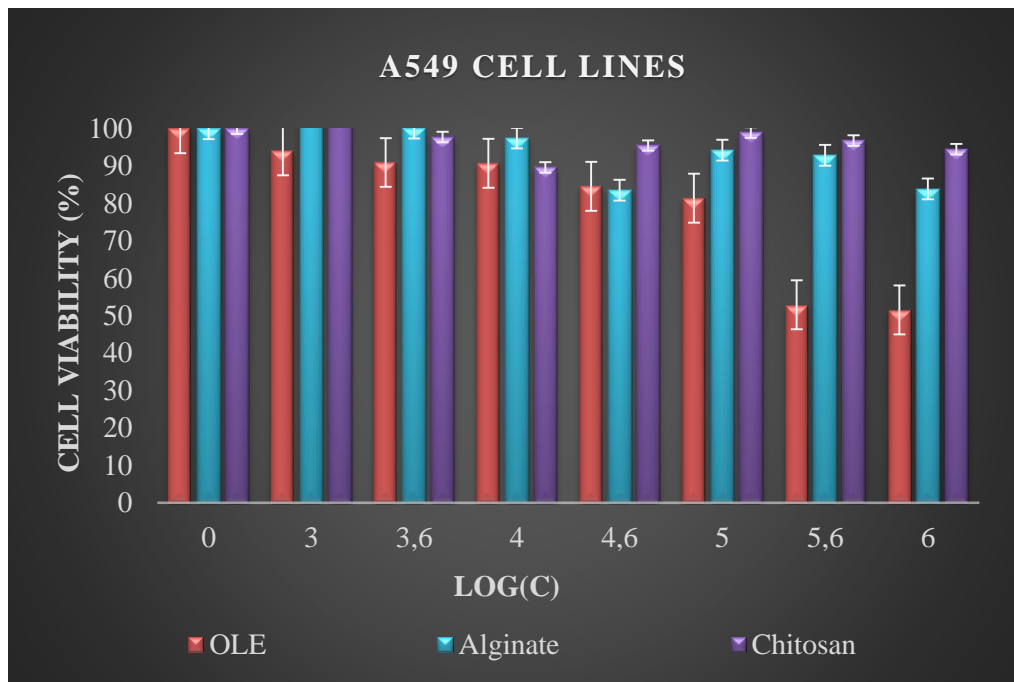
Figure 3.18. The cytotoxic effect of OLE, Alginate and CS on A549 cells.

According to these results OLE is effective at higher concentrations, however it loses its effectiveness when concentration of OLE goes down. In addition alginate and CS has no toxic effect even at highest concentration as expected. These results about cytotoxicity of CS and Alginate were predictable as all researches about these polymers indicate they are completely biocompatible since they are both derived from natural sources. Therefore, it can be concluded that these natural polymers could be effective for protecting OLE`s effectiveness.

With this respect, the cytotoxic effect of the empty capsules that were produced with these polymers are measured and results are represented in figure 3.19. with these concentration 1.0, 5.0, 10.0, 50.0, 100.0, 500.0 and 1000.0 µg/mL.
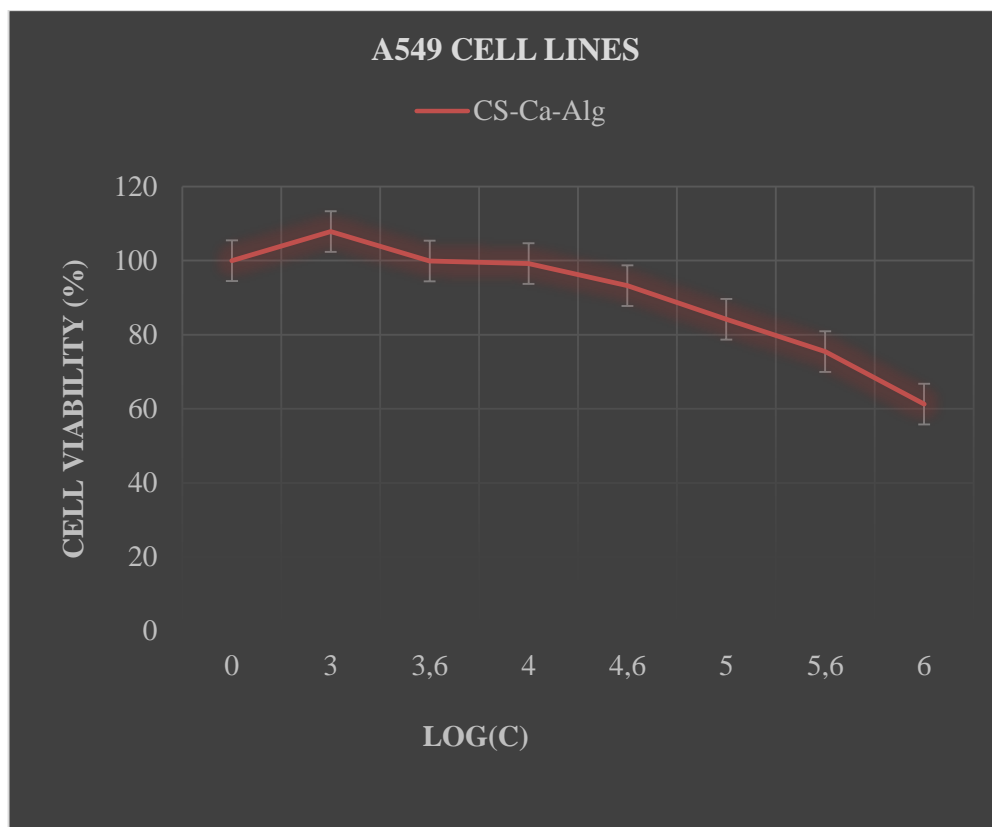
Figure 3.19. The cytotoxic effect of empty capsules.

As seen in figure 3.19. it can be clearly understood that empty capsules are not effective in A549 cell proliferation. With these results it can be assumed that these capsules may be used as an effective carrier for OLE.

With this purpose the cytotoxic effect of OLE, OLE loaded CS-Ca-Alg and Ca-Alg is compared and to determine the cytotoxic effect of them on A549 cells, IC_{50} value of compound was calculated from the [LOG(C) ng/mL]-Cell Viability graph as seen in Figure 3.20. Calculated IC_{50} value for OLE is 8, 96 ng/mL, for OLE-Ca-Alg is 6, 01 ng/mL, for OLE-CS-Ca-Alg is 4, 06 ng/mL for A549 cells.
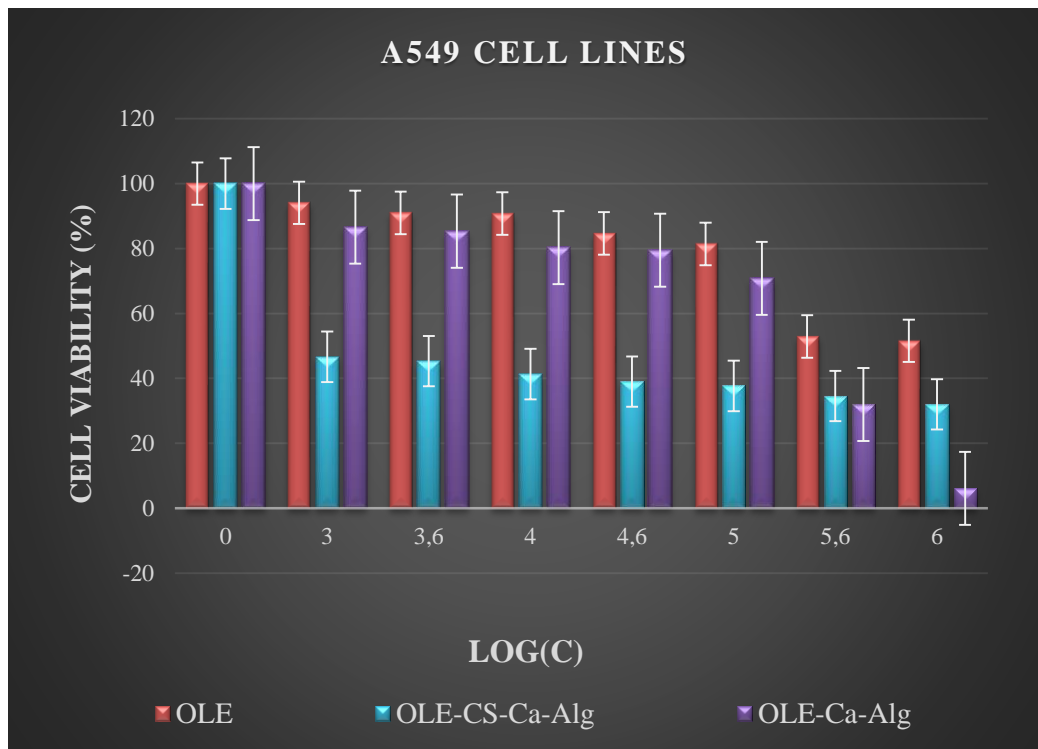
Figure 3.20. The cytotoxic effect of OLE and OLE loaded CS-Ca-Alg and Ca-Alg
capsules

Additionally, figure 3.20. indicates Ca-Alginate microcapsules in other word
uncoated capsules are more cytotoxic at higher concentrations. However there is not any
correlation between the concentration and the cytotoxicity. Thus, it is assumed that
uncoated capsules are not effective in controlled manner as it is expected because of its
porous nature which is shown before with the ESEM observations in chapter 3.1.2.2.3.

Due to these results it can be said CS coated microcapsules could be more
effective carrier for OLE, since there was a significant gradual cytotoxicity on A549 cell
lines proportional to concentrations. Moreover, with these results and some studies about
CS, it can be also assumed that CS coating may protect OLE from the gastrointestinal
tract. The study of Xiao-Yu Li and his friends, 2009, support this idea, as they claimed
that nonencapsulated egg yolk immunoglobulin (IgY) was rapidly hydrolyzed, and
antibody activity was almost completely lost during 2 hours of incubation. The stability
of IgY was significantly improved by encapsulation in alginate microcapsules (retaining
43.5% activity) and was further improved by including chitosan at any of the
concentrations assessed (retaining an average of 69.4% activity).

Therefore, chitosan–alginate microcapsules appear to be a more effective vehicle
for the oral delivery of OLE compared with alginate microcapsules. Because of this

reason the cytotoxicity of OLE loaded CS-Ca-Alg and free OLE are compared and represented in figure 3.21.
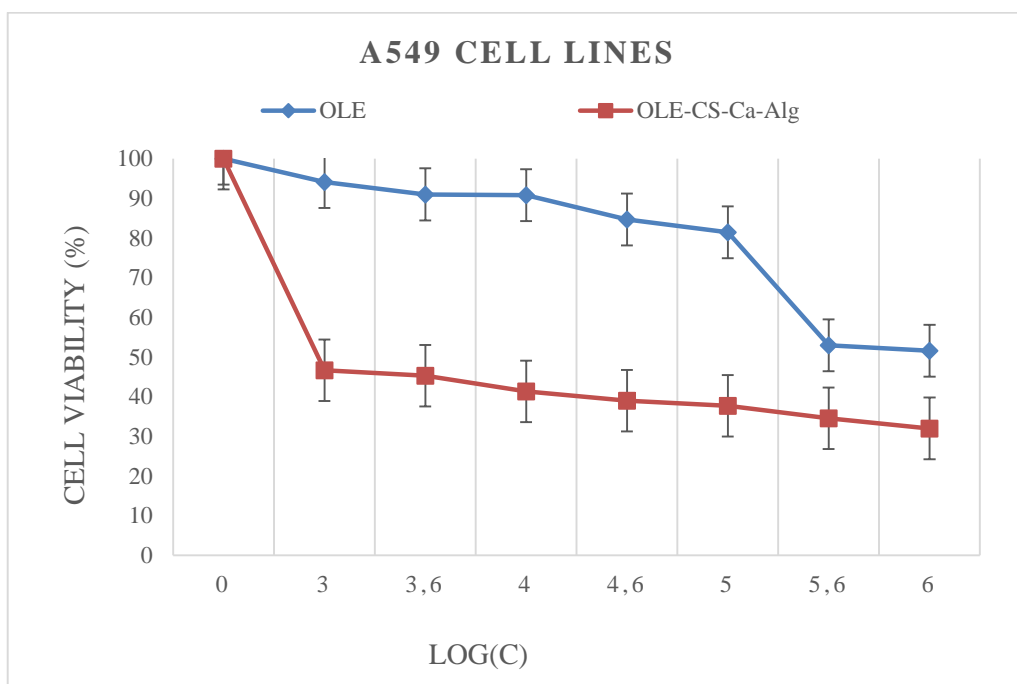
Figure 3.21. The cytotoxic effect of OLE and OLE loaded CS-Ca-Alg capsules.

To determine the cytotoxic effect of OLE-CS-Ca-Alg and OLE on A549 cells, IC_{50} value of compound was calculated from the Log [ng/mL]-Cell Viability graph as seen in Fig.3.21. IC_{50} value of OLE-CS-Ca-Alg was found as 4.06 ng/mL and IC_{50} of OLE was 8, 96 ng/mL for A549 cells.

With this comparison it is easy to say, OLE is much more effective when it is sealed in chitosan coated calcium alginate microcapsules. Thus, encapsulation enhances the bioavailability of OLE. With these results it can be definitely concluded that these capsules can supply a great protection for OLE.

Additionally, there were not any analytical researches encountered about cytotoxic effects of OLE loaded CS-Ca-Alg microcapsules, also there is no analytical epidemiological study that has evaluated the association between the components of the Mediterranean diet and lung cancer (Fortes, C. et al., 2003). Thus, our study can open a new window to enlighten the dark side of unknown relations.

3.1.2.3.1.2. Cytotoxicity Study on MCF-7 Cell Lines

The cytotoxicity of various concentrations (200.0, 300.0, 400.0, 600.0, 800.0, 900.0 and 1000.0 µg/mL) of the OLE-CS-Ca-Alg, OLE-Ca-Alg, CS-Ca-Alg, OLE, Alginate and CS were measured for breast cancer cells also, and results are represented.
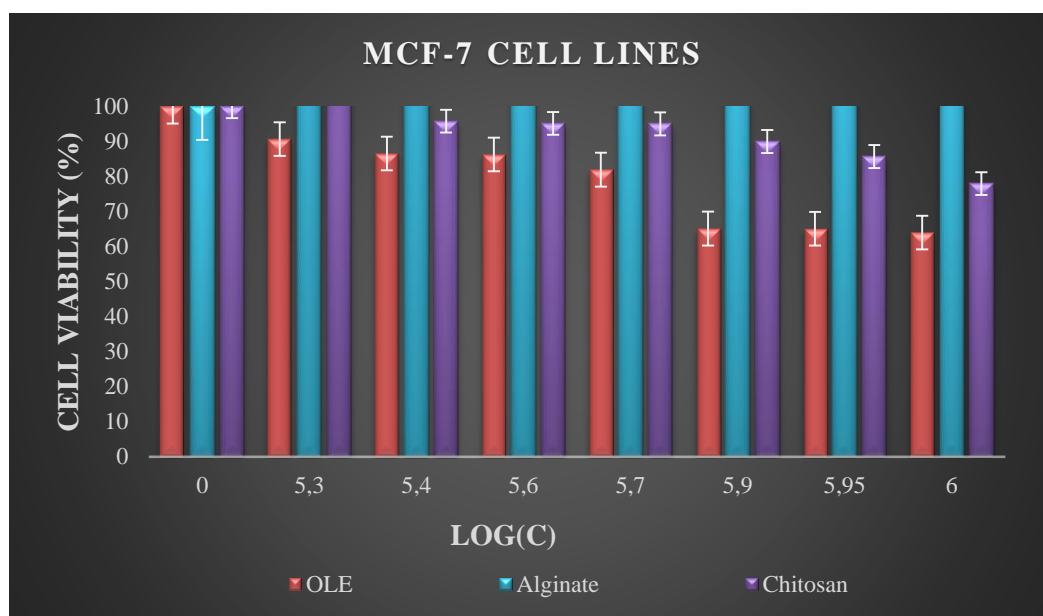
Figure 3.22. The cytotoxic effect of OLE, Alginate and CS on MCF-7 cells.

According to figure 3.22., neither alginate nor CS have cytotoxic effect on MCF-7 cells. These results are acceptable as there are numerous research about CS and alginate reveal that these polymers are biocompatible and can be used for several purposes like wound dressing, dental implanting, and encapsulating material (Ravi Kumar et al., 2004).

Moreover, when olive leaf extract applied freely, there was not a significant reduction in MCF-7 cell proliferation, except at highest concentration. Therefore it can be said there is a need for protection to enhance the activity of OLE. In addition to our results, in a study hydroxytyrosol rich olive leaf extract was applied and dose dependent manner inhibition (38.4%) in the MTT was observed. However in this study OLE was enriched and concentrations were high, 2000, 2220, 2400, 2800 and 30000 µg/ml (Zouhaier Bouallagui et al., 2010).

In another study, Junkyu Han and his friends, 2009, also worked about the cytotoxic effects of OLE on MCF-7 cell lines. Their results indicate cancer cell proliferation was inhibited to 60% and the other treatment concentrations did not show

cytotoxic effect. Thus, with our results and the data from other studies may prove that encapsulation is a promising approach to enhance and protect the effectiveness of OLE.

For this purpose, cytotoxic effects of empty capsules that were produced with the polymers were examined in order to understand whether they could be used for encapsulation of OLE. Results of MTT trials are represented in figure 3.23. for these concentrations 200.0, 300.0, 400.0, 600.0, 800.0, 900.0 and 1000.0 μg/mL.
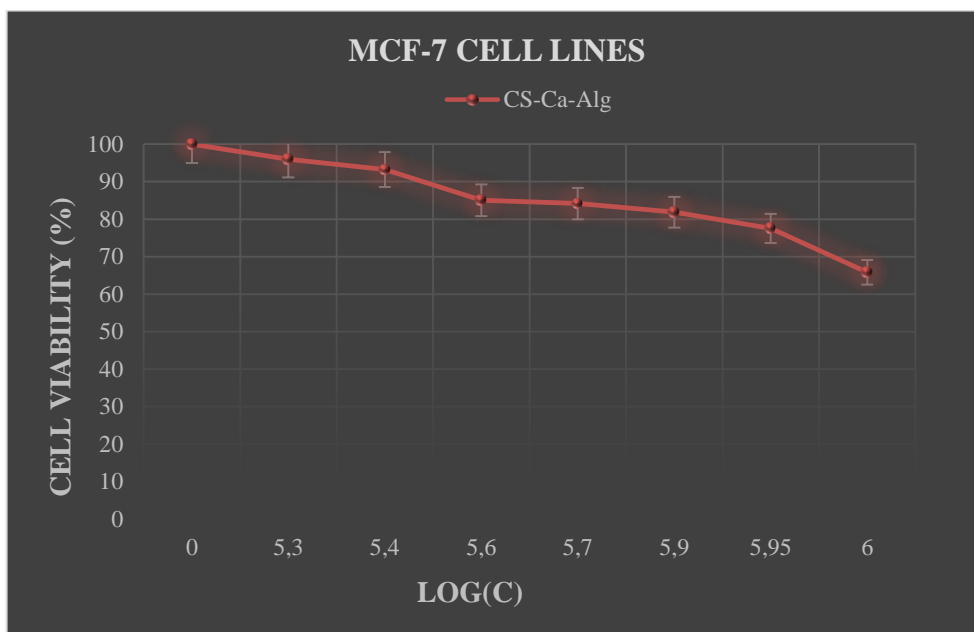
Figure 3.23.The cytotoxic effect of CS-Ca-Alg capsules on MCF-7 cells.

To determine the cytotoxic effect of CS-Ca-Alg capsules on MCF-7 cells, results were represented in [LOG(C) ng/mL]-Cell Viability graph as seen in Fig. 3.23. Thus, this result may show that CS-Ca-Alg capsules do not inhibit MCF-7 cell proliferation and these capsules can be used for protection of OLE and may enhance its effectiveness.

With this purpose comparison of cytotoxic effects of OLE, OLE-loaded capsules were made and represented in figure. 3.24.
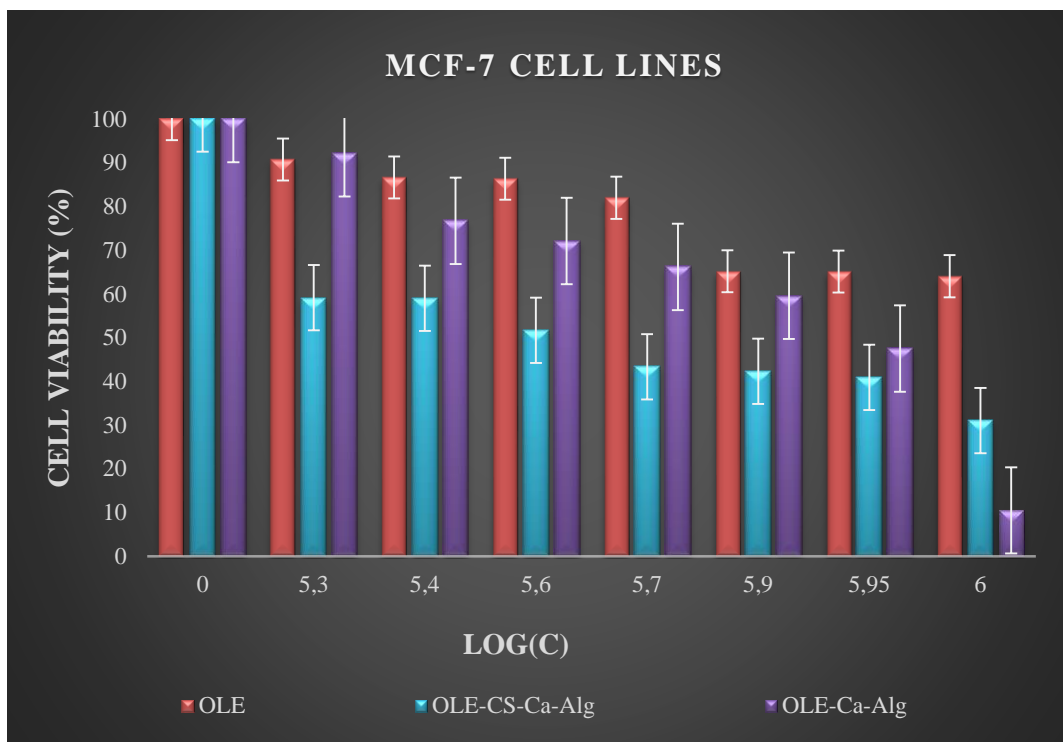
Figure 3.24. The cytotoxic effect of OLE, OLE-CS-Ca-Alg and OLE-Ca-Alg capsules on MCF-7 Cell Lines.

Log [c]-Cell Viability graph indicates that all of the compounds are effective in concentration dependent manner on MCF-7 cell lines. However, the results show that OLE is much more effective when it is encapsulated. Additionally, to protect and enhance OLE effectiveness, results indicates CS coated capsules are more effective then uncoated calcium alginate capsules. And it can be explained with the same reasons as mentioned in 3.1.2.3.1.1. chapter since these results correlates with the MTT results of A549 cell lines.

Consequently, both OLE loaded CS-Ca-Alg and Ca-Alg capsules are effective on MCF-7 cells. Yet, most importantly CS-Ca-Alg capsules are more suitable carriers for OLE. As Cs coated capsules are assumed to be more effective for protection of OLE, the effectiveness of OLE and OLE loaded CS-Ca-Alg capsules are compared and results are represented in figure 3.25.
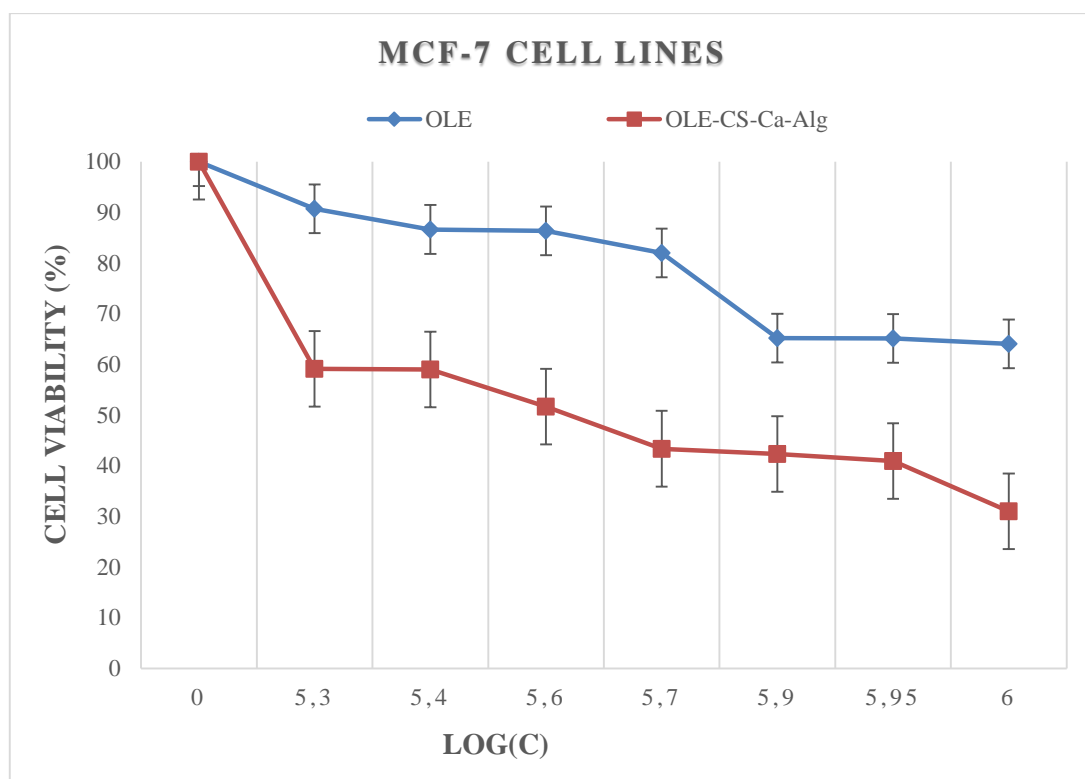
Figure 3.25. The cytotoxic effect of OLE and OLE-CS-Ca-Alg capsules on MCF-7 Cell
.   Lines

As figure 3.25. illustrates OLE-CS-Ca-Alg capsules are much more effective than
OLE and IC_{50} value of compounds also prove this data since it was found as 10,12 ng/mL
for OLE and 4,95 ng/mL for OLE-CS-Ca-Alg capsules. Thus OLE is much more
effective when it is sealed in CS-Alg capsules.

Although there are some studies about polyphenols encapsulation, encapsulation
of OLE is a new approach to try. And our results can show that OLE is much more
effective when it is sealed in chitosan coated calcium alginate microcapsules on MCF-7
cells. Thus, encapsulation enhances the bioavailability of OLE. With these results of ours
and in the light of the data that gained from literature, it can be definitely concluded that
these capsules can be used as an effective carrier for OLE.

3.1.2.3.1.3. Cytotoxicity Study on BEAS 2B Cell Lines

The cytotoxicity of various concentrations of the OLE, OLE loaded capsules were
measured for these concentrations 200.0, 300.0, 400.0, 600.0, 800.0, 900.0 and 1000.0
μg/mL and results are represented in figure 3.26., 3.27.and 3.28. separately.

Figure 3.26. The cytotoxic effect of OLE on BEAS 2B Cell Lines.

According to Log [OLE]-Cell Viability graph as seen in Fig. 3.26, it is easy to say OLE does not have cytotoxic effect on BEAS 2B cells even at the highest doses. Moreover, the figure illustrates that OLE has a positive effect on the cell proliferation for BEAS 2B lines.
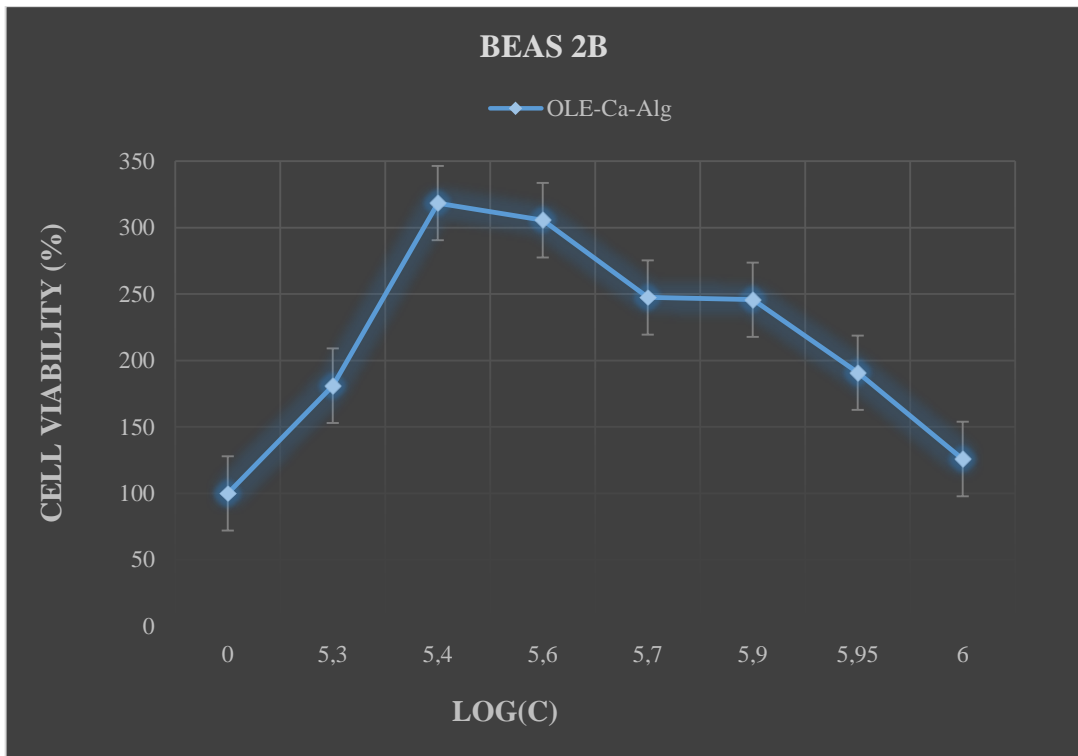
Figure 3.27. The cytotoxic effect of OLE loaded Ca-Alg capsules on BEAS 2B Cell Lines.
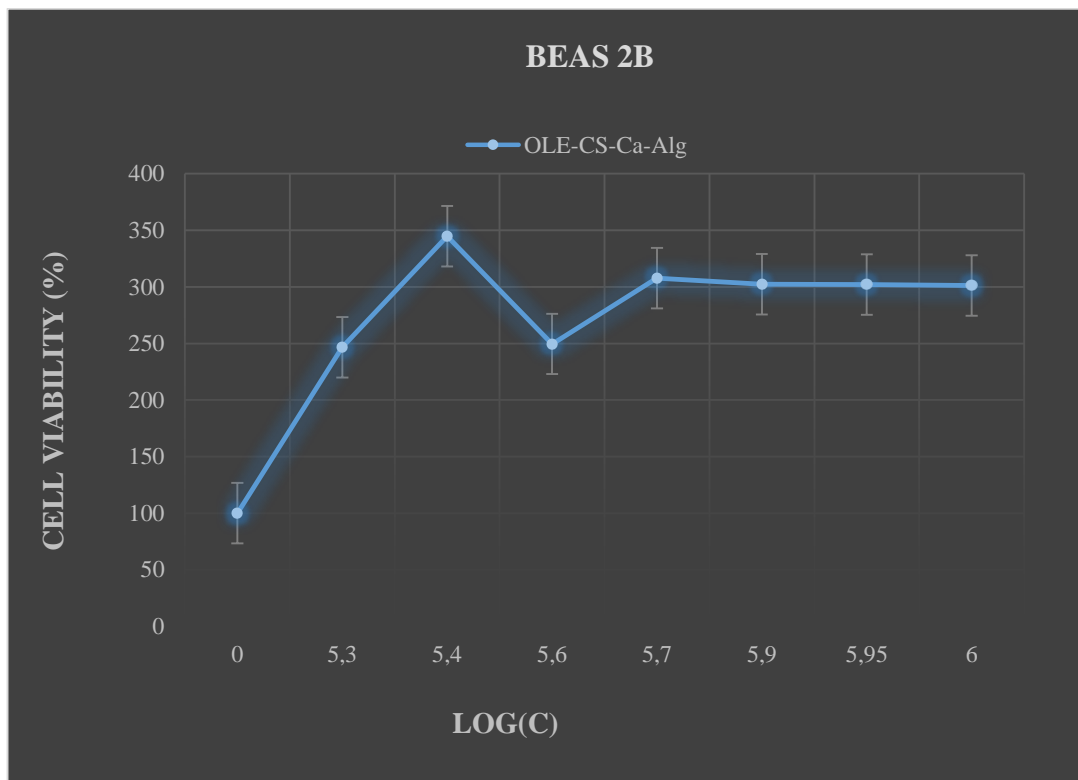
Figure 3.28. The cytotoxic effect of OLE loaded CS-Ca-Alg capsules on BEAS 2B Cell. Lines

As the figure 3.27. and figure 3.28. illustrate neither OLE-Ca-Alg nor OLE-CS-Ca-Alg have cytotoxic effect on BEAS 2B cells even they have positive effect on the cell proliferation for BEAS 2B lines. To see the whole picture, all results are represented in figure 3.29.
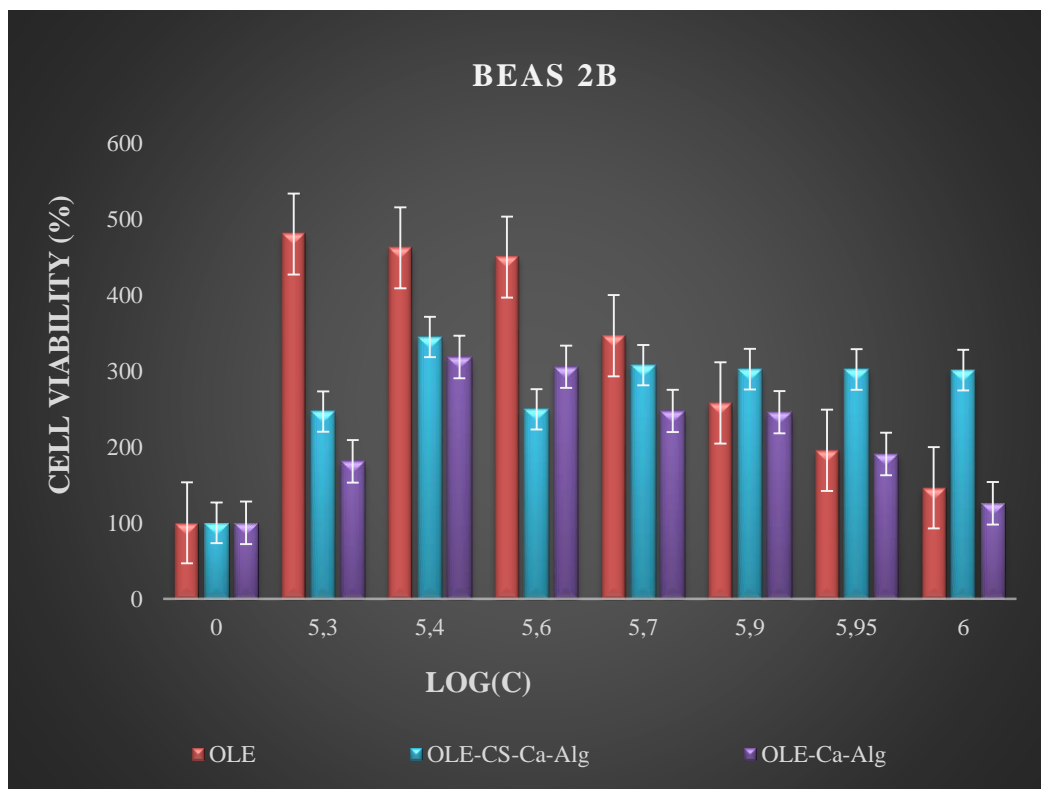
Figure 3.29. The cytotoxic effect of OLE, OLE loaded CS-Ca-Alg and capsules on BEAS 2B Cell Lines.

This comparison is not only show the compounds do not have cytotoxic effects but also reveal that they have positive effects on BEAS 2B cells proliferation. These results were expected since OLE have a great antioxidant activity which can eliminate free radical stress in cells. Therefore, it can be assumed that the compounds may have nutrition supplementary effects. Thus, our study can enhance the drug industry as it is a new approach for anticancer drugs and biocompatible material for biomedical applications.

3.1.2.3.2. Cell Cycle Analysis by Flow Cytometry

For examination to further investigate the mechanism underlying the anti-proliferative activity of the compounds, cell cycle analysis was carried out by analyzing the cell cycle distribution of the arrested cells using flow cytometry.

With this purpose, to examine in which step of cell cycle is effected by the compounds, both A549 and MCF-7 cells were treated with free OLE and OLE-CS-Ca-Alg capsules for different concentrations (50, 100, 500, 1000 μM) and untreated cells were analyzed as control group, this assay based on propidium iodide (PI) staining as explained in Chapter 2.

3.1.2.3.2.1. Cell Cycle Analysis on A549 Cell Lines

To investigate the effect of olive leaves extract and OLE loaded CS-Ca-Alg on cell cycle distribution in A549 cells. The cells were treated with OLE and OLE-CS-Ca-Alg capsules for different concentrations (50, 100, 500, 1000 μM) and untreated cells were performed as control group by using flow cytometry. Results (Table 3.1.) represent the average (%) of free OLE and OLE-CS-Ca-Alg capsules determinations.

Table 3.1. Cell phase composition (%) of OLE-CS-Ca-Alg and OLE effected cells for different concentrations.

Cell Cycle Distribution (%)			
OLE-CS-Ca-Alg (µg/mL)	G0/G1	S	G1/G2
Control	60,52	30,17	9,31
50	64,45	27,69	6,91
100	65,7	25,78	8,52
500	66,97	25,92	7,11
1000	67,99	24,37	7,64

Cell Cycle Distribution (%)			
OLE (µg/mL)	G0/G1	S	G1/G2
Control	60,52	30,17	9,31
50	55,88	38,6	5,52
100	59,39	31,89	8,72
500	62,84	29,01	8,15
1000	61,6	29,71	8,69

These results reveal that the cells treated with OLE-CS-Ca-Alg capsules show much more significant block of G1 to S phase indicated by the increase of cell number in G0/G1 phase. However free OLE induced less cell cycle arrest, as table indicates there was no significant difference between control and treated cells even at highest concentration. For this reason, their effectiveness is illustrated separately.

Effects of OLE-CS-Ca-Alg on cell cycle arrest in A549 cells are seen in figure 3.30. When all these results were compared with cell phase composition (%) of control cells, it was seen that there was an increase in G0/G1 phase from 60, 52 % to 67.99% with the increasing concentration of OLE-CS-Ca-Alg. In addition to the increasing of G0/G1 phase composition, the composition of S and G2/M phase was decreased as expected. Thus, OLE-CS-Ca-Alg capsules induce G1 cell cycle arrest since accumulation

of G1 arrested cells with the decrease of the proportion of cells in the S and G2/M phases are revealed.

Figure 3.30. Effects of OLE-CS-Ca-Alg on cell cycle arrest in A549 cells.

Effects of OLE on cell phase composition (%) against A549 cells are seen in figure 3.31. When the results were compared with cell phase composition (%) of control cells, it was seen that there were nearly no differences on cell phase composition (%) of OLE effected cells. The results were found nearly the same with control cells. It can be concluded from this result that free OLE is not effective on cell cycle disruption in A549 cells as expected.

Figure 3.31. Effects of free OLE on cell cycle arrest in A549 cells.

According to these results OLE-CS-Ca-Alg induce G1 cell cycle arrest since an increase at G0/G1 cell phase with the decrease of the proportion of cell in the S and G2/M phases were revealed and also encapsulated OLE is much more effective on perturbing cell cycle than free OLE. Thus, these results confirmed the cytotoxicity trials.

3.1.2.3.2.2. Cell Cycle Analysis on MCF-7 Cell Lines

To investigate the effects of free OLE and OLE-CS-Ca-Alg capsules on cell cycle distribution of the arrested MCF-7 cells, the cells were treated with OLE and OLE-CS-Ca-Alg capsules for different concentrations (50, 100, 500, 1000 µM) and untreated cells were performed as control group by using flow cytometry. The results are listed in Table 3.2.

Table 3.2. Cell phase composition (%) of OLE-CS-Ca-Alg and OLE effected cells for different concentrations.

Cell Cycle Distrubition (%)			
OLE-CS-Ca-Alg (µg/mL)	**G0/G1**	**S**	**G1/G2**
Control	55,33	33,88	10,79
50	58,53	27,34	14,13
100	62	25,81	12,19
500	61,22	24,01	14,77
1000	63,47	22,8	13,73

Cell Cycle Distrubition (%)			
OLE (µg/mL)	**G0/G1**	**S**	**G1/G2**
Control	53,33	33,88	10,79
50	53,22	34,2	12,58
100	53,6	33,86	12,53
500	54,34	34,55	11,1
1000	53,04	33,95	13,01

These results reveal that the cells treated with OLE-CS-Alg capsules show much more significant block of G1 to S phase indicated by the increase of cell number in G0/G1 phase. However free OLE induced less cell cycle arrest. For this reason, their effectiveness are illustrated separately.

Effects of OLE-CS-Ca-Alg on cell cycle arrest in MCF-7 cells are seen in Fig.3.32. When the results were compared with cell phase composition (%) of control cells, it was seen that in addition to increase in cell number at G2/M phase (from 10, 79 to 13, 73), there was a significant increase at G0/G1 phase from 55.33 % to 63.47% with the increasing concentration of OLE-CS-Ca-Alg. Thus, OLE-CS-Ca-Alg capsules induce G1 cell cycle arrest.
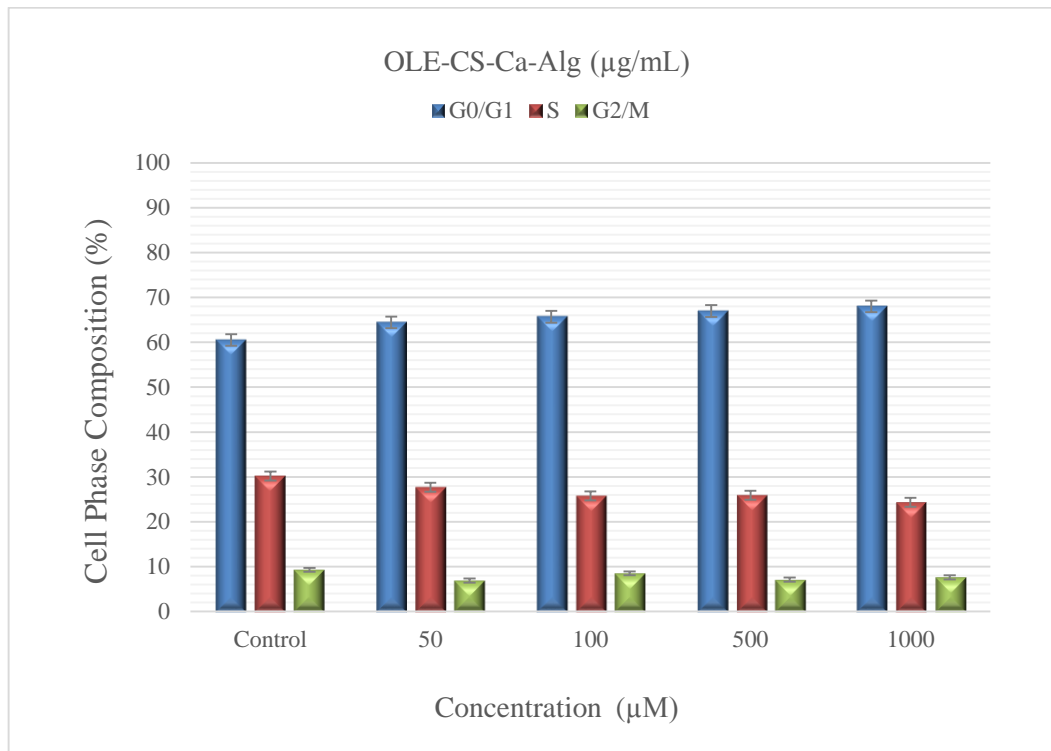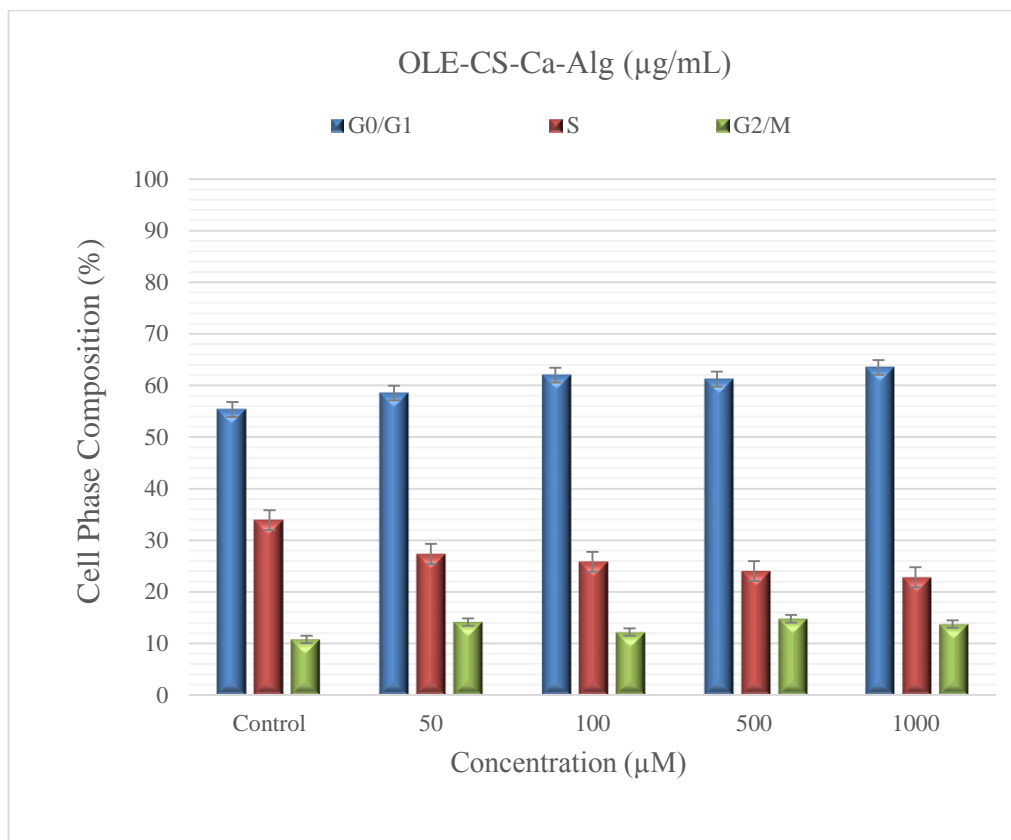
Figure 3.32. Effects of OLE-CS-Ca-Alg on cell cycle arrest in MCF-7 cells.

Effects of OLE on cell phase composition (%) against MCF-7 cells are seen in Fig.3.33. When the results were compared with cell phase composition (%) of control cells, it was seen that there were almost no differences on cell phase composition (%) of OLE effected cells. It can be concluded from this result that free OLE is not effective on cell cycle disruption in MCF-7 cells as expected.
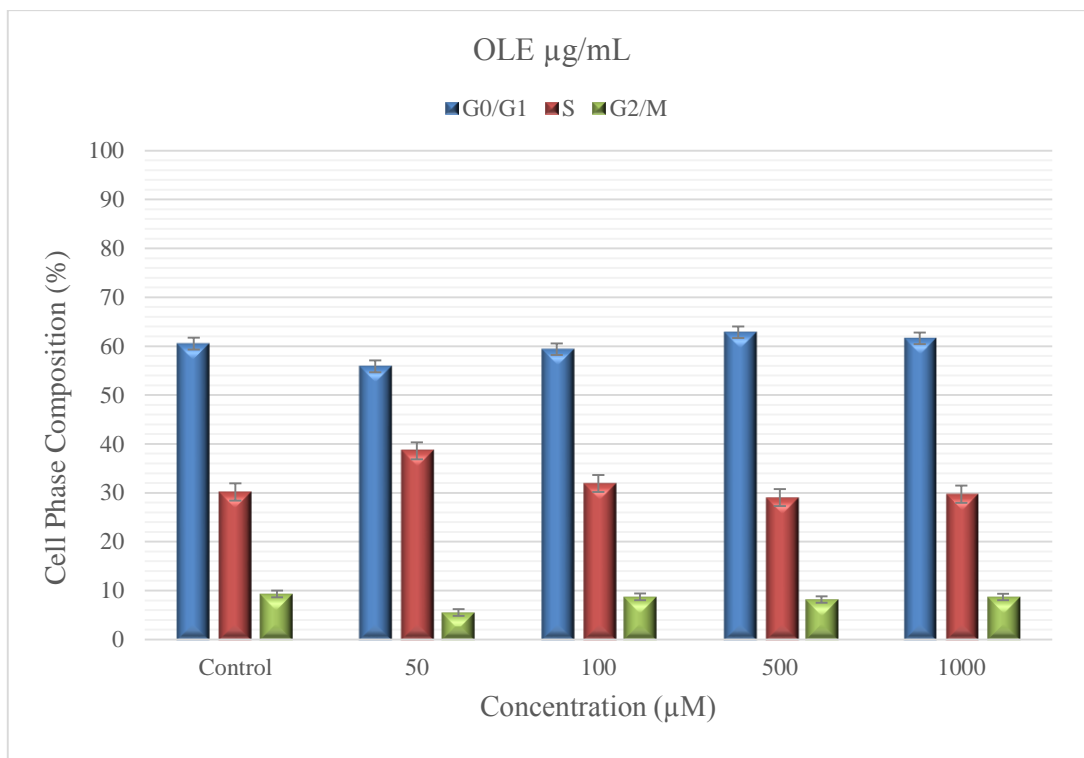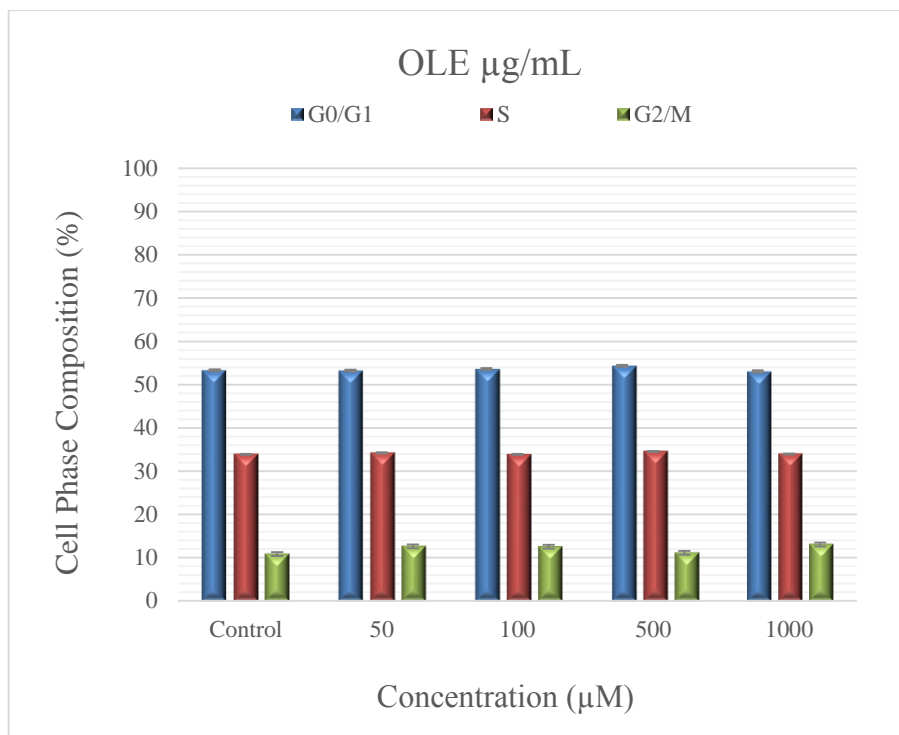
Figure 3.33. Effects of free OLE on cell cycle arrest in MCF-7 cells.

According to these results OLE-CS-Ca-Alg induce a cell cycle block in G1 and it is more effective on perturbing cell cycle than free OLE. Thus, these results confirmed the cytotoxicity and cell cycle results.

3.1.2.3.3. Apoptosis Analysis

Since G0/G1 cell cycle arrest has been shown to correlates with apoptosis, further studies were carried out to elucidate cytotoxic nature of the OLE-CS-Ca-Alg capsules on breast and lung cancer cell lines with different concentrations-50,100,500 and 1000 µg/mL by Annexin V/propidium iodide (PI) staining technique.

This method is an ideal way to detect cell apoptosis quantitatively and distinguish the cells with the characteristics. Because, neither Annexin V nor PI can stain the viable cells, early apoptotic cell are only stained with Annexin V and late apoptotic cell are stained with both Annexin V and PI. And also, the PI can be used to distinguish necrotic cells from apoptotic and living cells since necrotic cells are stained only with PI.

Therefore, by this way percentage of cell phase composition was calculated and represented as a result (Figure 3.34. and figure 3.35.).
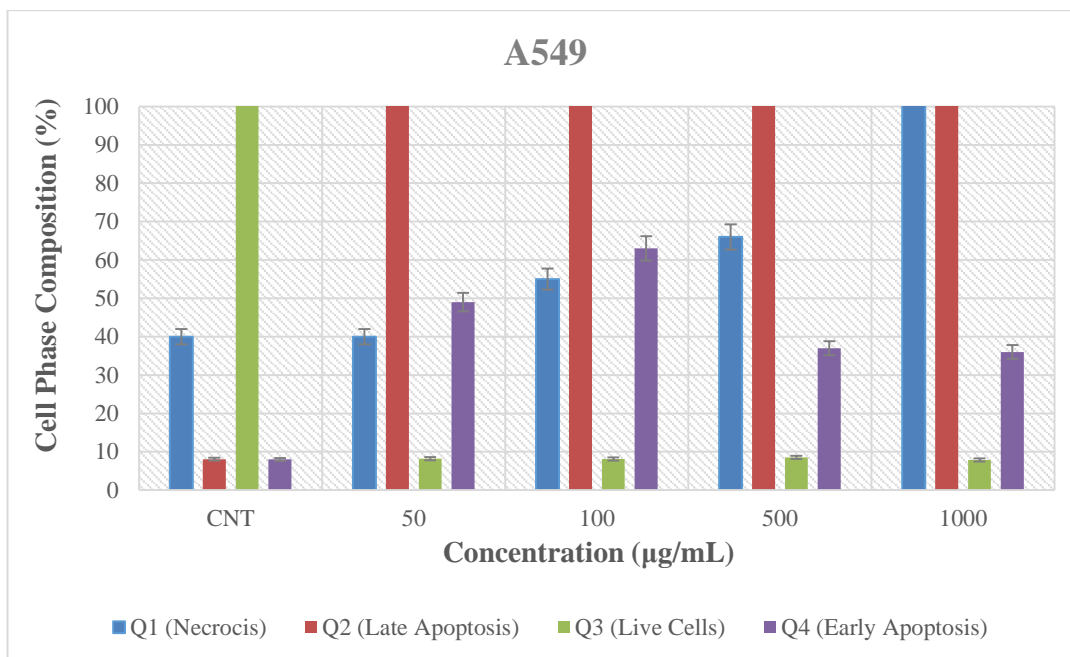
Figure 3.34. Apoptotic effects of OLE-CS-Ca-Alg against A549 cells.

When cell phase composition of control cells was compared with OLE-CS-Ca-Alg treated cells (figure 3.34.) it is clear that OLE loaded CS-Ca-Alg triggers cell death in lung cancer since there is a significant decrease in alive cell number with the increase in late apoptosis phase which is naturally occuring programmed and targeted cell death.
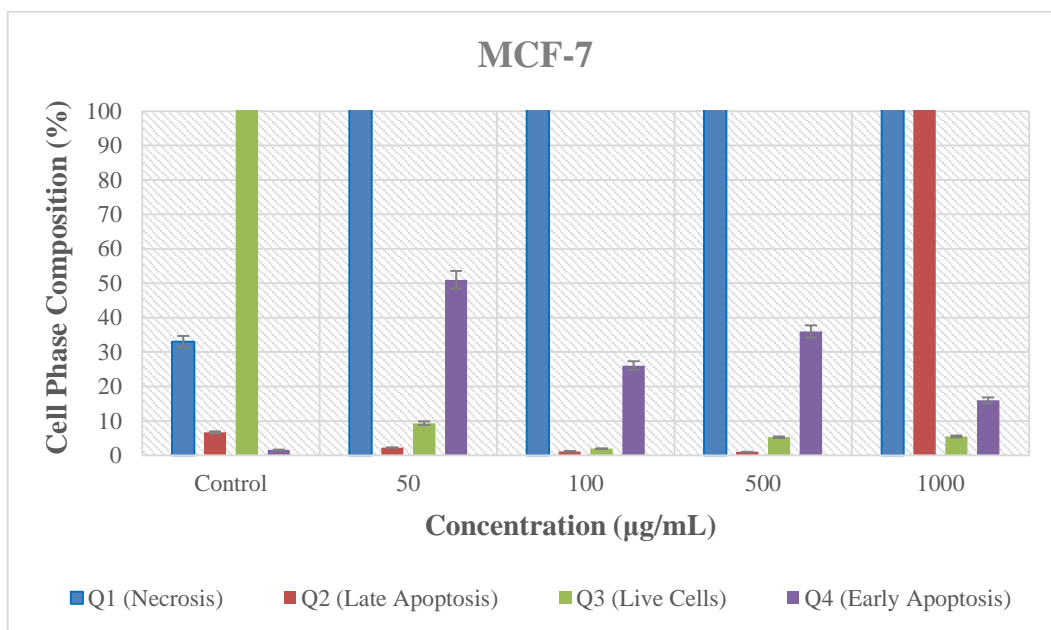
Figure 3.35. Apoptotic effects of OLE-CS-Ca-Alg against MCF-7 cells.

The apoptotic effects of OLE-CS-Ca-Alg on MCF-7 cells was shown in figure 3.35. According to this result, OLE-CS-Ca-Alg microcapsules were also effective on MCF-7 cells since the proportion of apoptosis can be considered as the sum of both early and late apoptosis phase and there was a clear decrease in alive cell number.

3.1.2.3.4. Imaging of Optical Microscopy

During the trials with cell cytotoxicity assays, morphological changes were determined. In order to examine whether the appearance of cancer cells would change before and after treatment of OLE, OLE-Ca-Alg and OLE-CS-Ca-Alg, optical microscopy was used. And results are showed in figure 3.36. for A549 and in figure 3.37. for MCF-7.
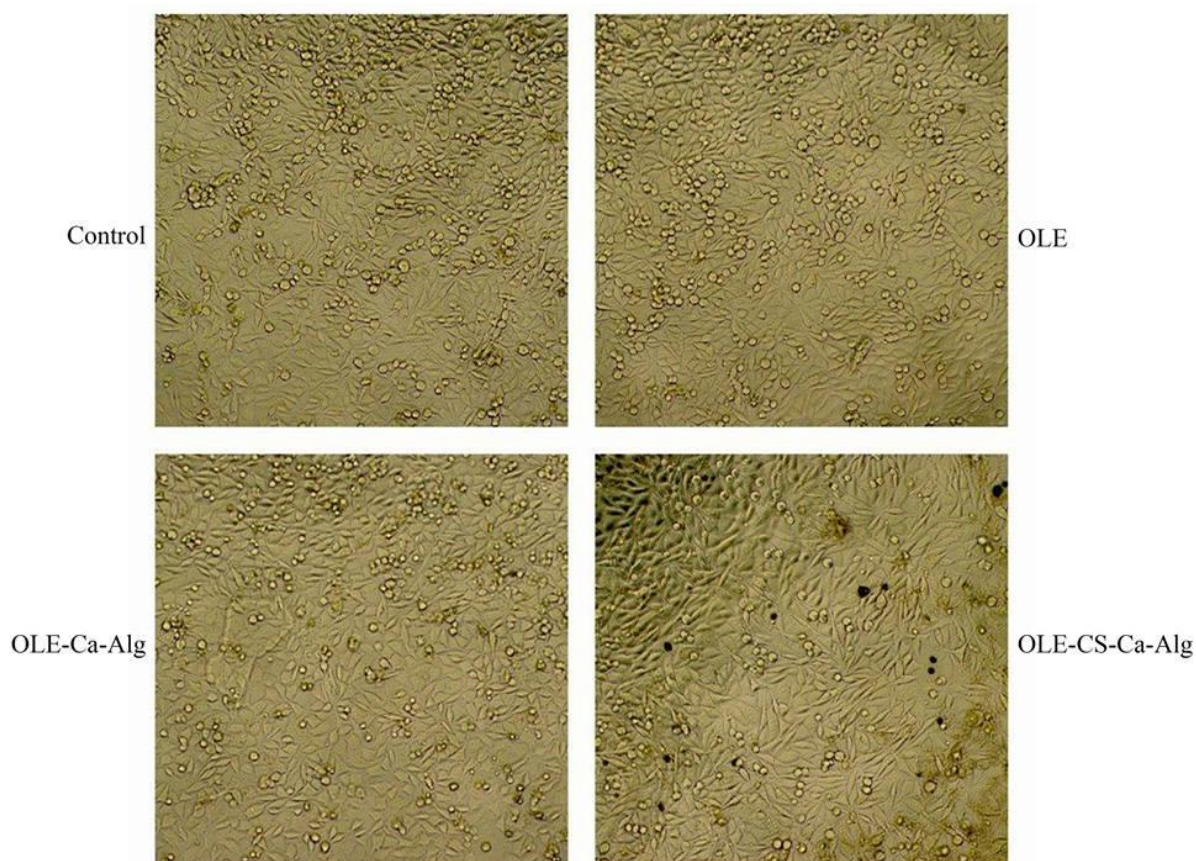
Figure 3.36. Optical microscopy images of A549 cells as control and 1000 µg/mL free OLE, OLE-Ca-Alg, OLE-CS-Ca-Alg capsules applied cells.

According to the figure 3.36., there were nearly no differences between free OLE applied cells and control cells of A549. In contrast, the results show that there is a

significant differences when the cells were treated with OLE-CS-Ca-Alg and OLE-Ca-Alg capsules as expected. There was a reduction in the cell number when the cells treated with OLE-Ca-Alg. Most importantly, morphological changes were observed significantly in the cells incubated with OLE-CS-Ca-Alg capsules, in addition to the reduction in the cell number. Cell shrinkage and formation of apoptotic bodies were clearly seen in OLE-CS-Ca-Alg capsules applied cells. Thus, these results fit with cytotoxicity results.

Additionally, same observations were carried out for MCF-7 cells (Figure 3.37).
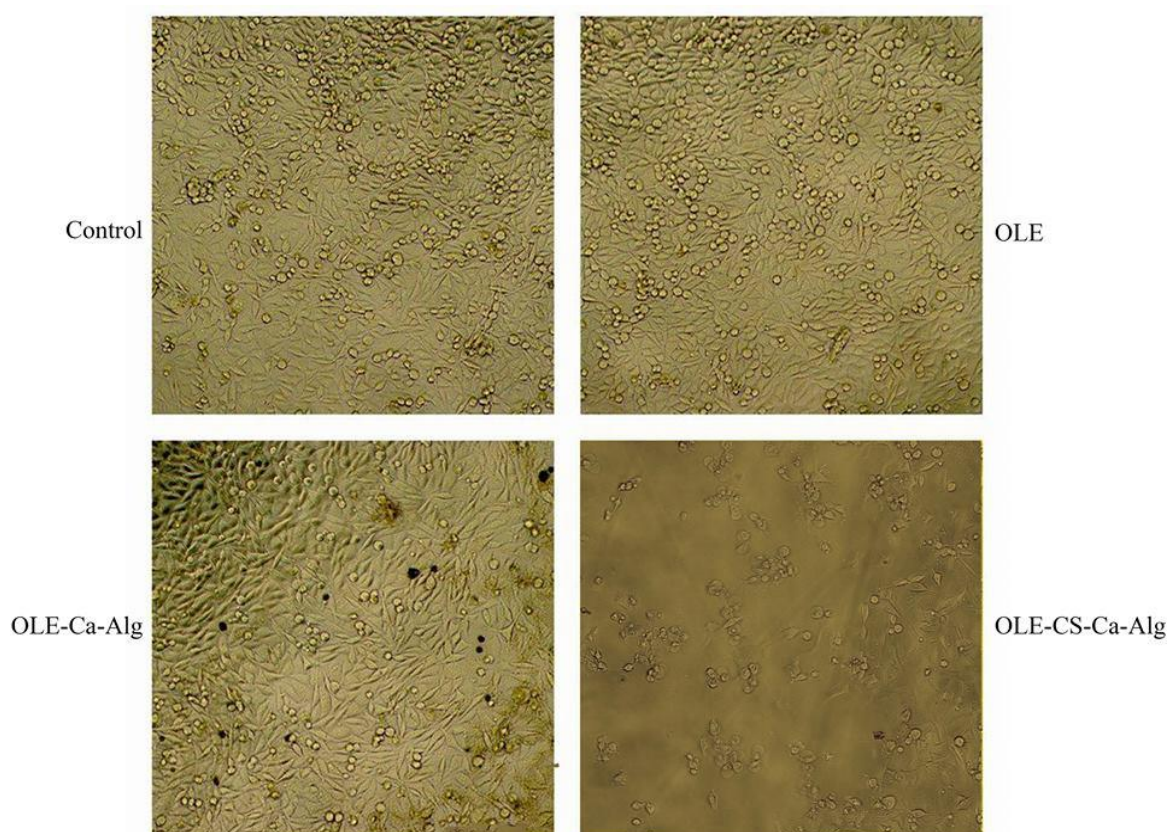
Figure 3.37. Optical microscopy images of MCF-7 cells as control and 1000 μg/mL free. OLE, OLE-Ca-Alg, OLE-CS-Ca-Alg capsules applied cells

While there was almost no differences on cells that treated with free OLE, significant morphological changes in the cells incubated in OLE loaded capsules were observed. Since OLE loaded Ca-Alg and CS-Ca-Alg capsules reduced the cell number and induce apoptotic body formation. Most importantly, it was clearly observed that there was a significant reduce in cellular density and exactly apoptotic body formation on OLE-CS-Ca-Alg applied cells.

With all these results of molecular biological and chemical studies showed that CS coating is an effective way to protect and enhance the ability of OLE polyphenols. However to have a better understanding further studies can be made.

CHAPTER 4

CONCLUSION

There are so many researches indicating that cancer incidence is increasing worldwide. However, Mediterranean diet which is an indicator type of diet of high consumption of olive tree products, may play an important role in cancer prevention. The main polyphenols, that function in olives and olive products, are hydroxytyrosol and oleuropein. Mainly, studies have revolved around the effects of these compounds against some types of cancers mostly because they have antioxidant properties. It is well known that polyphenols have some direct usage limitations, such as low solubility, bitter taste short life time and utilization of encapsulated polyphenols can effectively alleviate them. Therefore, encapsulation of olive leaf extract may improve the stability and effectiveness. Alginate is one of the most well know biopolymer and it is used in this work due to it bioavailability, biodegradability. However, calcium alginate beads are very porous thus they are not effective for retaining of encapsulated molecules and hence to limit the loss of encapsulated material, the microcapsules are sometimes coated with a polycationic polymer that forms a membrane at the bead surface. Chitosan is natually occuring biopolymer and is used in this study.

Olive leaf extract is kept into microcapsules, (OLE-Ca-Alg and OLE-CS-Ca-Alg) were produced and optimization studies were carried out. According to optimization studies, concentration ranges were chosen and the best loading capacity was observed at these concentrations; 3% for Alginate, 1.7 M for $CaCl_2$, 0.3M and pH 8.5 for TRIS buffer, 1% for Chitosan and 1.5% for OLE. According to these results OLE loaded capsules were produced. For characterization of these capsules were performed by ESEM and optical microscopy. With the results of these studies and in the light of some other scientific researches, our capsules were acceptable as disscused in Chapter 3.

Then, in order to understand whether encapsulated OLE has antiproliferative activitiy on different cancer cell lines, the effect of loaded capsules and free OLE were compared. Also, the effectiveness of the compounds were investigated against BEAS 2B to determine their biocompatibility and whether they damage the healthy cells. Moreover,

morfological differences of cells were examined in the presence and absence of the compounds by using optical microscopy.

In order to examine all of the effects for in vitro analysis on A549, MCF-7 and BEAS-2B cell lines, cell culture studies were performed. MTT assay was used to calculate cell viability and IC_{50} values of different conjugates, OLE-CS-Ca-Alg, OLE-Ca-Alg, containing different concentrations were assessed. Also, their effectivenes were compared with freely applied OLE. With these comparison, the effectiveness of OLE is much more effective when it is encapsulated. Since IC_{50} values were 8,96 ng/mL for OLE, 4,06 ng/mL for OLE-CS-Ca-Alg on A549 cells and on MCF-7 cell IC_{50} values were 10,12 ng/mL for OLE and 4,95 ng/mL for OLE-CS-Ca-Alg capsules. Moreover, MTT assay results of the polymers used, showed that they does not have cytotoxic effect on both A549 and MCF-7 cells since cell viability was approximately 90-100%.

There is not any analytical researches encountered about cytotoxic effects of OLE loaded CS-Ca-Alg microcapsules, also there is no analytical epidemiological study that has evaluated the association between the components of the Mediterranean diet and lung cancer (Fortes, C. et al., 2003). And also, Junkyu Han and his friends, 2009, also worked about the cytotoxic effects of OLE on MCF-7 cell lines. Their results indicate cancer cell proliferation was inhibited to 60% only. Thus, with our results and the data from other studies may prove that encapsulation is a promising approach to enhance and protect the effectiveness of OLE.

To understand which step of cell cycle were affected by the compounds cell cycle, analyses were performed by flow cytometer. The cells were prepared by plating 1×10^5 number of cells per well in a 6-well culture plate for one day before flow cytometry analysis. And results revealed that the cells treated with OLE-CS-Ca-Alg capsules show much more significant block of G1 to S phase indicated by the increase of cell number in G0/G1 phase. However free OLE induced less cell cyle arrest in G0/G1 phase. And our results consist with the study of Junkyu Han et al., since their results were when cells were treated for 24 h with changed concentration ranges, the cell cycle arrest at G1 phase was evident (41.1 and 45.7% in oleuropein and hydroxytyrosol treated cells, respectively), accompanying a decrease in G2/M phase when compared with the untreated control cells (27.1% in untreated cells) (Junkyu Han et al., 2009).

Moreover, further studies were carried out to elucidate apoptotic effects of the OLE-CS-Ca-Alg capsules on breast and lung cancer cell lines with different concentrations-50,100,500 and 1000 μg/mL by Annexin V/propidium iodide (PI) staining

technique. According to apoptosis analysis results, OLE loaded CS-Ca-Alg triggers cell death in lung and breast cancer cells which fits with the results of MTT assay as expected.

We also demonstrated the optical imaging results for each cell group treated by different conjugates with optic microscope. In results, OLE-CS-Ca-Alg beads showed more cytotoxicity than other conjugate while there was almost no difference on OLE applied cells morphology. Also in optical imaging, cell number decrease and morfological changes are seen on cancerous cells. These result was expected and they support the cytotoxicity results.

It can be concluded that our results will enhance the drug industry as it is a new approach for anticancer drugs and biocompatible material for biomedical applications. To provide a deeper understanding of OLE loaded microcapsules activity in cancerous cells, protein profiling studies may be performed as further steps.

REFERENCES

Andre, F., Broglio, K., Roche, H., Martin, M., Mackey, J.R., Penault-Llorca, F., Hortobagyi, G.N., Pusztai, L., 2008. Estrogen receptor expression and efficacy of docetaxel-containing adjuvant chemotherapy in patients with node-positive breast cancer: results from a pooled analysis, J. Clin. Oncol., 26 (16), 2636–2643.

Agrawal P, Strijkers GJ, Nicolay K., 2009. Chitosan-based systems for molecular imaging. Adv Drug Deliv Rev; 62:42–58.

Aude Munin and Florence Edwards-Lévy, 2011. Encapsulation of Natural Polyphenolic Compounds; a Review. Pharmaceutics, 3,793-829; doi: 10.3390/pharmaceutics3040793.

Badve, S., Nakshatri, H., 2009. Oestrogen-receptor-positive breast cancer: towards bridging histopathological and molecular classifications. J. Clin. Pathol. 62 (1), 6–12.

Barclay, L. R. C.; Locke, S. J.; MacNeil, J. M., 1985. Autoxidation in micelles - Synergism of Vitamin-C with Lipid-Soluble Vitamin-E and Water-Soluble Trolox. Can. J. Chem. 63 (2): 366–374.

Benavente-Garcia J, Castillo J, Lorente A, Ortuno A, Del Rio JA., 2000. Antioxidant activity of phenolics extracted from Olea europaea L. leaves. Food Chem. 2000;68:457–462.

Berhoune M, Banu E, Scotte F, Prognon P, Oudard S, et al., 2008. Therapeutic strategy for treatment of metastatic non-small cell lung cancer. Ann Pharmacother 42: 1640–1652.

Bors, W., Hellers, W., Michel, C. & Saran, M., 1990. Radical chemistry of avonoid antioxidants. In Emerit, Antioxidants in therapy and preventive. Plenum Press. medicine Vol. 1, pp. 165±170.

Bors, W., Hellers, W., Michel, C. & Saran, M., 1990. Flavonoids as antioxidants: determination of radical-scavenging efficiencies. In L. Packer & A. N. Glazer, Methods in enzymology. CA: Academic Press. Vol. 186, pp. 343± 355

C. Alvarez-Lorenzo, R. Duro, J.L. Gomez-Amoza, R. Martin- Pacheco, C. Souto, A. Concheiro, 1999. Degradation of hydroxypropylcellulose by rhizomucor: effects on release from theophylline hydroxypropylcellulose tablets. Int. J. Pharm. 180 105.

Cancer multidrug resistance, 2000. Nat Biotechnol 18 Suppl: IT18–20.

Chen, L., Remondetto, G. E., & Subirade, M., 2006. Food proteinbased materials as nutraceutical delivery systems. Trends in Food Science & Technology, 17, 272e283.

Chu Q, Vincent M, Logan D, Mackay JA, Evans WK., 2005. Taxanes as firstline therapy for advanced non-small cell lung cancer: a systematic review and practice guideline. Lung Cancer 50:355–374.

Clarke, M., Collins, R., Darby, S., Davies, C., Elphinstone, P., Evans, E., Godwin, J., Gray, R., Hicks, C., James, S., MacKinnon, E., McGale, P., McHugh, T., Peto, R., Taylor, C., Wang, Y., 2005. Early Breast Cancer Trialists' Collaborative Group. Effects of radiotherapy and of differences in the extent of surgery for early breast cancer on local recurrence and 15-year survival: an overview of the randomised trials. Lancet 366 (9503), 2087–2106.

Clarke, M., Collins, R., Darby, S., Davies, C., Elphinstone, P., Evans, E., Godwin, J., Gray, R., Hicks, C., James, S., MacKinnon, E., McGale, P., McHugh, T., Peto, R., Taylor, C., Wang, Y., 2005. Early Breast Cancer Trialists' Collaborative Group. Effects of radiotherapy and of differences in the extent of surgery for early breast cancer on local recurrence and 15-year survival: an overview of the randomised trials. Lancet 366 (9503), 2087–2106.

Concept of Genetic by William S. Klug, Michael R. Cummings, Charlotte A. Spencer, Michael A. Palladino.

Delgado-Pertinez M, Gomez-Cabrera A, Garrido A., 2000. Predicting the nutritive value of olive leaf (Olea europaea): digestibility and chemical composition and in vitro studies. 187-201).

D. Baycın., E. Altıok., S. Ulku., O. Bayraktar., 2007. Adsorption of Olive Leaf (Olea europaea L.) Antioxidants on Silk Fibroin. Journal of Agricultural and Food Chemistry., 55., 1227-1236.

D. Teomim, A. Nyska, A.J., 1999. Domb, Ricinoleic acid based biopolymers, J. Biomed. Mater. Res. 45 258.

Desai, K. G. H., & Park, H. J., 2005. Recent developments in microencapsulation of food ingredients. Drying Technology, 23, 1361e1394.

Fabiani R, De Bartolomeo A, Rosignoli P et al., 2002. Cancer chemoprevention by hydroxytyrosol isolated from virgin olive oil through G1 cell cycle arrest and apoptosis. Eur J Cancer Prev 11:351 358.

Fabiani R, Bartolomeo AD, Rosignoli P et al., 2006. Virgin olive oil phenols inhibit proliferation of human promyelocytic leukemia cells (HL60) by inducing apoptosis and differentiation. J Nutr 136:614–619.

Fisher, B., Anderson, S., Bryant, J., Margolese, R., Deutsch, M., Fisher, E., Jeong, J., Wolmark, N., 2002. Twenty-year follow-up of a randomized trial comparing total mastectomy, lumpectomy, and lumpectomy plus irradiation for the treatment of invasive breast cancer. Engl. J. Med. 347, 1233–1241.

Fortes, C., Forastiere, F., Farchi, S., Mallone, S., Trequattrinni, T., Anatra, F., Schmid, G., and Perucci, C. A., 2003. The Protective Effect of the Mediterranean Diet on Lung Cancer., Nutrition and Cancer., 46, 30–37.

G. Crini, G. Torri, M. Guerrini, M. Morcellet, M. Weltrowski, B. Martel, 1997. NMR characterization of N-benzyl sulfonated derivatives of chitosan. Carbohydr. Polym. 33 145.

Gibbs, B. F., Kermasha, S., Alli, I., & Mulligan, C. N., 1999. Encapsulation in the food industry: a review. International Journal of Food Sciences and Nutrition, 50, 213e224.

Giulia, C., Monica, D., Alessandra, I., David, V., Assunta Dessı', M., Spencer, Jeremy P.E., 2007. Inhibition of p38/CREB phosphorylation and COX-2 expression by olive oil polyphenols underlies their anti-proliferative effects. Biochem. Biophys. Res. Commun. 362, 606–611.

Hamdi K, Hamdi RC., 2005. Oleuropein, a non-toxic olive iridoid, is an anti-tumor agent and cytoskeleton disruptor. Biochem Biophys Res Commun 334:769–778.

Hanasaki, Y.; Ogawa, S.; Fukui, S., 1994. The correlation between active oxygens scavenging and antioxidative effects of flavonoids. Free Radic. Biol. Med., 16, 845–85.

Heidge Fukumasu, Arina L. Rochetti, Pedro R. L. Pires, Edson R. Silva, Ligia G. Mesquita, Ricardo F. Strefezzi, Daniel D. De Carvalho, Maria L. Dagli, 2014, Constitutive Androstane Receptor Ligands Modulate the Anti-Tumor Efficacy of Paclitaxel in Non-Small Cell Lung Cancer Cells.

Hengartner, M. O., 2000. The biochemistry of apoptosis. Nature, 407(6805), 770-776. doi: 10.1038/35037710.

Huguet ML, Dellacherie E., 1996. Calcium-alginate beads coated with chitosan: Effect of the structure of encapsulated Materials on their release. Process Biochem; 31:745-51.

Jacobson, M. D., Weil, M., & Raff, M. C., 1997. Programmed cell death in animal development. Cell, 88(3), 347-354.

Jemal, A., Bray, F., Center, M.M., et al., 2011. Global cancer statistics. CA Cancer J. Clin. 61 (2), 69–90.

Junkyu Han, Terence P. N. Talorete, Parida Yamada, Hiroko Isoda, 2009. Anti-proliferative and apoptotic effects of oleuropein and hydroxytyrosol on human breast cancer MCF-7 cells. Springer Science+Business Media, 51,52.

K.D. Yao, T. Peng, J.J. Yu, M.X. Xu, M.F.A., 1995. Goosen, Microcapsules/microspheres related to chitosan, J.M.S. - Rev. Macromol. Chem. Phys. C35 155.

K.E. Uhrich, S.M. Cannizzaro, R.S. Langer, K.M. Shakesheff, 1999. Polymeric systems for controlled drug release, Chem. Rev. 99 3181.

Keys et al. 1981. The diet and all-causes death rate in the seven countries study, 58–61.

Key, T.J., Schatzkin, A., Willett, W.C., Allen, N.E., Spencer, E.A., Travis, R.C., 2004. Diet, nutrition and the prevention of cancer. Public Health Nutr. 7 187–200.

Keita Kashima and Masanao Imai, 2012, Advanced Membrane Material from Marine Biological Polymer and Sensitive Molecular-Size Recognition for Promising Separation Technology, DOI: 10.5772/50734.

Kreso A, OBrien CA, Van Galen P, Gan O, Notta F, et al., 2013. Variable clonal repopulation dynamics influence chemotherapy response in colorectal cancer. Science 339: 543–548.

L. Illum, 1998. Chitosan and its use as pharmaceutical excipient. Pharm. Res. 15 1326.

Leopoldini, M.; Russo, N.; Toscano, M., 2011. The molecular basis of working mechanism of natural polyphenolic antioxidants. Food Chem., 125, 288–306.

Leist, M., & Jaattela, M., 2001. Four deaths and a funeral: from caspases to alternative mechanisms. Nat Rev Mol Cell Biol, 2(8), 589-598. doi: 10.1038/35085008.

M. N. V. Ravi Kumar, R. A. A. Muzzarelli,C. Muzzarelli,H. Sashiwa, and A. J. Domb, 2004, Chitosan Chemistry and Pharmaceutical Perspectives, Chem. Rev. 104, 6017-6084.

Majeti N.V. Ravi Kumar, 2000. A review of chitin and chitosan applications, Reactive & Functional Polymers 46 1–27.

Maha H. Elamin, Maha H. Daghestani, Sawsan A. Omer, Mai A Elobeid, Promy Virk, Ebtesam M. Al-Olayan, Zeinab K. Hassan, Osama B. Mohammed, Abdelilah Aboussekhra, 2012. Olive oil oleuropein has anti-breast cancer properties with higher efficiency on ER-negative cells. Food and Chemical Toxicology 53 310–316.

Meera George, T. Emilia Abraham, 2006. Polyionic hydrocolloids for the intestinal delivery of protein drugs: Alginate and chitosan — a review, Journal of Controlled Release 114 1–14.

Meier, P., Finch, A., & Evan, G., 2000. Apoptosis in development. Nature, 407(6805), 796-801. doi: 10.1038/35037734.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods, 65(1-2), 55-63.

N. Pellegrini., M. Serafini., B. Colombi., D. D. Rio., S. Salvatore., M. Bianchi., F. Brighenti. 2003. Total Antioxidant Capacity of Plant Foods, Beverages and Oils

Consumed in Italy Assessed by Three Different In Vitro Assays., The Journal of Nutrition., 133: 2812–2819.

Nasti, A., Zaki, N.M., De Leonardis, P., Ungphaiboon, S., Sansongsak, P., Rimoli, M.G.,Tirelli, N., 2009. Chitosan/TPP and chitosan/TPP-hyaluronic acid nanoparticles:systematic optimisation of the preparative process and preliminary biologicalevaluation. Pharm. Res. 26, 1918–1930.

P. Madhavan, 1983. Chitin, Chitosan and their Novel Appli cations. Therm. Anal. 28 189.

R.L. Reis, A.M. Cunha, 1995. Characterization of two biodegradable polymers of potential applications within the biomaterial field, J. Mater. Sci.: Mater. Med. 6786.

Rice-Evans, C.A.; Miller, N.J. Paganga, G., 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic. Biol. Med., 20, 933–956.

Roberts GAF., 1992. Structure of chitin and chitosan. In: Roberts GAF, editor. Chitin chemistry. Houndmills: Macmillan p. 1–53.

Rouzier, R., Perou, C.M., Symmans, W.F., Ibrahim, N., Cristofanilli, M., Anderson, K., Hess, K.R., Stec, J., Ayers, M., et al., 2005. Breast cancer molecular subtypes respond differently to preoperative chemotherapy. Clin. Cancer Res. 11 (16), 5678–5685.

Saad, E.D., 2011. Endpoints in advanced breast cancer: methodological aspects & clinical implications. Indian J. Med. Res. 134 (4), 413–418.

S. Isık et al., 2012. Proliferative and apoptotic effects of olive extracts on cell lines and healthy human cells. Food Chemistry. 134. 29–36.

Schrooyen, P. M. M., van der Meer, R., & De Kruif, C. G., 2001. Microencapsulation: its application in nutrition. Proceedings of the Nutrition Society, 60, 475e479.

Sedef N El and Sibel Karakaya, 2009. Olive tree (Olea europaea) leaves: potential beneficial effects on human health. International Life Sciences Institute, 632.

Shahidi, F., & Han, X. Q., 1993. Encapsulation of food ingredients. Critical Reviews in Food Science and Nutrition, 33, 501e547.

S.J. Kim, S.S. Kim, Y.M. Lee, 1994. Synthesis and characterization of ether type chitin derivatives. Macromol. Chem. Phys. 195 () 1687.

S. Nishimura, O. Kohgo, K. Kurita, H. Kuzuhara, 1991.Chemospecific manipulations of a rigid polysaccharide synthesis of novel chitosan derivatives with excellent solubility in common organic solvents by regioselective chemical modifications. Macromolecules 24 4745.

Tonnesen HH, Karlsen J., 2002. Alginate in drug delivery systems. Drug Dev Ind Pharm;28:621–30.

Vilstrup, P., 2001. Microencapsulation of food ingredients. Surrey: Leatherhead Pub.

Visioli F, Galli C, Plasmati E et al., 2000. Olive phenol hydroxytyrosol prevents passive smokinginduced oxidative stress. Circulation 102:2169–2171.

Visioli, F., Bellomo, G., & Galli, C., 1998. Free-radical-scavenging properties of olive oil polyphenols. Biochemical and Biophysical Research Communications, 247, 60±64.

Visioli, F, Bellosta, S, & Galli, C.,1998. Oleuropein, the bitter principle of olives enhances nitric oxide production by mouse macrophages. Life Sciences, 62, 541±546.

Vaux, K., 1999. Law and lamb: AKEDAH and the search for a deep religious symbol for an ecumenical bioethics. Christ Bioeth, 5(3), 213-219. doi: 10.1076/chbi.5.3.213.6893.

Yi H, Wu LQ, Bentley WE, Ghodssi R, Rubloff GW, Culver JN, Payne GF., 2005. Biofabrication with chitosan. Biomacromolecules;6:2881–94.

Zhang P., Gao W. Y., Turner S., Ducatman S. B., 2003. Gleevec (STI-571) inhibits lung cancer cell growth (A549) and potentiates the cisplatin effect in vitro, Molecular Cancer, 2:1.

APPENDIX A

MEDIAS

A.1. RPMI-1640 Growth Medium

Roswell Park Memorial Institute – 1640 (RPMI 1640) growth medium, fetal bovine serum (FBS) and gentamicin sulfate were obtained from Gibco, BRL.

50 ml FBS (~10% of all volume) and 50 μg/ml gentamicin sulfate (~1%) were added into 500 ml RPMI-1640 then mixed at room temperature.

APPENDIX B

CHEMICALS, REAGENTS AND SOLUTIONS

Table B.1. Chemicals and Reagents Used in Experiments.

NO	CHEMICALS	COMPANY
1	Absolute Ethanol	AppliChem
2	ABTS reagent	Fluka
3	Acetic Acid Glacial	Sigma
4	Alginic Acid (Biochemica)	AppliChem
5	Annexin-V Apoptosis Detection Kit I	BD Pharmingen
6	Calcium chloride	Sigma
7	Chitosan (high molecular weight)	Aldrich
8	Dimethyl Sulfoxide (DMSO)	Sigma
9	Fetal Bovine Serum (FBS)	Gibco
10	Folin Reagent	Merck

Table B.1. (Cont)

11	Gentamicine Sulfate	Gibco
12	$K_2S_2O_8$	Merck
13	MTT Reagent (should not be exposed to light)	Sigma
14	Phosphate Buffered Saline (PBS)	Invitrogen
15	Phosphoric Acid	AppliChem
16	RNase	Thermo
17	Sodium Citrate	Merk
18	Sodium Hydroxyde (NaOH)	Sigma
19	Tris	Amresco
20	Triton X-100	Sigma
21	Trypan Blue Dye	Sigma
22	Trypsin	Sigma

B.2. MTT Reaction Solution

For preparing a sufficient reaction solution for a 96-well plate 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT solution was prepared in PBS to obtained a concentration of 5mg/ml. % 10 MTT solution was prepared with RPMI.

APPENDIX C

CALCULATIONS OF CHARACTERIZATION OF OLIVE LEAF EXTRACT

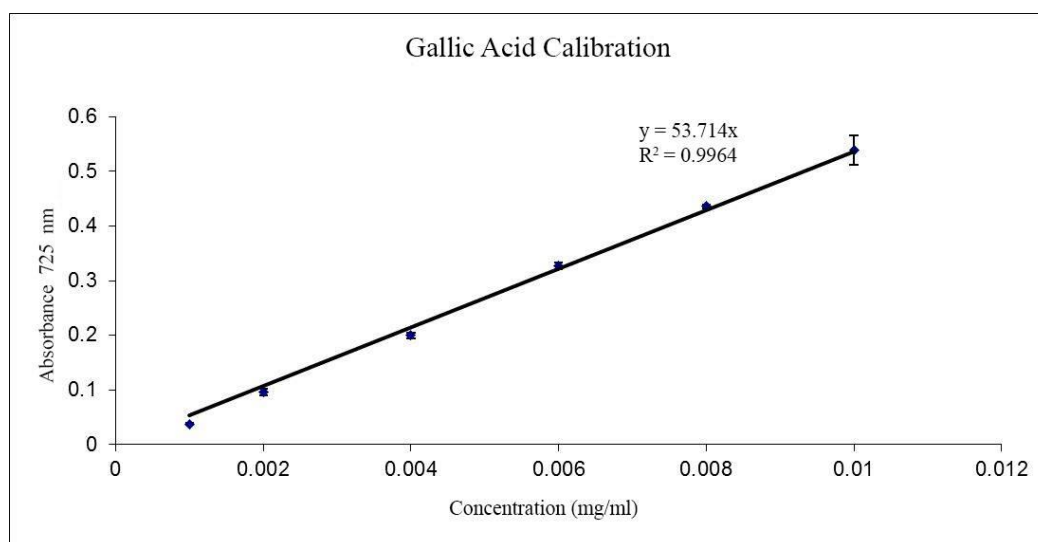## C.1. Calibration Curve for Total Phenolic Compound Content:

Figure C.1. Calibration curve for total phenol content as gallic acid equevalant.

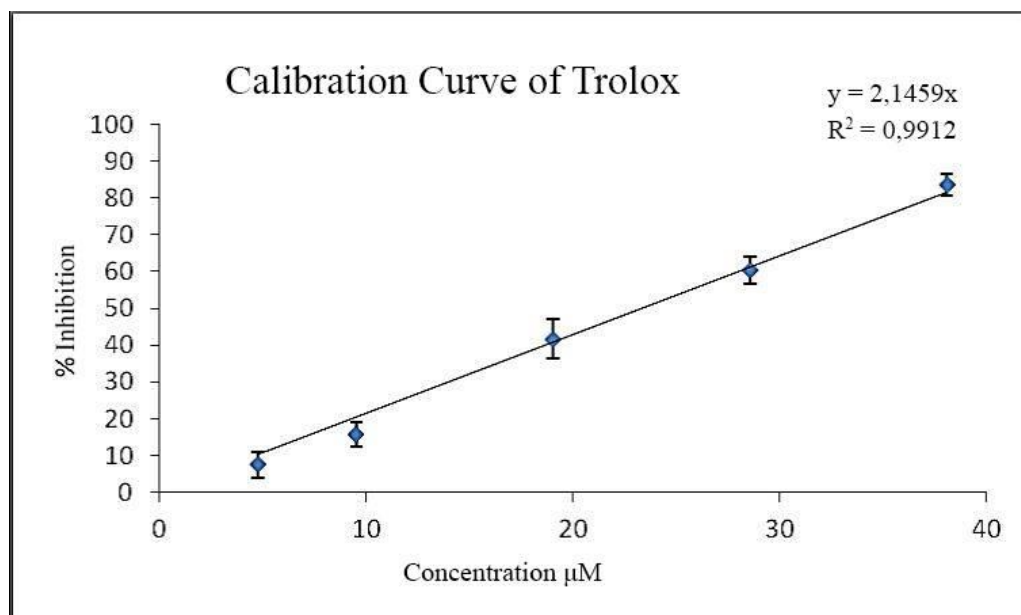## C.2. Sample Calculation for Percentage Inhibition:

Figure C.2. Calibration curve for total antioxidant capacity as mmol TEAC/g OLE.

Olive leaf extract solution was added to ABTS radical cation solution and the absorbance values were taken at each 1 minute during 60 minutes. Average of the first and last absorbance was taken for two diluted OLE solution and its decrease from the absorbance value of ABTS radical cation solution was calculated in order to find out percentage inhibition.

The measured absorbance values after adding the olive leaf extract solution were, 0.4924 and 0.6016 for first absorbance and 0.298244 and 0.57723 for last absorbance. % inhibition was calculated as:

$$\% \ 39.43 = [1-(0.4924/0.2982)]*100$$

and

$$\% \ 4.05 = [1-(0.6016/0.57723)]*100$$

The concentrations of two solutions were calculated as 1.67 and 2.68 mM and the average was 2.18 mM per g of OLE. The result was concluded 2.18 mmol TEAC/ g of OLE.

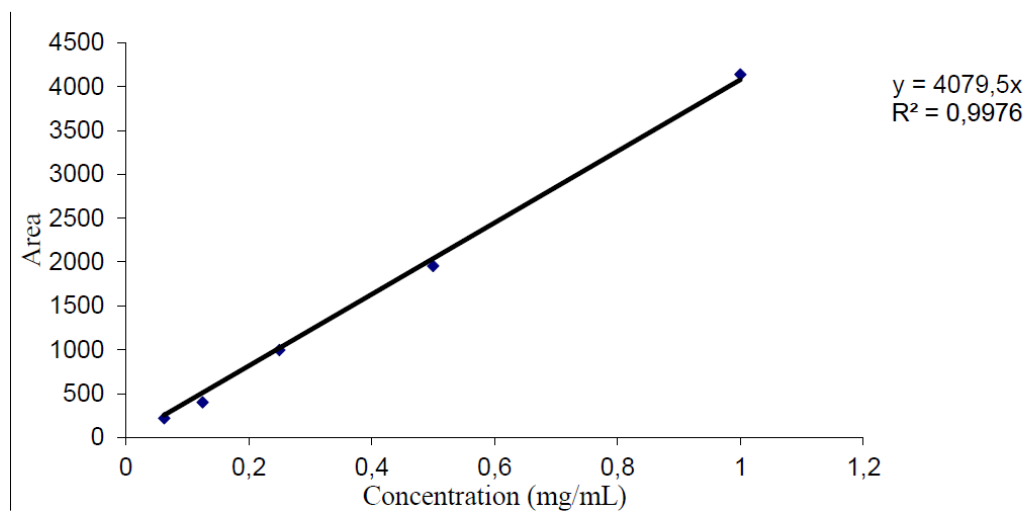## C.3. Calculation for Analysis of Total Phenolic Compounds with HPLC:

Figure C.3. Calibration curve of oleuropein.

In order to find out the amount of oleuropein in olive leaf extract, the responses of HPLC in terms of areas were recorded. By using these areas and calibration curves, concentration of oleuropein was calculated. the HPLC response for oleuropein was 944.4.

The equation of external calibration curve for oleuropein is:

$$y = 4079.5 \ x$$

Where, y = area x = concentration 944.4 = 4079.5 x x = 0.231 µg/mL = 0.00023 mg/mL.