PREPARATION AND EVALUATION OF CHITOSAN MICROSPHERES FOR ERADICATION OF *HELICOBACTER PYLORI*

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Duygu ALTIOK

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We approve the thesis of **Duygu ALTIOK**

Prof. Funda TIHMINLIOĞLU
Supervisor

Prof. Serdar ÖZÇELİK
Committee Member

Prof. Özlem YILMAZ
Committee Member

Assoc. Prof. Oğuz BAYRAKTAR
Committee Member

Assoc. Prof. Volga BULMUŞ ZAREIE
Committee Member

12 July 2011

Prof. Mehmet POLAT
Head of the Department of
Chemical Engineering

Prof. Durmuş Ali DEMİR
Dean of the Graduate School of
Engineering and Sciences

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ABSTRACT

PREPARATION AND EVALUATION OF CHITOSAN MICROSPHERES FOR ERADICATION OF *HELICOBACTER PYLORI*

The main focus of this dissertation is to develop chitosan based microspheres loaded with antibiotic and essential oil to eradicate *Helicobacter pylori* by maintaining the constant drug level and prolonged gastric retention. The primary objective was to show that novel essential oil loaded microspheres are promising in the treatment of *H. pylori* infection as an alternative to conventional antibiotic therapy. In this context, firstly, the minimum inhibitory concentration of clarithromycin and five essential oils namely lemongrass oil, thyme oil, clove leaf oil, lemon oil and cinnamon bark oil on *H. pylori* was investigated. Among five essential oils, cinnamon bark oil showed the strongest anti-*H. pylori* activity.

After choosing the most effective essential oil on *H. pylori*, we focused on the preparation of clarithromycin and cinnamon bark oil loaded microspheres by spray drying technique. Spray drying conditions were optimized by reponse surface methodology. Genipin, a natural crosslinker, was used as a crosslinking agent to achieve controlled drug release.

Finally, it was aimed to investigate the clarithromycin and essential oil release in buffer solution and their antibacterial activity on *H. pylori* when released from microspheres. Higuchi equation well described the release characteristics. Drug release from microspheres was diffusion controlled. Cinnamon bark oil and clarithromycin released from the microspheres inhibited the growth of *H. pylori* resulting that the antibacterial activity of cinnamon bark oil and clarithromycin was maintained during the microsphere manufacturing. In conclusion, clarithromycin and cinnamon bark oil loaded chitosan microspheres have a great potential to be used as a control release system in treatment of *H. pylori* infection.

ÖZET

HELICOBACTER PYLORI'NİN ERADİKASYONU İÇİN KİTOSAN MİKROKÜRELERİN HAZIRLANMASI VE DEĞERLENDİRİLMESİ

Bu tezin odak noktası *Helicobacter pylori'*nin eradikasyonu için sabit ilaç seviyesini sağlayan ve midede tutulma süresinin uzatan antibiyotik ve uçucu yağ yüklü kitosan bazlı mikroküreleri geliştirmektir. Antibakteriyel ajanların mukoyapışkan ilaç taşıyıcı sistemlerden kontrollü salımı, önemlidir. Tezin ana amacı uçucu yağ yüklü mikroküre lerin *H. pylori* infeksiyonunun tedavisinde geleneksel antibiyotik tedavilerine alternatif olarak kullanılabilecek, umut vaad eden, orijinal bir sistem olduğunu göstermektir. Bu kapsamda ilk olarak klaritromisin ile limonotu, kekik, karanfil, limon ve tarçın uçucu yağlarının *H. pylori* üzerine minimum inhibisyon konsantrasyonları (MİK) araştırıldı. Beş uçucu yağ arasında en güçlü anti-*H. pylori* aktivitesini tarçın yağı gösterdi.

H. pylori üzerine en etkili uçucu yağ seçildikten sonra klaritromisin ve tarçın yağı yüklü mikroküre lerin püskürtmeli kurutma tekniğiyle eldesine odaklanıldı. Püskürtmeli kurutma koşulları tepki yüzey yöntemiyle optimize edildi. Kontrollü ilaç salımını sağlamak için doğal bir çapraz bağlama ajanı olan genipin kullanıldı.

Son olarak klaritromisin ve tarçın yağının mikroküre lerden tampon çözeltiye salımının ve mikroküre lerden ortama salınan klaritromisin ve tarçın yağının *H. pylori* üzerine antibakteriyel aktivitelerinin araştırılması hedeflendi. Salım karakteristiğini Higuchi denklemi iyi açıkladı. Mikroküre lerden ilaç difüzyon kontrollü salım olarak belirlendi. Mikroküre lerden salınan klaritromisin ve tarçın yağının *H. pylori* üremesini inhibe etmesi klaritromisin ve tarçın yağının antibakteriyel aktivitelerinin mikroküre üretimi sırasında korunduğunu gösterdi. Sonuç olarak klaritromisin ve tarçın yağı yüklü mikroküre lerin *H. pylori* infeksiyonunun tedavisinde kullanılabilecek potansiyel bir kontrollü salım sistemi olduğu belirlendi.

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CHAPTER 1

INTRODUCTION

Helicobacter pylori (*H. pylori*) is one of the most common patogens in the world and it may be very important risk factor on human life unless its infection is treated immediately. Although the microorganism is susceptible many antimicrobial agents, the eradication rate is very low due to poor permeability of the antibiotics across the mucus layer, poor stability of the drug in the acidic pH of the gastric fluid, short residence time of antibiotic in the stomach and development of resistance to antimicrobial agents. Triple therapy consisting of combined use of antibiotics, such as amoxicillin, clarithromycin or metronidazole, and a proton pump inhibitor gives better eradication rate than the monotherapy, and is now frequently used for clinical treatment of *H. pylori* associated gastroduodenal disease. However, harmful side effects, cost of therapy, and the lack of willingness to take many different drug products are the major drawbacks of triple therapy (Majithiya and Murthy, 2005; Zheng et al., 2006). For this reason, considerable interest has focused recently on the new drug delivery systems that deliver the antibiotic locally in the stomach by increasing residence time of antibiotics at infected site and biologically active compounds including antioxidants from plants and other natural sources as an alternative to antibiotics (Rajinikanth et al., 2008).

One way to improve the efficacy in eradicating the infection is to develop mucoadhesive drug carriers which may prolong the residence time in the gastrointestinal tract (GI) because they can adhere to the mucus surface, resulting in an effective localized drug concentration (Rajinikanth et al., 2008). Among several mucoadhesive polymers, chitosan is gaining attention in the pharmaceutical field for a wide range of drug delivery since it is known to be a natural, biocompatible, biodegradable and nontoxic. Clarithromycin is a macrolide and widely used in a standard eradication treatment of *H. Pylori* infection combined with a second antibiotic and an acid-suppressing agent (Rajinikanth et al., 2008). It has the highest eradication rate of *H. pylori* in monotherapy *in vivo* and hence was selected as a model drug in this study.

Another way to increase eradication rate is to use biologically active compounds such as essential oils as an alternative to antibiotics or as food additives to complement present therapies. Although essential oils have a broad spectrum of activity, not all of them are able to kill all bacteria. There has been an increased interest in looking at antimicrobial properties of extracts from aromatic plants particularly essential oils (Bergonzelli et al., 2003; Prabuseenivasan et al., 2006). Since there are not any assignments about the resistance of *H. pylori* to essential oils, the use of essential oils to eradicate the antibiotic-resistant strain of *H. pylori* and for patients having antibiotic allergy is promising (Shaik et al., 2005). There are also limited number of studies on the production of essential oil loaded microspheres and their controlled release profiles. Although, there is a study about citronella oil encapsulation by chitosan using o/w emulsion method, there is not any study on the *H. pylori* eradication with essential oil loaded microspheres (Hsieh et al., 2006).

The main purpose of this dissertation was to develop chitosan based microspheres loaded with antibiotic and essential oil to eradicate *H. pylori*. This stomach-specific delivery system would increase the gastric residence time and allow more of the antibiotic to penetrate through the gastric mucus layer and act locally at the infectious site. First of all, the minimum inhibitory concentration (MIC) of clarithromycin and five essential oils namely lemongrass oil, thyme oil, clove leaf oil, lemon oil and cinnamon bark oil on *H. pylori* was determined by agar dilution method (Clinical and Laboratory Standards Institute, 2007) that shows the potential use of essential oils for the eradication of *H. pylori*. The composition of essential oils was determined by GC-MS. Trolox equivalent antioxidant capacity of essential oils was calculated from the percentage inhibition curves by using ABTS. Since the cinnamon bark oil exhibited highest anti-*H. pylori* activity, clarithromycin and cinnamon bark oil loaded chitosan microspheres were obtained by spray drying. The significance of some parameters (inlet air temperature, drug concentration and feed flow rate) on spray drying efficiency and properties of clarithromycin and cinnamon bark oil loaded microspheres in terms of the water activity and morphology, and also the optimum conditions were determined by Central Composite Design (CCD) and Response Surface Methodology (RSM). The surface morphology, thermal properties, swelling and degradation properties and the structures of resultant microspheres produced according to the optimum conditions were investigated by SEM, DSC and TGA, phase contrast microscopy and ^{1}H-NMR spectroscopy, respectively. To achieve controlled release,

genipin crosslinked microspheres were manufactured by changing the genipin concentration and crosslinking time. The stability of clarithromycin in different pH solutions was examined. Clarithromycin and cinnamon bark oil release from the microspheres were carried out in phosphate buffer solution and model equations were used to explain the release mechanism. Finally, the antibacterial activity of clarithromycin and cinnamon bark oil released from microspheres were tested on *H. pylori*.

CHAPTER 2

HELICOBACTER PYLORI

Helicobacter pylori (*H. pylori)* is a spiral-shaped, microaerophilic, gram-negative bacteria which colonize gastric mucosa. *H. pylori* is related to a number of upper gastrointestinal diseases, such as chronic gastritis, duodenal and gastric ulcer, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma and has been classified as a class 1 carcinogen by "International Agency for Cancer Research (IACR)'' in 1994 (Graham and Graham, 2002). Considering gastroduodenal diseases related to *H. pylori* infection, this bacterium has the important role in terms of public health. *H. pylori* is localized in gastric mucus-secreting cells found in or under a layer of mucus and target the gastric epithelial cells (Wallis-Crespo and Crespo, 2004).

H. pylori has been found in the stomachs of humans in all parts of the world. In developing countries, 70 to 90% of the population carries *H. pylori*; almost all of these acquire the infection before the age of 10 years. In developed countries, the prevalence of infection is lower, ranging from 25 to 50% (Dunn et al., 1997).

Although *H. pylori* is sensitive to a wide range of antibiotics in vitro, they all fail as monotherapy in vivo. In infected patients, the most effective single drug is clarithromycin, which leads to an approximate eradication rate of 40% when given twice daily for 10 to 14 days. Dual therapies, combining twice-daily-dosed PPI with, in particular, amoxicillin, are stil in use in some countries, but dual therapies have mostly been replaced by triple therapies. Tetracycline, amoxicillin, imidazoles (predominantly metronidazole and tinidazole), and a few selected macrolides (in particular clarithromycin, sometimes azithromycin) are probably the drugs most widely used for *H. pylori* eradication therapy. The use of these drugs has resulted in effective therapies against *H. pylori*, with consistent eradication rates over 80%. Various treatment durations, doses, and drug combinations have been studied, but none have consistently reached eradication levels in excess of 90 to 95%. Failures are in particular related to insufficient therapy adherence, often because of side effects, and to the presence of antimicrobial resistance. Such resistance is common in patients who have had previous antibiotic treatment, including failed eradication therapies (Kusters et al., 2006). The

reported failures of antibiotic therapy could be due to poor permeability of the antibiotics across the mucus layer or due to poor stability of the drug in the acidic pH of the stomach. Moreover, conventional tablets or capsules do not remain in the stomach long. Therefore, antibiotics might not have enough time to diffuse into the mucosa layer, and the antibiotic concentrations in the gastric mucus can not reach minimum inhibitory concentrations (Majithiya and Murthy, 2005; Zheng et al., 2006).

2.1. History

H. pylori was first identified in 1983 by Robin Warren (pathologist) and Barry Marshall (gastroenterologist) and it has been understood that *H. pylori* infection is the main cause of human gastritis and peptic ulcer. *H. pylori* infection during childhood may lead stomach cancer in old ages. So, *H. pylori* has been classified by WHO (the World Health Organization) as a "class I human carcinogen" in 1994 (Kusters et al., 2006; Radosz-Komoniewska et al., 2005). In 2005, the Nobel Prize in Physiology or Medicine was attributed to B. Marshall and R. Warren who showed the public health importance of the discovery of *H. pylori* and its role in stomach diseases (Megraud and Lehours, 2007).

2.2. Morphology

H. pylori is a gram-negative bacterium, measuring 2 to 4 mm in length and 0.5 to 1 mm in width. Although usually spiral-shaped, the bacterium can appear as a rod, while coccoid shapes appear after prolonged in vitro culture or antibiotic treatment. The organism has 2 to 6 unipolar, sheathed flagella of approximately 3 mm in length, which often carry a distinctive bulb at the end. The flagella confer motility and allow rapid movement in viscous solutions such as the mucus layer overlying the gastric epithelial cells. In contrast to many other pathogens of the gastrointestinal tract, it lacks fimbrial adhesins. *H. pylori* is urease, catalase, and oxidase positive, characteristics which are often used in identification of *H. pylori*. *H. pylori* can catabolize glucose, and both genomic and biochemical information indicates that other sugars cannot be catabolized by *H. pylori* (Kusters et al., 1997).

2.3. *H. pylori* Infections

Helicobacter pylori colonizes in the stomach of humans and causes of chronic gastritis, peptic ulcer disease (PUD), gastric cancer, and mucosa-associated lymphoid tissue lymphoma (MALToma). Once established, it may reside in the gastric mucosa for years, possibly for the life of the host, because the immunological defense mechanisms of the host fail to eliminate it (Rudi et al., 1998). Although most bacteria quickly die in stomach acid (pH 2) *H. pylori* thrive in the stomach. Under acidic conditions, *H. pylori* use a type of enzyme called urease to convert urea, found in low levels in stomach, into ammonia and carbondioxide. The resulting ammonia neutralizes stomach acid, making the bacteria's microenvironment hospitable. *H. pylori* travel by whipping their tail-like flagella. Upon entering the stomach, the bacteria propel themselves through the mucus gel that lines and protects the stomach's epithelial cells. Under the mucus, the bacteria attach to the epithelial cells and continue to use urease to deacidify their microenvironment. Without urease, *H. pylori* can not colonize the stomach (Rudi et al., 1998, Salyers and Whitt, 2002, Stingl et al., 2008).

2.4. Clarithromycin

In the eradication of *H. pylori* infections, the macrolides of clarithromycin, amoxicilline or nitroimidazole group antibiotic of metronidazole with proton pump inhibitor (PPI) were used for 7 and 14 days therapy. Bacterial resistance to antimicrobial agents is currently considered as an increasing problem in the treatment of infectious diseases in general. Resistance of *H. pylori* to clarithromycin is regarded as a particular dilemma, since this drug is a part of established therapy regimen. Thus, macrolide resistance is a frequent cause for failure of *H. pylori* eradication therapy and secondary clarithromycin resistance rates in patients with a history of failed eradication attempts frequently exceed 50%. Also in the general population the prevalence of macrolide-resistant *H. pylori* has been increasing over the years, primarily in the industrial countries. Primary clarithromycin resistance rates reach up to 10% in Europe (Juttner et al., 2004). Among the macrolides, clarithromycin is currently widely used because of its low MIC's (minimum inhibitory concentration which is able to inhibit the

growth of bacteria) against *H. pylori* and its good pharmacokinetic properties (Megraud, 1997; Rimbara et al., 2011).

2.4.1. Structure

The macrolides are a group antibiotics whose activity stems from the presence of 14-16-membered macrocyclic lactone ring to which one or more deoxy sugars, usually cladinose and desosamine, may be attached (Figure 2.1) Clarithromycin (6-O-methylerythromycin) is synthesized by substituting a methoxy group for the C-6 hydroxyl group of erythromycin. This substitution creates a more acid-stable antimicrobial and prevents the degradation of the erythromycin base to the hemiketal intermediate. The increased acid stability of clarithromycin results in improved oral bioavailability and reduced gastrointestinal intolerance (Dinos et al., 2003).

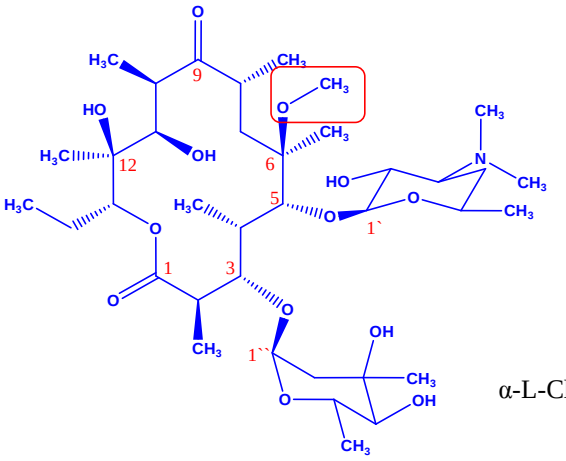

Figure 2.1. Chemical structure of clarithromycin
(Source: Kusters et al., 2006).

2.4.2. Mechanism of Action

It has been well documented that macrolides bind to the large ribosomal subunit in the vicinity of the peptidyl transferase center and cause cell growth arrest due to inhibition of protein synthesis. The precise mechanism of protein synthesis inhibition by macrolides depends on the specific chemical structure of the drug molecule. This affects its interaction with the ribosome as well as the mode of the inhibitory action. Four

modes of inhibition of protein synthesis have been ascribed to macrolides: 1) Inhibition of the progression of the nascent peptide chain during early rounds of translation; 2) Promotion of peptidyl tRNA dissociation from the ribosome; 3) Inhibition of peptide bond formation; and 4) Interference with 50S subunit assembly. All of these mechanisms have some correlation with the location of the macrolide binding site on the ribosome (Gaynor and Mankin, 2005). Clarithromycin exerts its antibacterial effect by reversibly binding to the peptidil transferase zone in 50S ribosomal subunit of *H. pylori* by hydrogen bonding with the cladinose sugar connected to C_3 with α-glicosidic bond of clarithromycin (Zuckerman, 2004; Gerrits et al., 2006).

Clarithromycin is a kind of bacteriostatic antibiotic (Gibreel et al., 2005; Mckenna and Evans, 2001). The minimum inhibitory concentration (MIC), the smallest concentration of antibiotics which is able to inhibit the growth of bacteria, of clarithromycin was reported as 0.03-0.06 μg/mL (Mckenna and Evans, 2001). Safak et al. 2009, have determined the MIC_{50} and MIC_{90} values as 0.064 μg/mL and 2.0 μg/mL, respectively, by agar dilution method (Safak et al., 2009). Among 106 patients *H. pylori* isolates, MIC_{90} was determined as 1 μg/mL (Kohanteb et al., 2007). Vega et al. 2009, have reported the MIC of *H. pylori* NCTC 11638 standard strain as 0.125 mg/L (Vega et al., 2009). The MIC's of clarithromycin resistant *H. pylori* strains having A2143G and A2142G mutations have been reported as 0.5-4 μg/mL and 8-32 μg/mL, respectively (Taylor, 2000). *H. pylori* strains associated with A2143G, A2142G and A2142C mutations had MIC's in a range of 64-128 μg/mL (Gibreel et al., 2005).

2.4.3. Stability

There are several possible explanations for the absence of a truly successful *H. pylori* eradication regimen. These include the drug delivery problems associated with directing antibacterial agents to the protective residential microenvironment in the gastric mucosa, the presence of 'sanctuary sites' for *H. pylori* such as extragastric sites, regions of the stomach sites such as gastric pits, and the chemical stability of antibacterial agents (Zheng et al., 2006). To inhibit *H.pylori*, minimum inhibitory concentrations of the drug should be available around the adherent mucus layer and gastric epithelial cells where *H. pylori* resides. The maintenance of inhibitory concentrations in the gastric milieu after intravenous drug administration depends on the

ability of the drug to be transferred to the gastric milieu from the blood circulation and the solubility of the drug in the mucus layer around *H. pylori*. After oral adminstration, the drug must be able to diffuse through the gastric mucus in sufficient amounts to generate antibacterial activity at sites of *H. pylori* residence (Erah et al., 1997).

Although metronidazole is known to be stable between pH 3.9 and 6.6, amoxycillin and clarithromycin are unstable at low pH, and the instability of these drugs at the normal gastric pH (pH 1–2) may affect their delivery to the sites where *H. pylori* resides in the stomach. The optimum stability of clarithromycin have been found over the pH range 5.0–8.0. At pH values below 5.0, the degradation rate increased markedly and 90.2% and 41.1% of clarithromycin was calculated to have been lost from the aqueous samples at pH 1.0 and pH 2.0, respectively, within the first hour of the experiment (Erah et al., 1997). The use of omeprazole, which is a potent proton-pump inhibitor which markedly reduces the secretion of acid in the stomach, hence increasing gastric pH towards neutrality to raise the pH of the stomach in patients receiving oral clarithromycin, has been reported to be necessary for the desired therapeutic effect. (Erah et al., 1997).

Clarithromycin undergoes hepatic metabolism; at least eight metabolites are recovered. 14-Hydroxyclarithromycin is the only metabolite shown to be both microbiologically active and present in the plasma at high concentrations. Approximately 20%-30% of a dose is recovered in the urine as unchanged clarithromycin. Another 10%-15% is recovered in the urine as 14-hydroxyclarithromycin. The mean elimination half-lives of clarithromycin and 14-hydroxyclarithromycin are 2.7-4.8 hours and 4.1-8.7 hours, respectively (Rodvold and Piscitelli, 2003).

CHAPTER 3

ESSENTIAL OILS AGAINST *HELICOBACTER PYLORI*

Essential oils are natural, volatile, complex, multi-component systems composed mainly of terpenes in addition to some other non-terpene components. They are used in the preservation of foods and as antimicrobial, analgesic, sedative, anti-inflammatory, spasmolytic and locally anesthetic remedies (Gupta and Kumar, 2000; Liu et al., 2000)

Essential oils are liquid, limpid and rarely colored, lipid soluble and soluble in organic solvents with a generally lower density than that of water. They are synthesized by all plant organs, i.e. buds, flowers, leaves, stems, twigs, seeds, fruits, roots, wood or bark, and are stored in secretory cells, cavities, canals, epidermic cells or glandular trichomes. According to climate, soil composition, plant organ, age and vegetative cycle stage, extraction product can vary in quality, quantity and in composition. At present, approximately 3000 essential oils are known, 300 of which are commercially important especially for the pharmaceutical, agronomic, food, sanitary, cosmetic and perfume industries. Essential oils having antimicrobial activity are listed in Table 3.1.

Table 3.1. Essential oils having antimicrobial activity

Essential oils	
Bergamot oil *(Citrus Bergamia)*	Juniper oil, *(Juniperus communis)*
Black pepper oil, *(Piper nigrum)*	Lavender oil, *(Lavandula officinalis)*
Cajeput oil, *(Melaleuca cajuputi)*	Lemon oil, *(Citrus limonum)*
Camphor oil, *(Cinnamomum camphora)*	Lemongrass oil, *(Cymbopogan citratus)*
Cardamom oil, *(Eletteria cardamomum)*	Lime oil, *(Citrus aurantiflora)*
Chamomile oil, *(Matricaria chamomilla)*	Mandarin (red) oil, *(Citrus reticulate)*
Citronella oil, *(Cymopogan nardus)*	Melissa oil, *(Melissa officinalis)*
Cypress, *(Cupessus sempervirens)*	Myrrh oil, *(Commiphora myrrha)*
Eucalyptus oil *(Eucalyptus globules)*	Orange oil (sweet), *(Citrus sinensis)*
Fennel oil, *(Foeniculum vulgare)*	Oregano oil, (Origanum vulgare)
Frankincense oil, *(Boswellia frereana)*	Peppermint oil, *(Mentha piperita)*
Geranium oil, *(Geranium pretense)*	Pine oil, *(Pinus nigra)*
Helichrysum oil, *(Helichrysum italicum)*	Tangerine oil, *(Citrus reticulate)*
Jasmine oil, *(Jasminum grandiflorum)*	Tea *(Melaleuca alternifolia)*

Essential oils or some of their components are used in perfumes and make-up products, in sanitary products, in dentistry, in agriculture, as food preservers and additives, and as natural remedies. Major components of essential oils that exhibit antibacterial properties are tabulated in Table 3.2. Moreover, some essential oils appear to exhibit particular medicinal properties that have been claimed to cure one or another organ disfunction or systemic disorder (Bakkali et al., 2007).

Several techniques can be used to extract essential oils from different parts of the aromatic plant, including water or steam distillation, solvent extraction, expression under pressure, supercritical fluid and subcritical water extractions (Edris, 2007).

Essential oils, as natural sources of phenolic components, attract investigators to evaluate their activity as antioxidants or free radical scavengers. The essential oils of basil, cinnamon, clove, nutmeg, oregano and thyme have been proven radical-scavenging and antioxidant properties in the DPPH radical assay at room temperature (Tomaino et al., 2005). The antioxidant activity of essential oils cannot be attributed only to the presence of phenolic constituents; monoterpene alcohols, ketones, aldehydes, hydrocarbons and ethers also contribute to the free radical scavenging activity of some essential oils. It is clear that essential oils may be considered as potential natural antioxidants and could perhaps be formulated as a part of daily supplements or additives to prevent oxidative stress that contributes too many degenerative diseases. The suppression effects of essential oils on glioma, colon cancer, gastric cancer, human liver tumor, pulmonary tumors, breast cancer, leukemia and others have been tested on human cancer cells (Edris, 2007).

The phenolic compounds also show inhibitory effects to many microorganisms. The mechanism by which phenolic compounds affect the growth of *H. pylori* is unknown, but different theories have been proposed, for example, inhibition of the urease activity, adhesion to human gastric mucus, disintegration of the outer membrane, and inhibition of VacA citotoxin activity which causes the development of inflammation and ulceration in patients (Romero et al., 2007). Abascal and Yarnell indicated that plant based materials having antimicrobial properties may be used as an alternative or combined to the antibiotics yielding lowered antimicrobial resistance (Abascal and Yarnell, 2002).

Essential oils showing inhibitory effect on *H. pylori* were summarized in Table 3.3. Among these essential oils, lemongrass and lemon verbana essential oils have shown the inhibition both in vitro and in vivo conditions (Ohno et al., 2003). The

activities of 60 essential oils against *H. pylori* were evaluated and the results showed that 30 oils were able to affect the growth *in vitro*, and 15 showed strong activity. Among the individual constituents of these oils, carvacrol, isoeugenol, nerol, citral and sabinene exhibited the strongest anti-*H. pylori* effects (Bergonzelli et al., 2003). Moreover, there are many essential oils having antimicrobial activity for different kind of microorganisms that are tabulated in Table 3.2. Therefore, these essential oils may be potential *H. pylori* inhibitors.

Table 3.2. Major components of essential oils that exhibit antibacterial properties

Common name of EO	Latin name of plant source	Major components	Approximate % composition
Cilantro	*Coriandrum sativum* (immature leaves)	Linalool	26%
		E-2-decanal	20%
Coriander	*Coriandrum sativum* (seeds)	Linalool	70%
		E-2-decanal	–
Cinnamon	*Cinnamomum zeylandicum*	Trans-cinnamaldehyde	65%
Oregano	*Origanum vulgare*	Carvacrol	Trace-80%
		Thymol	Trace-64%
		γ-Terpinene	2–52%
		p-Cymene	Trace-52%
Rosemary	*Rosmarinus officinalis*	α-pinene	2–25%
		Bornyl acetate	0–17%
		Camphor	2–14%
		1,8-cineole	3–89%
Sage	*Salvia officinalis* L.	Camphor	6–15%
		α-Pinene	4–5%
		β-pinene	2–10%
		1,8-cineole	6–14%
		α-tujone	20–42%
Clove (bud)	*Syzygium aromaticum*	Eugenol	75–85%
		Eugenyl acetate	8–15%
Thyme	*Thymus vulgaris*	Thymol	10–64%
		Carvacrol	2–11%
		γ-Terpinene	2–31%
		p-Cymene	10–56%

Bergonzelli et al. screened 60 essential oils for their anti-*H. pylori* properties. It was identified that 30 essential oils were able to inhibit *H. pylori* growth in vitro and 15 of them had great bactericidal activities. Among these essential oils, carrot seed, cinnamon bark, manuka and savory oils displayed the strongest bactericidal potential against *H. pylori*. Cinnamon oil was used as a positive control since the oil and its extracts had already been showed as active against *H. pylori* in previous studies. Moreover, it was reported to be better determining the antibacterial activities of

essential oil components rather than that of oil in case the composition may change in different climates and regions (Bergonzelli et al., 2003). Ohno et al. stated that thirteen essential oils used in their study completely inhibited the growth of *H. pylori* in vitro at a concentration of 0.1% (v/v). *Cymbopogon citratus* (lemongrass) and *Lippia citriodora* (lemon verbena) were bactericidal against *H. pylori* at 0.01% at pH 4.0 and 5.0. In vivo studies demonstrated the density of *H. pylori* in the stomach of mice treated with lemongrass was significantly reduced compared with untreated mice. Regarding these results, the use of essential oils has been asserted as new and safe antimicrobial to overcome the resistance problem in eradication of *H. pylori* (Ohno et al., 2003). On the contrary to increase in antibiotic resistance, there has been no indication of resistance to plants and plant based drugs. Shikov et al. determined the MIC of *Chamomilla recutita* oil as 125 mg/ml. At this concentration urease enzyme production has stopped and the morphological property of *H. pylori* has changed. Thus, it was stated that the *Chamomilla recutita* oil may be used in the treatment of stomach ulcers and duodenal intestinal diseases and to eradicate the antibiotic resistant strains of *H. pylori* (Shikov et al., 2008).

Table 3.3. Essential oils effective in *H. Pylori* eradication

Essential Oils	**Results in vitro/in vivo**	**Reference**
Lemongrass lemon verbena	+/+	(Ohno et al. 2003)
Carrot seed, Cinnamon bark, Clove Caraway, Eucalyptus, Pink grapefruit, White grapefruit, Lemongrass, Manuka, Oregano (vulgaris) Sage, Savory, Tarragon, Thyme (red), Thyme (vulgaris), Vervein	+/-	(Bergonzelli et al. 2003)
Chamomilla recutita	+/n.s	(Shikov et al. 2008)
Dittrichia viscosa	+/n.s	(Miguel et al. 2008)
Garlic Oil, Garlic Powder	+/n.s	(O'gara et al. 2000)
Origanum, cassia, cumin, lavender oils, myrtle and bay leaf pumpkin seed and sage, olive oil	+/n.s *olive oil didn't prevent growth	(Preuss et al. 2005)
Olive oil	+/n.s	(Romero et al. 2007)
Myrtus communis	+/n.s	(Deriu et al. 2007)

n.s: not studied

3.1. Lemongrass Oil

Lemongrass *(Cymbopogon citratus)* belongs to a family of fragrant grasses, of Indian origin, which includes palmarosa *(Cymbopogon martinii)* and citronella *(Cymbopogon nardus);* lemongrass is a tall grass which grows up to 5 feet high within a short period of time. It produces a network of roots and rootlets that rapidly exhaust the soil. It is now a cultivar of many tropical countries including central Africa, Sri Lanka, Brazil, Madagascar, Vietnam and Malaysia.

Lemongrass oil is extracted from the fresh or partly dried leaves by steam distillation. It has a lemony, sweet smell and is dark yellow to amber and reddish in color, with a watery viscosity. The main chemical components of lemongrass oil are myrcene, citronellal, geranyl acetate, nerol, geraniol, neral and traces of limonene and citral (Ohno et al., 2003).

The therapeutic properties of lemongrass oil are analgesic, anti-depressant, antimicrobial, antipyretic, antiseptic, astringent, bactericidal, carminative, deodorant, diuretic, febrifuge, fungicidal, galactagogue, insecticidal, nervine, nervous system sedative and tonic. It is a great overall tonic for the body and it boosts the parasympathetic nervous system, which is a boon when recovering from illness, as it also stimulates glandular secretions. It is useful with respiratory infections such as sore throats, laryngitis and fever and helps prevent spreading of infectious diseases. It is helpful with colitis, indigestion and gastro-enteritis. Lemongrass essential oil has anti microbial property which makes it inhibit microbial and bacterial growth in the body, internally or externally. It is seen to be effective in inhibiting bacterial infections in colon, stomach, urinary tracts, wounds, respiratory system etc. and helps cure diseases resulting from bacterial or microbial infections such as typhoid, food poisoning, skin diseases, malaria (caused by protozoon) etc (Maizura et al., 2007). It has been shown that lemongrass inhibited the growth of all *H. pylori* strains studied (either antibiotics-susceptible or –resistant strains), even at a concentration of 0.01% (v/v) (Ohno et al., 2003).

3.2. Clove Leaf Oil

Clove essential oil is extracted from Eugenia caryophyllata (also known as Syzygium aromaticum, Eugenia aromatica, E. carophyllus) of the Myrtaceae family.

Clove oil has a warm, strong, spicy smell and the oil is colorless to pale yellow with a medium to watery viscosity. Clove oil can be extracted from the leaves, stem and buds. We sell clove leaf oil, which is extracted by water distillation, containing the desired lower percentage of eugenol. The main chemical components of clove oil are eugenol, eugenol acetate, iso-eugenol and caryophyllene (Lawless, 1995).

The therapeutic properties of clove oil are analgesic, antiseptic, antispasmodic, anti-neuralgic, carminative, anti-infectious, disinfectant, insecticide, stimulant, stomachic, uterine and tonic (Fu et al., 2009). Clove oil can be used for acne, bruises, burns and cuts, keeping infection at bay and as a pain reliever. It helps with toothache, mouth sores, rheumatism and arthritis. It is beneficial to the digestive system, effective against vomiting, diarrhea, flatulence, spasms and parasites, as well as bad breath. Clove oil is valuable for relieving respiratory problems, like bronchitis, asthma and tuberculosis. The disinfecting property is useful in cases of infectious diseases. It is considered safe in very small quantities (<1500 p.p.m.) as a food additive (Bruneton, 1995).

3.3. Lemon Oil

Lemon essential oil is extracted from the Citrus limonum (also known as Citrus Limon), of the Rutaceae family and is also known as cedro oil (which refers to terpeneless oil). Lemon oil is extracted from the fresh fruit peel by cold expression. Lemon oil has a sharp, fresh smell, is pale greenish-yellow in color and is watery in viscosity. The main chemical components of lemon oil are a-pinene, camphene, b-pinene, sabinene, myrcene, a-terpinene, linalool, b-bisabolene, limonene, trans-a-bergamotene, nerol and neral. It is non toxic (Hayes and Markovic, 2002).

The therapeutic properties of lemon oil are anti-anemic, antimicrobial, anti-rheumatic, anti-sclerotic, antiseptic, bactericidal, carminative, cicatrisant, depurative, diaphoretic, diuretic, febrifuge, haemostatic, hypotensive, insecticidal, rubefacient, tonic and vermifuge. Lemon oil can be very beneficial to the circulatory system and aids with blood flow, reducing blood pressure and helping with nosebleeds. It can help bring down fever, helps relieve throat infections, bronchitis, asthma and flu. It boosts the immune system and cleanses the body, improves the functions of the digestive system, and it is helpful with constipation, dyspepsia and cellulite. Lemon oil soothes and

relieves headaches and migraines and is helpful for rheumatism and arthritis. It is also used for clearing acne, cleaning greasy skin and hair, as well as removing dead skin cells, easing painful cold sores, mouth ulcers, herpes and insect bites (Duccio et al., 1998).

3.4. Thyme Oil

Thyme oil is obtained from Thymus vulgaris (also known as *Thymus aestivus, T. ilerdensis* and *T. velantianus*) of the Labiatae family and is also known as common or red thyme. It is extracted from the fresh or partly dried flowering tops and leaves of the plant by water or steam distillation and the yield is 0.7 -1.0 %. It has a rather sweet, yet strongly herbal smell and is reddish-brown to amber in color. The oil is mainly located in small glands on the leaves and contains thymol, paracymene & linalool. Its chemical components are a-thujone, a-pinene, camphene, b-pinene, p-cymene, a-terpinene, linalool, borneol, b-caryophyllene, thymol and carvacrol. The essential oil of common thyme (*Thymus vulgaris*) is made up of 20-54% thymol. Thymol, an antiseptic, has been extensively documented for its antibacterial, antiviral, and anti-fungal action. According to Valnet, 1990, thyme oil kills the anthrax bacillus, the typhoid bacillus, meningococcus, and the agent responsible for tuberculosis and is active against salmonella and staphylococcus bacteria. In addition, this component has been studied for its effects on gingivitis and plaque-caused organisms in the mouth (Valnet, 1990). It has also been shown to be effective against the fungus that commonly infects toenails (Ramsewak et al., 2003).

The therapeutic properties of thyme oil are antirheumatic, antiseptic, antispasmodic, bactericidal, bechic, cardiac, carminative, cicatrisant, diuretic, emmenagogue, expectorant, hypertensive, insecticide, stimulant, tonic and vermifuge. Thyme oil strengthens the nerves, aids memory and concentration, can help with the feeling of exhaustion and combats depression, while it fortifies the lungs and helps with colds, coughs, asthma, laryngitis, sinusitis, sore throats and tonsillitis. Thyme oil is beneficial to boost the immune system and can help fight colds, flu, infectious diseases and chills and as a urinary antiseptic, it is very helpful for cystitis and urethritis (Bruneton, 1995).

3.5. Cinnamon Bark Oil

Cinnamon, which has the scientific name *Cinnamomum zeylanicum*, has originated from tropical Asia, especially Sri Lanka and India. Now the shrub is grown in almost every tropical region of the world. The herb, owing to its vast medicinal uses, had found a prominent position in traditional medicines, especially Ayurveda (the traditional Indian medicinal system). Traditionally cinnamon, due to its medicinal properties, has been used in many cultures for treating a variety of health disorders including diarrhea, arthritis, menstrual cramps, heavy menstruation, yeast infections, colds, flu, and digestive problems. The most widely used part of cinnamon is its bark. Steam distillation is the mostly adopted technology for production of cinnamon bark oil. However, there are occasional moves for the extraction with Super Critical Carbon dioxide (CO_2 Extracts) for quality markets.

Today cinnamon bark oil is one of the most powerful antibacterial, antifungal, antiviral oils of all-even stronger than most antibiotics (Tung et al., 2010; Cava et al., 2007). This oil is a powerful antioxidant. It may also be beneficial for circulation, infections, coughs, exhaustion, respiratory infections, digestion, rheumatism, and warts. This is a warming oil for the spirit as well as for the body. It restores wakefulness in the presence of fatigue. It is vitalizing, refreshing as well as warming. The warm glow of cinnamon helps transform sorrow into happiness. For emotional healing, it is used to encourage invigoration, benevolence, strength, and energy. Cinnamon bark oil possesses the delicate aroma of the spice with a sweet and pungent taste. Cinnamon bark oils major constituent is cinnamaldehyde but other, minor components impart the characteristic odour and flavour that is world renowned. It is employed mainly in the flavoring industry where it is used in meat and fast food seasonings, sauces and pickles, baked goods, confectionery, cola-type drinks, tobacco flavors and in dental and pharmaceutical preparations (Rose, 1992).

CHAPTER 4

CHITOSAN IN DRUG DELIVERY SYSTEMS

4.1. Sources, Structure, and Physicochemical Properties of Chitosan

Chitin, which is the most abundant biopolymer in nature after cellulose, is a principal component of exoskeletons of crustaceans and insects as well as of cell walls of some bacteria and fungi. Chitosan is derived from of the chitin and is produced by partial alkaline deacetylation of chitin to form the polymer of acetyl glucosamine. The primary unit of chitosan is 2-amino-2-deoxy-D-glucose and with β→1-4 glucosidic linkages polymer chain is formed. Depending on its degree of deacetylation, the structure of chitosan includes 2-acetamido-2-deoxy-D-glucose. The chemical structure of chitosan is given in Figure 4.1.

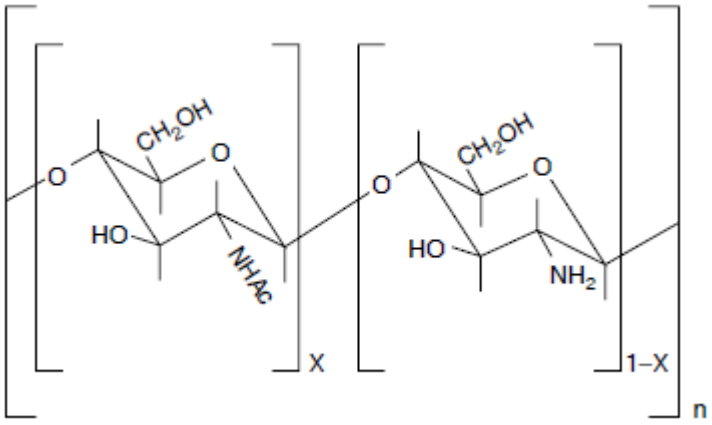

Figure 4.1. Chemical structure of chitosan with X=degree of deacetylation and n=number of sugar units per polymer (Source: Dodane and Vilivalam, 1998).

Chitosan is a polycationic polymer that has one amino group and two hydroxyl groups in the repeating glucosidic residue. The carbohydrate backbone is very similar to cellulose, which consists of β→1-4 -linked D-glucosamine. An important difference to cellulose is that chitosan is composed of acetylamino groups on the structure (Figure 4.2) (Dash et al., 2011).

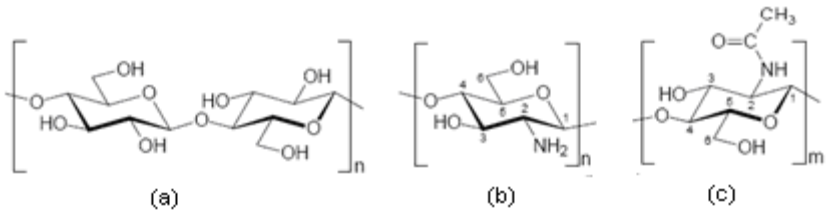

Figure 4.2. Chemical structures of cellulose (a), Chitosan (b) and chitin (c) (Source: Jayakumar et al., 2010).

Chitin is second most abundant polymer in nature. However, applications of chitin are limited compared to chitosan because chitin is chemically inert. Due to its poor solubility in aqueous solution and organic solvents, it does not find practical applications whereas chitosan as an artificial variant of chitin is more suitable for useful bioapplications. So, chemically modified chitosan has multidimensional properties and a wide range of applications in biomedical and other industrial areas.

Acetamide group of chitin can be converted into amino group to give chitosan, which is carried out by treating chitin with concentrated alkali solution. The treatment of chitin with an aqueous 40–45% (w/v) NaOH solution at 90–120 ºC for 4–5 h results in deacetylation of chitin (Dash et al., 2011). The conditions used for deacetylation determines the polymer molecular weight and the degree of deacetylation.

Chitin and chitosan represent long-chain polymers having molecular mass up to several million Daltons. Chitosan is relatively reactive and can be produced in various forms such as powder, paste, film, fiber, etc. (Agnihotri et al., 2004). Commercially available chitosan has an average molecular weight ranging between 3800 and 20,000 Daltons and is 66% to 95% deacetylated.

The active primary amino groups on the molecule being reactive provide sites for a variety of side group attachment employing mild reaction conditions (Figure 4.3). Chitosan, being a cationic polysaccharide in neutral or basic pH conditions, contains free amino groups and hence, is insoluble in water. In acidic pH, amino groups can undergo protonation thus, making it soluble in water. Solubility of chitosan depends upon the distribution of free amino and N-acetyl groups. Usually 1–3% aqueous acetic acid solutions are used to solubilize chitosan. Chitosan is also soluble in dilute nitric and hydrochloric acids, marginally soluble in 0.5% phosphoric acid and insoluble in sulfuric acid at room temperature.

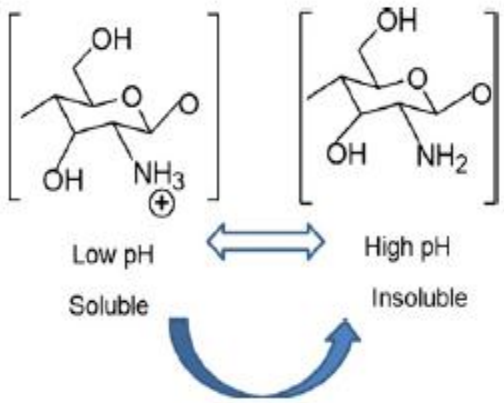

Figure 4.3. Schematic illustration of chitosan's versatility. At low pH (less than about 6), chitosan's amine groups are protonated (because amine group has a pKa value of 6.3) conferring polycationic behavior to chitosan. At higher pH (above about 6.5), chitosan's amines are deprontonated and reactive (Source: Dash et al., 2011).

Chitosan a linear polyelectrolyte at acidic pH, is soluble in variety of acids and interacts with polyanionic counterions. It forms gels with a number of multivalent anions. It has a high charge density i.e. one charge per glucosamine unit. Since many minerals carry negative charges, the positive charge of chitosan interacts strongly with negative surfaces. Chitosan is a linear polyamine where amino groups are readily available for chemical reactions and salt formation with acids.

The attached side groups on chitosan provide versatile materials with specific functionality, alter biological properties or modify physical properties. For example, the characteristic features of chitosan such as being cationic, hemostatic and insoluble at high pH, can be changed by sulfating the amine group which makes the molecule anionic and water-soluble (Suh and Matthew, 2000).

The important characteristics of chitosan are its molecular weight, viscosity, deacetylation degree (DA) crystallinity index, number of monomeric units (n), water retention value, pKa and energy of hydration.

A linear unbranched structure and high molecular chitosan is an excellent viscosity enhancing agent in acidic environments. The viscosity of chitosan solution increases with an increase in the concentration of chitosan, decrease in temperature and with increasing degree of deacetylation. Viscosity also influences biological properties such as wound-healing properties (Altiok et al., 2010) and osteogenesis enhancement as well as biodegradation by lysozyme (Dash et al., 2011).

4.2. Biological Properties of Chitosan

Chitosan has been widely used in medical and pharmaceutical applications because they have interesting properties that make them suitable for use in the biomedical field, such as biocompatibility, biodegradability and non toxicity. Moreover, other properties such as analgesic, antitumor, hemostatic, hypocholesterolemic, antimicrobial, and antioxidant properties have also been reported (Aranaz et al., 2009, Gobin et al., 2005, Gonzalez et al., 1999). The majority of the biological properties are related to the cationic behaviour of chitosan, the parameter with a higher effect is the degree of deacetylation. However, in some cases (Dash et al., 2011), the molecular weight has a predominant role.

Chitosan can be degraded by several proteases (lysozyme, papain etc.). Besides, chitosan degrades under the action of fermentation and it is nontoxic and easily removable from the organism without causing concurrent side reactions. Their biodegradation leads to the release of non-toxic oligosaccharides of variable length which can be subsequently incorporated to glycosaminoglycans and glycoproteins, to metabolic pathways or be excreted (Aranaz et al., 2009). The degradation of chitosan by enzymatic reaction depends on the degree of deacetylation and length of the chain (molecular weight of the chitosan), because the number and distribution of acetyl groups affects biodegradability.

Chitosan is biocompatible with living tissues since it does not cause allergic reactions and rejection. It breaks down slowly to harmless products (amino sugars), which are completely absorbed by the human body (Dash et al., 2011). However, fast degradation rates will cause accumulation of amino sugars and hence cause inflamatory response (Aranaz et al., 2009). Chitosan absorbs the proton ion released in the inflammatory area and thus has the main analgesic effect. The free amino groups of chitosan can protonate in the presence of proton ion and the reduction in pH is the main cause of the analgesic effect.

Chitosan possesses antimicrobial property and absorbs toxic metals like mercury, cadmium, lead, etc. The polycationic structure of chitosan is responsible for the antimicrobial activity of chitosan. As environmental pH is below the pKa of the chitosan, electrostatic interactions between the polycationic structure of the chitosan and anionic components of the microorganisms' surface plays a primary role (Kong et al.,

2010). Thus, the number of amino groups in chitosan structure is important in electrostatic interaction, which strongly depends on the degree of deacetylation. Large amount of amino groups are able to enhance the antimicrobial activity. As environmental pH is above pKa, hydrophobic and chelating effects are responsible for antimicrobial activity. In another study, Aranaz et al. (2009) reported that the degradation product of the chitosan has important role to inhibit the microorganisms and has more antimicrobial activities than the chitosan.

Chitosan has shown a significant scavenging capacity against different radical species, the results being comparable to those obtained with commercial antioxidants. Samples prepared from crab shell chitin with degree of deacetylation of 90, 75 and 50% where evaluated on the basis of their abilities to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, hydroxyl radical, superoxide radical and alkyl radical. The results revealed that chitosan with higher DD exhibited the highest scavenging activity (Park et al., 2004). The chelation of metal ions is one of the reasons why chitosan may be considered as a potential natural antioxidant for stabilizing lipid containing foods. Both degree of deacetylation and molecular weight of the chitosan affect on the antioxidative property of the chitosan and it increases by increasing the number of amino groups in chitosan structure.

An antitumor activity of chitosan has been claimed by inhibition of the growth of tumor cells mainly due to an immune stimulation effect. Jeon and Kim (2002) have found that chitosan oligomers possess antitumor activities tested both *in vitro* and *in vivo*. Studies carried out using mice that had ingested low-molecular weight chitosan revealed significant antimetastatic effects of chitosan against Lewis lung carcinoma.

Chitosan is one of the mucoadhesive polymers that may fulfill the desirable features of a prolonged residence time at the site of drug absorption owing to increased contact with the mucosa, resulting in localization in specified regions to improve the bioavailability of drugs (Pedro et al., 2009). The mucus is composed of a glycoprotein called mucin, which is rich in negative charges since it has sialic acid residues (He et al., 1998). In the stomach, chitosan is positively charged due to the acidic environment and, therefore, it can interact with mucin by electrostatic forces. This mucoadhesive property of the chitosan lets use it as drug delivery systems in gastrointestine. The extent of this union depends on the amount of sialic acid present in the mucin (He et al., 1998) and on the molecular weight and degree of deacetylation of chitosan. It has been found that when the molecular weight of chitosan increases, the penetration in the

mucin layer also increases and hence the mucoadhesion is stronger (Lehr et al., 1992). On the other hand, a higher degree of deacetylation leads to an increase in charge density of the molecule and the adhesive properties become more relevant (He et al., 1998).

Additionally, because of the beneficial properties of the chitosan, it has found wide applications in many industries such as in cosmetics as a fungicide and viscosity enhancer, in opthalmology for contact lenses, in environmental protection as the aid for heavy metal removal, and color removal from textile mill effluents, in food as antimicrobial and antioxidative agent,

4.3. Biomedical Applications of Chitosan

Due to its high biocompatibility, chitosan has been employed in drug delivery systems, surgical dressings, implantable and injectable systems such as orthopaedic and periodontal composites, wound healing management and scaffolds for tissue regeneration.

4.3.1. Wound Dressing Material

Chitosan oligomers have also exhibited wound-healing properties, it is suggested that their wound-healing properties are due to their ability to stimulate fibroblast production by affecting the fibroblast growth factor. Subsequent collagen production further facilitates the formation of connective tissue (Kweon et al., 2003, Wittaya-areekul and Prahsarn., 2006, Ishihara et al., 2002). Recently, the use of natural bioactive compounds, such as essential oils have been used in wound healing application. Chitosan's wound healing property together with both thyme oil's antimicrobial activity and the role of thymol (active compound in thyme oil) in wounds make it possible to have functional wound healing material (Altiok et al., 2010).

4.3.2. Drug Delivery Systems

An important application of chitosan in industry is the development of drug delivery systems such as nanoparticles, hydrogels, microspheres, films and tablets. If degree of deacetylation and molecular weight of chitosan can be controlled, then it would be a material of choice for developing micro/nanoparticles. Chitosan has many advantages, particularly for developing micro/nanoparticles. These include;

- its ability to control the release of active agents, it avoids the use of hazardous organic solvents while fabricating particles since it is soluble in aqueous acidic solution,
- it is a linear polyamine containing a number of free amine groups that are readily available for crosslinking,
- its cationic nature allows for ionic crosslinking with multivalent anions,
- it has mucoadhesive character, which increases residual time at the site of absorption,
- it prolongs the duration of drug activity and improves therapeutic efficiency,
- it reduces side effects,
- it has very low toxicity; LD50 of chitosan in laboratory mice is 16 g/kg body weight, which is close to sugar or salt.
- it is possible to use various sterilization methods such as ionizing radiation, heat, steam and chemical methods can be suitably adopted for sterilization of chitosan in clinical application.

In view of the above-mentioned properties, chitosan is extensively used in developing drug delivery systems. Particularly, chitosan has been used in the preparation of mucoadhesive formulations (Nascimento et al., 2001; Obara et al., 2005; Falk et al., 2004; Guo et al., 2007), improving the dissolution rate of the poorly soluble drugs (Gobin et al., 2005; Jain et al., 2007, Bigia et al., 2002), drug targeting (Ekici and Saraydin, 2007; Yuan et al., 2007) and enhancement of peptide absorption (Obara et al., 2005; Falk et al., 2004; Bigia et al., 2002).

The achievement of predictable and reproducible release of an agent into a specific environment over an extended period of time has much significant subject. It creates a desired environment with optimum response, minimum side-effects and

prolonged efficiacy (Figure 4.4). Controlled release dosage forms enhance the safety, efficacy and reliability of drug theraphy.

Generally, a drug is administered in a high dose at a given time only to repeat that dose several hours or days later (Figure 4.4). This conventional dosage forms often leads to wide swings in serum drug concentrations. Most of the drug content isi released soon after administration, causing drug levels in the body to rise rapidly, peak and decline sharply. Those sharp fluctuations often cause unacceptable side effects at the peaks, followed by inadequate theraphy. On the other hand, controlled release of the drug can eliminate the overdose of the drug in plasma, and accordingly side effects, so effective theraphy is observed. Besides this, many drugs have beneficial effects when they are delivered slowly over a prolonged period. As a consequence, increasing attention has been focused on methods of giving drugs continually for prolonged time periods and in a controlled fashion.

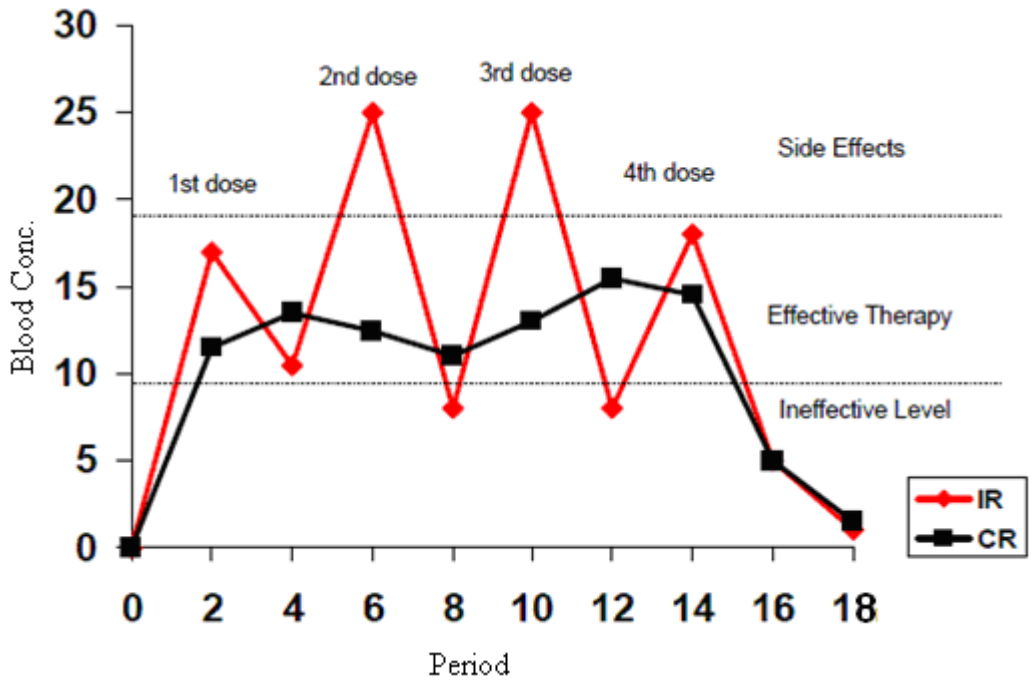

Figure 4.4. Comparison of the high dose administration (IR) and prolonged controlled release (CR) of the drug. Effective theraphy is achieved without any side effects by CR.

The primary method of accomplishing this controlled release has been through incorporating the drugs within polymers. The optimal carrier would preserve the biological activity of drug and prevent its rapid release. Because of the beneficial

biological and physical properties of the chitosan, it can be good candidate for the drug carrier systems.

The primary hydroxyl and amine groups located on backbone of chitosan allow chemical modification to control its physical properties. When the hydrophobic moiety is conjugated to a chitosan molecule, the resulting amphiphile may form self assembled nanoparticles that can encapsulate a quantity of drugs and deliver them to a specific site (Park et al., 2010). Chemical attachment of the drug to the chitosan throughout the functional linker may produce useful systems, exhibiting the appropriate biological activity at the target site. Additionally, mucoadhesive and absorption enhancement properties of chitosan increase the in vivo residence time of the dosage form in the gastrointestinal tract and improve the bioavailability of various drugs.

Chitosan is used in drug delivery system in many forms, such as hydrogels, tablets, and transdermal delivery forms and as microcapsules and nanoparticules.

4.3.2.1. Microsphere Production Techniques

Different methods have been employed to prepare chitosan particulate systems. Figure 4.5. summarizes the methods most widely applied to produce microspheres. For selection of any of the methods one should take into consideration factors such as particle size requirement, thermal and chemical stability of the active agents, reproducibility of the release kinetic profiles, stability of the final product, residual toxicity associated with the final products, the nature of the active molecule as well as the type of the delivery device.

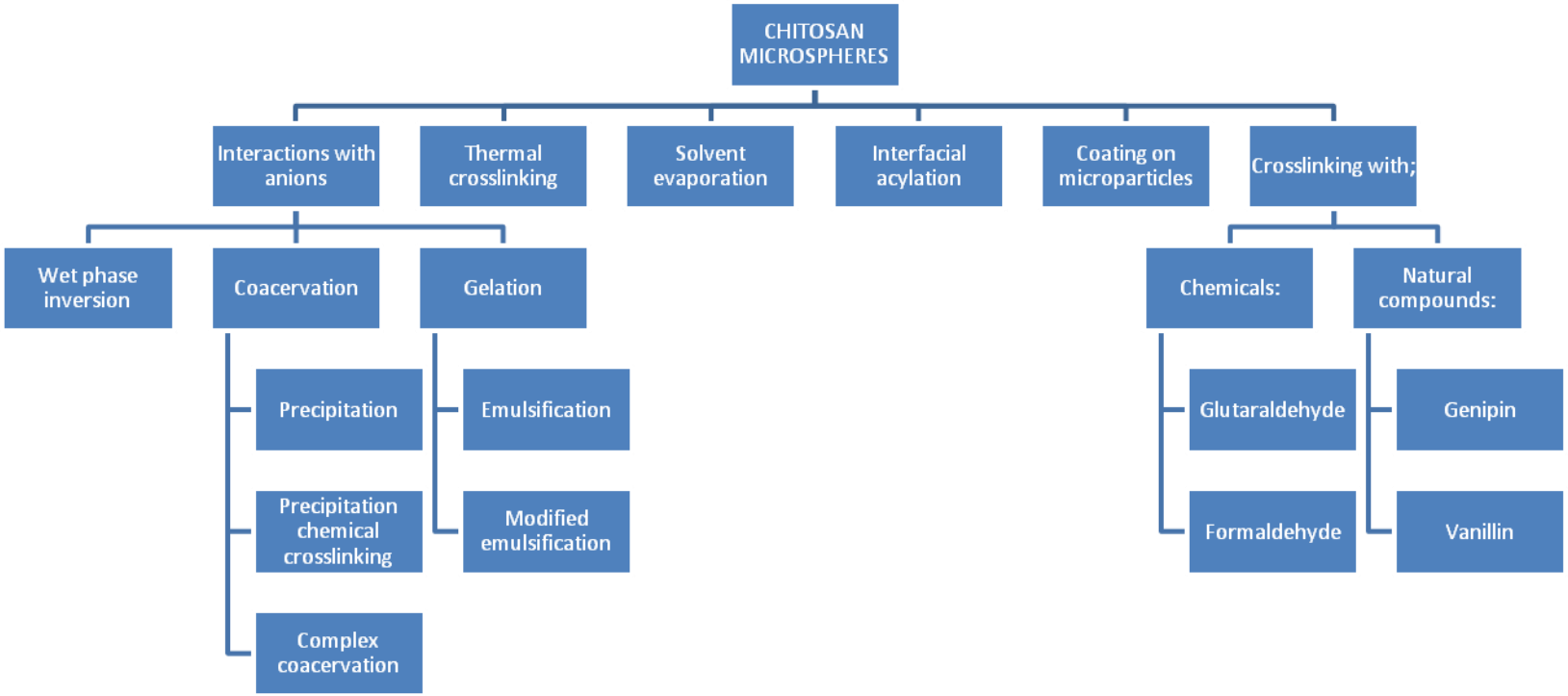

Figure 4.5. Methods for preparation of chitosan microspheres.

4.3.2.1.1. Ionotropic gelation

In this method, chitosan solutions in acetic acid are prepared and extruded drop wise through a needle into different concentrations of aqueous solutions of magnetically stirred counterions such as tripolyphosphate, alginate, lauryl sulphate etc. The beads are removed from the counter ion solution by filtration, washed with distilled water, dried by an air jet and further air dried at ambient temperature (Agnihotri et al., 2004).

4.3.2.1.2. Wet Phase Inversion

In this method of preparation, chitosan solution in acetic acid is dropped into an aqueous solution of a counterion sodium tripolyphosphate through a nozzle. Microspheres formed are allowed to stand for 1 h, washed and crosslinked with 5% ethylene glycol diglycidyl ether. Finally, the microspheres are washed and freeze-dried to form porous chitosan microshperes (Mi et al., 1999). Changing the pH of the coagulation medium could modify the pore structure of the chitosan microsphere.

4.3.2.1.3. Emulsification and Ionotropic Gelation

This method exploits the reactive functional amine group of chitosan to cross-link with the available reactive groups of the cross-linking agent. A suitable surfactant is used to stabilize the aqueous droplets. In this method the dispersed phase, which consists of an aqueous solution of chitosan, is added to a non-aqueous continous phase (emulsifer) to form a water-in-oil (w/o) emulsion. Sodium hydroxide solution is then added at different intervals leading to ionotropic gelation. The microspheres thus formed are removed by filtration, washed and dried. This method, is helpful in controlling the size of the particles by controlling the size of aqueous droplets. However, the particle size of final product is dependent on the extent of cross-linking agent used while hardening along with the speed of stirring.

The emulsion cross-linking method involves a few drawbacks. Besides being tedious it uses harsh cross-linking agents, which might possibly induce chemical

reactions with the active agent. Moreover, complete removal of the unreacted cross-linking agent may be a challenge.

4.3.2.1.4. Coacervation and Complex Coacervation

In this process, the polymer is solubilized to form a solution. This is followed by addition of a solute, which forms insoluble polymer derivative and precipitates the polymer. This process avoids the use of toxic organic solvents and glutaraldehyde used in the other methods of preparation of chitosan microspheres. Chitosan microparticles can also be prepared by complex coacervation. Sodium alginate, sodium carboxymethylcellulose, κ-carregeenan and sodium polyacrylic acid can be used for complex coacervation with chitosan to form microspheres (Sinha et al., 2004). These microparticles are formed by interionic interaction between oppositely charged polymers. Formulation of coacervate capsules of chitosan–alginate and chitosan–κ-carrageenan was carried out by interaction of the above-mentioned solutions with potassium chloride and calcium chloride, respectively. The obtained capsules were hardened in the counterion solution before washing and drying.

The physicochemical property of chitosan is utilized in this method since it is insoluble in alkaline pH medium, thus precipitates/coacervates when it comes in contact with alkaline solution. Chitosan solution is blown into an alkali solution like sodium hydroxide, NaOH–methanol or ethanediamine using a compressed air nozzle to form coacervate droplets (Dash et al., 2011). Separation and purification of particles are performed by filtration/centrifugation followed by successive washing with hot and cold water. The method is schematically represented in Figure 4.6. Variation in compressed air pressure or spray-nozzle diameter can be done to control the size of the particles. The drug release can be controlled by using appropriate cross-linking agent.

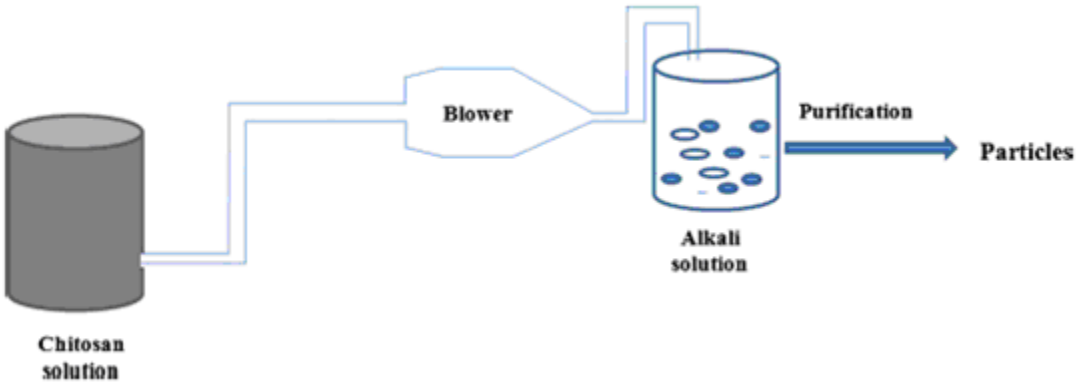

Figure 4.6. Schematic representation of preparation of chitosan particulate systems by coacervation/precipitation method (Source: Sinha et al., 2004).

4.3.2.1.5. Coating by Chitosan

In this method, previously formed microparticles are coated with chitosan. Microspheres are prepared and added to various concentrations of chitosan acetic acid solutions and mixed; the chitosan treated microspheres are filtered and dried.

4.3.2.1.6. Thermal Crosslinking

In this method, crosslinking agent is added to an aqueous acetic acid solution of chitosan and chitosan crosslinked solution is cooled to 0 ºC. Than, this emulsion is added to liquid paraffin oil maintained at 120 ºC and crosslinking is performed (Koradia et al., 2009). The microspheres obtained are filtered and washed with diethyl ether, dried at room temperature.

4.3.2.1.7. Solvent Evaporation Technique

The solvent evaporation technique to produce microcapsules is applicable to wide variety of core materials. This method involves the formation of an emulsion between polymer solution and an immiscible continuous phase whether aqueous (o/w) or non-aqueous (w/o). Chitosan dissolved in an aqueous acetic acid solution. The solution is added to toluene and sonicated to form a w/o emulsion. Glutaraldehyde solution in toluene is added to the emulsion and stirred at room temperature to give

cross-linked microspheres. The suspension is centrifuged. Following evaporation of the solvent, the microspheres are separated, washed with distilled water and dried. Here system is warmed to 50 ºC and the pressure is reduced. When the solvent is evaporated completely the microspheres are separated, washed with sodium hydroxide solution, distilled water and diethyl ether and dried.

4.3.2.1.8. Spray Drying

Spray drying is one of the microencapsulation technique in which an active material is dissolved or suspended in a melt or polymer solution and becomes trapped in the dried particle. Although several methods are available for the preparation of microparticles, spray drying is widely used in the pharmaceutical industry and has been used to produce dry powders, granules, or agglomerates from drug-excipient solutions and suspensions (Gonzalez et al. 1999). This technique can be used either for both heat-resistant and heat-sensitive drugs or for both water-soluble and water-insoluble drugs or for both hydrophilic and hydrophobic polymers. The main advantage of spray drying is the ability to handle labile materials because of the short contact time in the dryer, in addition, it is a one-stage continuous process, easy to scale-up, and only slightly dependent upon solubility of drug and polymer. The particle size of the microspheres prepared by the spray-drying method ranged from a micron to several tens of microns and had a relatively narrow distribution (Desai and Park 2005).

In a spray drying process, a liquid feed is dried with a hot gas. This hot gas is generally air which is replaced by nitrogen for drying sensitive materials such as pharmaceuticals, and solvents like ethanol. The liquid feed may be a solution, colloid or suspension.The method is based on drying of atomized droplets in a stream of hot air. Briefly, chitosan is dissolved in aqueous acetic acid solution, drug is then dissolved or dispersed in the solution followed by the addition of a suitable cross-linking agent. This solution or dispersion is then atomized in a stream of hot air that leads to the formation of small droplets, from which solvent evaporates instantaneously leading to the formation of free flowing particles (Figure 4.7).

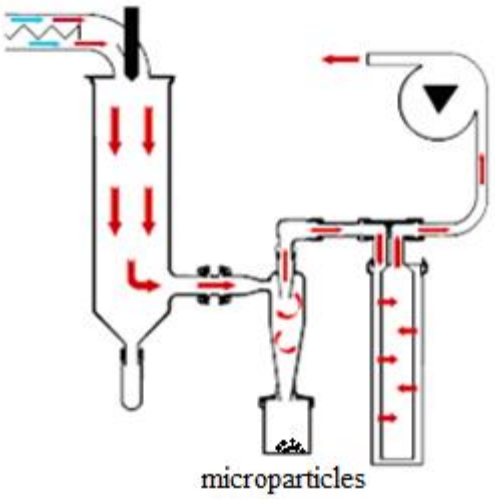

Figure 4.7. Schematic representation of preparation of particulate system by spray drying metod.

Selection of the atomiser (nozzle) is one of the most important choices in spray dryer design and has a significant effect upon the size distribution of the final dried particles. The most important atomiser characteristics from the standpoint of product quality are uniformity of drop-size, control of drop-size distribution, and homogeneity of the spray. Atomisers common forms are pressure atomisers, centrifugal (wheel) atomisers and pneumatic (two-fluid) atomisers (Re, 1998). Drying process conditions, such as dryer inlet and outlet air temperatures, drying air velocity, solution feed rate, dryer feed temperature, humidity of the dryer inlet air etc. directly influence on the either physicochemical properties (shape, mean particle size, integrity, porosity, bulk volume ec.) of the final microparticles (Masters, 1985) and biological activities of encapsulated active materials. Accordingly, properties of encapsulated materials (molecular weight, vapor pressure, concentration in the emulsion), properties of the capsule material (type and molecular weight, chemical interactions with the active compounds), properties of the emulsion (dissolved solids content, viscosity, oil droplet size distribution etc.) should influence the final microspheres' properties (Re, 1998).

In comparison with a wide variety of microencapsulation techniques, spray drying may offer the advantage of realising the microencapsulation process in one step. In addition, it seems to come close to the properties desired for an ideal method of preparation of controlled release microparticles: spray drying offers rapidity, little dependency on the solubility characteristics of the drug and polymer, and is easy to scale up (Re, 1998). From these considerations, the evaluation of the spray drying as a method for encapsulation of drugs has been a research area of increasing interest.

A number of articles have been published describing the preparation of microspheres by such spray drying methods. For example, microspheres composed of chitosan were prepared for the delivery of cimetidine, famotidine, nizatidine (He et al., 1999) and vitamin C (Desai et al., 2006). Glutaraldehyde was used in most of the studies as a crosslinking agent (He et al., 1999; Desai et al., 2006). In Table 4.1, the drug release studies were summarized in which the microspheres were formed by spray drying. The particle size of the microspheres prepared by spray drying method ranged from a few microns to several tens of microns, and had a relatively narrow distribution. The microspheres were positively charged in order to enhance the mucoadhesive properties and make these suitable for delivery of drugs via the nasal or gastrointestinal routes of delivery (He et al., 1999).

Table 4.1. Microspheres prepared by spray drying for drug release

Polymer	**Crosslinker**	**Drug Released**	**Reference**
Chitosan chondroitin sulphate	formaldehyde	metoclopramide hydrochloride	Gonzalez et al., 1999
Chitosan	glutaraldehyde or formaldehyde	cimetidine, famotidine, nizatidine	He et al., 1999
Gelatin	glutaraldehyde	salmon calcitonin	Desai et al., 2006
Chitosan, hydroxypropyl-methylcellulose, hydroxypropylcellulose, sodium alginate	-	Levocetirizine dihydrochloride	Rathananand et al., 2007
5-methylpyrrolidinone chitosan	-	metoclopramide hydrochloride	Gavini et al., 2008
chitosan/b-cyclodextrin	-	theophylline	Zhang et al., 2008
sodium hyaluronate	-	piroxicam	Piao et al., 2007
bovine serum albumin	glutaraldehyde	gentamicin	Haswani et al., 2006
hydroxypropyl methylcellulose, chitosan, carbopol 934P	-	Propranolol HCl	Harikarnpakdee et al., 2006
chitosan	tripolyphosphate	Vitamin C	Desai et al., 2006

There are number of articles describing the oil encapsulation by spray drying with different wall materials. In these studies, encapsulations of oils by spray drying were performed in order to preserve aroma components of them for food applications. However, it would be very beneficial to investigate these studies since, in most of these studies, spray drying conditions were investigated and effects of drying conditions were analyzed. These studies were summarized in Table 4.2. Generally, 180 °C of inlet temperature was chosen for microsphere production but some studies pointed out the effect of inlet temperature on microsphere's properties (Turchiuli et al., 2005; Klinkesorn et al., 2006; Fuchs et al., 2006; Drusch and Berg, 2008). Feed rate and oil/ polymer ratio were significantly different in these studies. The optimization of these parameters strongly depends on the oil used as a model drug material.

Table 4.2. Spray drying studies for oil encapsulation

Polymer	**Oil Type**	**Spray Drying Conditions**	**Reference**
Chitosan and corn syrup solid	Tuna oil (5%)	Feed rate: 2.2 L/h Inlet T: 165, 180, 195 °C	(Klinkesorn et al. 2006)
nOSA starch and glucose syrup	Fish oil (30% and 50%)	Inlet T: 160, 210 °C Outlet T: 60, 90 °C	(Drusch and Berg 2008)
maltodextrin and acacia gum	Vegetable oil (5%(w/w dry matter)	Feed rate: 22, 68 ml/min Inlet T: 200, 220°C Outlet T: 100, 130°C	(Turchiuli et al. 2005)
maltodextrin and acacia gum	Vegetable oil (5%(w/w)	Feed rate: 24, 68 ml/min Inlet T: 200, 220°C Outlet T: 100, 130°C	(Fuchs et al. 2006)
whey protein isolate/ maltodextrin	Avocado oil 50% (w/w)	Feed rate: 1 kg/h Inlet T: 100°C Outlet T: 80°C	(Bae and Lee 2008)
starch, gum arabic, sugar beet pectin, sodium caseinate, glucose, syrup	Fish oil 40% (w/w)	Feed rate: 1-7 kg/h Inlet T: 180°C Outlet T: 70°C	(Drusch et al. 2007)
Methylcellulose Maltodextrin	Fish oil Wall:core ratio (3:1) (1.5:1)	Feed rate: 15.0 g/min Inlet T: 160°C Outlet T: 65°C	(Kolanowski et al. 2006)

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Table 4.2. (cont.)

modified starch	Fish oil Oil:starch ratio (0.5:1), (1:1) (1.5:1)	Inlet T: 150°C Outlet T: 80°C	(Tan et al. 2005)
maltodextrin modified starch whey protein concentrate	Fish oil 1:4 (core:wall)	Feed rate: 3kg/min Inlet T: 180°C Outlet T: 65°C	(Jafari et al. 2008)

Although a dehydration process is successfully employed for the drying of oils to preserve the antioxidant activity, antimicrobial effect and aroma effect of oil's active components, spray drying can also be used as an oil encapsulation method for therapeutic purpose when it entraps active material within a protective matrix.

4.4. Crosslinking of Chitosan

For the preparation of chitosan particles, several techniques are available such as emulsion, ionotropic gelation, reverse micellar, solvent evaporation, spray drying, coacervation, and sieving methods (Desai and Park, 2005). A variety of hydrophilic and hydrophobic drugs can be loaded into the chitosan particles during the preparation of the microparticles, in which the loading efficiency of the drug may depend on its physicochemical characteristics and the preparation method.

The cross-linked microparticles have often been prepared using the chitosan and its derivatives by exploiting their cationic nature, in which the amino groups of the chitosan backbone can interact with glutaraldehyde, sodium sulphate, tripolyphosphate (Park et al., 2010) and natural crosslinkers: genipin (Muzzarelli, 2009), vanillin (Peng et al., 2010) etc. The crosslinking may be achieved in acidic, neutral or basic environments depending on the method applied.

Ionotropic gelation can be achieved by reaction of the amine group of the chitosan with tripolyphosphate, or hydrophobic counterions (e.g. alginate, carragenan etc.) and or high molecular weight ions (e.g. octyl sulphate, lauryl sulphate etc.) (Sinha et al., 2004). The chitosan solution in acetic acid is extruded dropwise through a needle into different concentrations of aqueous solutions of magnetically stirred tripolyphosphate or some other anion. The beads are removed from the counter ion solution by filtration, washed with distilled water and dried. Alternatively, wet phase

invasion, emulsification, coacervation methods can be used to obtain chitosan microspheres.

Crosslinking agents such as glutaraldehyde and genipin have been widely used in most of the studies in literature for the preparation of chitosan microspheres. Possible chemical reactions between the chitosan-glutaraldehyde and chitosan-genipin are shown in Figures 4.5 and 4.6, respectively (Mi et al., 2001).

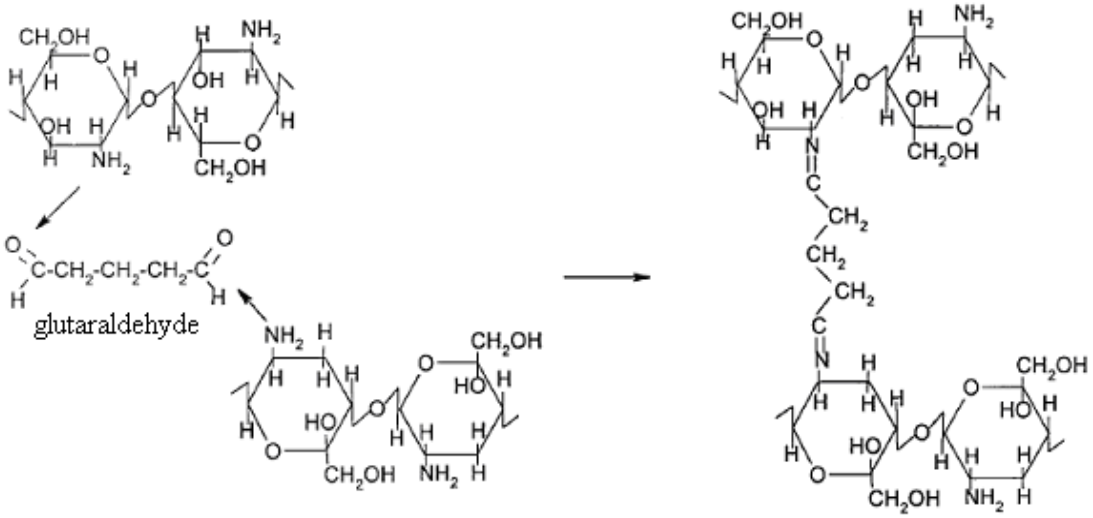

Figure 4.5. Chitosan- glutaraldehyde crosslinking mechanism (Source et al., 2001).

Glutaraldehyde is by far the most widely used agent in spite of some local cytotoxicity, due to its efficiency of stabilization of many biomaterials. Glutaraldehyde is easily available, inexpensive and its aqueous solutions can effectively crosslink collagenous tissues in a relatively short period. Glutaraldehyde reacts with chitosan (Figure 4.5) and it cross-links in an inter- and intra- molecular fashion through the formation of covalent bonds mainly with the amino groups of the polysaccharide (Genta et al., 1998). However, if released into the host due to biodegradation, glutaraldehyde is toxic. This is the reason of the increasing demand for a crosslinking agent able to form stable and biocompatible crosslinked products, without added citotoxicity problems (Bigia et al., 2002; Shalaby and Burg, 2004).

Genipin is obtained from its parent compound, geniposide, via enzymatic hydrolysis with glucosidase. Geniposide is isolated from the fruits of *Genipa americana* and *Gardenia jasminoides Ellis* and constitutes about 4–6% of dried fruit. *Genipa*

americana is found in tropical America, from Mexico and the Caribbean to Argentina, where it is widely planted for its shade and fruit. The fruits, with a taste similar to quinces, are eaten raw, used to make a sour, refreshing drink, cooked with sugar to flavor liquor, and used as a diuretic and as a remedy for respiratory ailments. *Gardenia jasminoides* plants are grown in the Far East, and their fruits have long been used in Chinese medicine for their anti-inflammatory, diuretic, choleretic, and haemostatic properties. Extracts from the fruits have also been used to form brilliant blue pigments via the reaction of genipin with primary amines in the presence of oxygen, which are used as a food dye commonly known as gardenia blue. These blue pigments are of particular importance because they are highly stable to heat, pH, and light. Initial observation of the formation of dimers of genipin in the presence of glycine led to the suggestion that genipin could be used to covalently crosslink proteins containing residues with primary amine groups. Subsequently, it has been used in studies of tissue fixation to crosslink collagen and gelatin, in foodstuff to crosslink soy protein isolates, and in studies of drug delivery, where it has been used to crosslink chitosan. Figure 4.6 indicates the chemical reaction between the genipin and amino group of the chitosan.

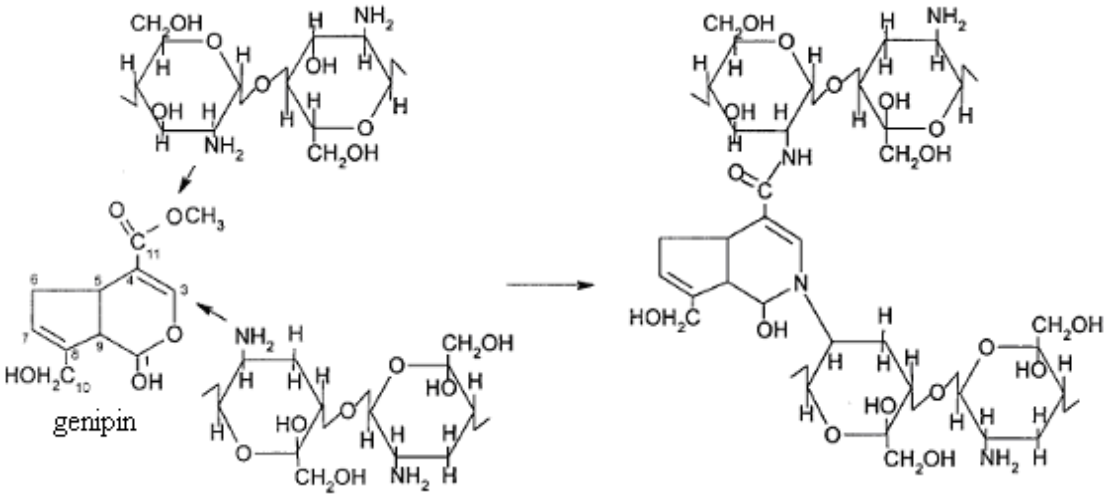

Figure 4.6. Chitosan-genipin crosslinking mechanism
(Source: Mi et al., 2000).

Varying amount of crosslinking agent directly effects the crosslinking density (Sinha et al., 2004). Additionally, it was found that the swelling ability of the crosslinked chitosan microspheres increased with the decrease of reaction time (Mi et al., 2000). This can be explained by the requirement of the time for the reaction between

the functional groups of the chitosan and croslinkers. Buttler et al. (2003) indicated the significance of the reaction time for achieving crosslinked genipin-chitosan structure. The lower swelling ability of chitosan gel is attributed to the increased intermolecular or intramolecular linkage of the $–NH_2$ sites in chitosan, which could be achieved by a more complete crosslinking reaction. By maintaining the same reaction time for crosslinking and comparing the equilibrium swelling ratio of genipin crosslinked chitosan microsphere to that of the traditional glutaraldehyde-crosslinked one, it was found that the genipin-crosslinked chitosan microsphere has the higher swelling ability (Mi et al., 2000). The result is ascribed to the slower gelation rate of genipin-crosslinked chitosan. Moreover, by measuring the viscosity changes in the chitosan solution during gelation, it was found that the viscosity of glutaraldehyde crosslinked chitosan solution increased significantly faster than that of the genipin-crosslinked one. Consequently, crosslinking of chitosan strictly depends on the crosslinker used, concentration of the crosslinker, crosslinking time and pH of the medium.

Within the view of many applications, genipin can be chosen as crosslinking agent because of its markedly lower cytotoxicity as compared with alternative crosslinker, glutaraldehyde. It was found that genipin is about 5000–10,000 times less cytotoxic than glutaraldehyde (Yuan et al., 2007). Genipin has been widely used in drug release systems due to its promising properties. A number of studies were summarized in Table 4.3.

Table 4.3. Studies with genipin as a crosslinker

Polymers	**System**	**Application Area**	**Reference**
Chitosan	Film	Edible Film	(Mi et al., 2006)
Chitosan / poly (vinyl pyrrolidone)	Hydrogel	Characterization	(Khurma et al., 2005)
Chitosan / poly (vinyl pyrrolidone)	Hydrogel	Characterization	(Khurma et al., 2006)
chitosan/ poly (ethylene oxide)	Film	Characterization	(Jin et al., 2004)
Chitosan	Film	Characterization	(Mi et al., 2005)

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Table 4.3. (cont.)

Chitosan	Hydrogel	Drug delivery	(Mi et al., 2008)
Chitosan	Microsphere	Intramuscularly implantable drug-delivery-vehicle	(Mia et al., 2008)
Chitosan/ gelatin	Microsphere	Drug- and immunotargeting	(Mi, 2005)
Chitosan/ alginate	Beads	Drug delivery	(Mi et al., 2002)
Gelatin	Film	Characterization	(Bigi et al., 2002)
Gelatin	Hydrogel	Characterization	(Yao et al., 2004)
Gelatin/ Fe3O4	Magnetic Hydrogel	Drug delivery	(Liu et al., 2006)

To eradicate the *H. pylori*, many studies have been performed and are listed in Table 4.4. Amoxicillin, tetracycline and clarithromycin were used as antibiotics.

Table 4.4. Antibiotic release systems for *H. pylori* theraphy.

Polymer	**System**	**Used Technique**	**Crosslinking Agent**	**Drug**	**Source**
Chitosan Polyacrylic Acid	Hydrogel	Spray drying	-	Amoxicillin	(Torre et al., 2003)
Chitosan	Microsphere	Emulsification	Glutaraldehyde	Amoxicillin	(Patel and Patel, 2007)
Chitosan	Microsphere	Emulsification	Glutaraldehyde	Clarithromycin	(Majithiya and Murthy, 2005)
Chitosan Polyacrylic Acid	Hydrogel	Spray drying	-	Amoxicillin	(Torre et al., 2005)
Chitosan	Microsphere	Ionic crosslinking/ Precipitation	-	Tetracycline	(Hejazi and Amiji, 2002)

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Table 4.4. (cont.)

Chitosan	Microsphere	Chemical crosslinking/ Precipitation	Glyoxal	Tetracycline	(Hejazi and Amiji, 2004)
Chitosan Poly (ethylene oxide)	Hydrogel	Spray drying	Glyoxal	Amoxicillin metranidazole	(Patel and Amiji, 1996)
Chitosan Polyacrylic Acid	Hydrogel	Spray drying	-	Amoxicillin	(Torrado et al., 2004)

Patel ve Amiji (1996) aimed to develop a pH sensitive antibiotic release system for stomach using chitosan and poly(ethylen oxide) by crosslinking. Amoxicillin and metranidazole were used as model drugs and their release from the freeze dried hydrogels into SGF was studied. Within 2 hours, release of 65% amoxicillin and 59% metranidazole were successfully achieved. Hejazi ve Amiji (2002) tried to develop a stomach-specific drug delivery system to increase the efficacy of tetracycline against *Helicobacter pylori*. Chitosan microspheres were prepared by ionic cross-linking and precipitation with sodium sulfate. Maximum drug loading efficiency was achieved as 69% (w/w). They found that thirty percent of tetracycline either in solution or when released from microspheres was found to degrade at pH 1.2 in 12 h. In another study by the same authors the effect of chemical crosslinking of chitosan microspheres on the gastric residence and local tetracycline concentrations was examined. Results of this study show that chitosan microspheres prepared by chemical crosslinking provide a longer residence time in the stomach than either tetracycline solution or microspheres prepared by ionic precipitation (Hejazi and Amiji, 2004). Chitosan-based mucoadhesive microspheres of clarithromycin was developed to provide prolonged contact time for drug delivery of antibiotics to treat stomach ulcers. Microspheres were prepared by emulsification technique using glutaraldehyde as a crosslinking agent and drug entrapment upto 74% was obtained. Extent of cross-linking exhibited an inverse relation to drug release rate as well as mucoadhesion, whereas polymer concentration exhibited an inverse correlation with drug release while linear relationship with mucoadhesion (Majithiya and Murthy, 2005).

There are limited number of studies on the production of essential oil loaded microspheres and their controlled release profiles. Although there is no study for the *H.*

pylori eradication with essential oil loaded microspheres, citronella oil was encapsulated by chitosan using o/w emulsion method (Hsieh et al., 2006). The changes in concentration of chitosan affected the encapsulation efficiency of the volatile citronella oil. Furthermore, it was found that, the particle size of chitosan microcapsules decreased as the emulsification stirring speed increased.

CHAPTER 5

THEORY OF CONTROLLED DRUG DELIVERY

5.1. General Aspects

Controlled drug delivery is the release of drug or other active agent in a predesigned manner from a material combined with them. The release of the active agent may be constant or cyclic over a long period, or it may be triggered by the environment. The purpose of controlled drug delivery is to achieve more effective therapies while eliminating the potential for both under- and overdosing. Other advantages of using controlled drug delivery systems are maintenance of optimum therapeutic drug concentration in the blood or in a cell, predictable and reproducible release rates for extended periods of time, enhancement of activity duration for short half-life drugs, the elimination of side effects, frequent dosing, and waste of drug, optimized therapy and better patient compliance. The ideal drug delivery system should be inert, biocompatible, mechanically strong, comfortable for the patient, capable of achieving high drug loading, safe from accidental release, simple to administer and remove, and easy to fabricate and sterilize (Boateng et al., 2007).

In recent years, more sophisticated controlled drug delivery systems have been developed which are able to respond to changes in the biological environment and deliver drugs based on these changes. Also, the studies focusing on the targeting delivery systems in which the drug is to be delivered in a targeted cell, tissue or site have been carried on. (Lin et al., 2005; Polnok et al., 2004; Chena et al., 2004).

The selection of a biomaterial for a specific application depends on the physicochemical properties and durability of the material, the nature of the physiological environment at the organ or tissue, adverse effects in case of failure, cost and production ease, biocompatibility, mechanical strength, sterilization requirement, etc. Typically, inorganic (metals, ceramics, and glasses) and polymeric (synthetic and natural) materials have been used for such items as artificial heart-valves, (polymeric or carbon-based), synthetic blood-vessels, artificial hips (metallic or ceramic), medical adhesives, sutures, dental composites, and polymers for controlled slow drug delivery.

One area of intense research activity has been the use of polymers for controlled drug delivery. Among them, the biodegradable polymers which are broken down into biologically acceptable molecules that are metabolized and removed from the body via normal metabolic pathways have been widely used for sutures, controlled drug delivery and tissue engineering. During the last two decades, significant advances have been made in the development of biocompatible and biodegradable materials for biomedical applications (Sinha et al., 2004).

5.2. Classification of Drug Delivery Systems

Drug formulation researchs have focused on the systems that delay the release of drugs after their administration. For this reason, many drug delivery systems have been developed to achieve active and effective treatment. Table 5.1 summarizes the developed release systems, mostly used in the area of controlled drug delivery.

Table 5.1. Classification of Drug Delivery Systems

Drug delivery Systems	**Mechanisms**
Diffusion Controlled Systems	Oral
	Matrix-type system
	Reservoir-type system
	Expandable system
	Floating system
	Transdermal
	Mucoadhesive system
	Inert matrix system
	Semisolid/reservoir matrix system
Dissolution Controlled Systems	Based on dissolution controlled release of;
	solid particles
	coated technologies
	matrix technologies
Osmotic Controlled Systems	Osmotic delivery systems for solids;
	Type I: single compartment
	Type II: multiple compartments
	Osmotic delivery systems for liquids
Biodegradable Polymeric Systems	Microparticles
	Nanoparticles
	Implants
Programmable Delivery Systems	Pulsatile systems
	Feedback-controlled systems
Stimulus Responsive	Physically modulated: Temperature
	Chemically modulated: pH dependent

Among them, oral administration is the main way of delivering immediate release of drugs because of easy delivery, better acceptance by patients and cost effective manufacturing. After investigation of sustained release technology, different strategies and technologies have been developed with the view to achieve controlled drug release. The oral administration of therapeutics leads to internalization by the matrix at the mouth, stomach, small intestine and colon. Using drug delivery systems, it is possible to target these different tissue systems for local drug action within the gastrointestinal tract. Chitosan is one of the most widely used polymers in the gastrointestinal release systems (Patel and Amiji, 1996).

Chitosan based delivery systems are advantageous because of its pH sensivity and mucoadhesive property. The fluctuation of pH through the gastrointestinal tract (GI) is significant, ranging from 1 to 7.5 (Gupta and Kumar, 2000). This offers significant drug release targeting based on pH shifts by regulating the swelling response. Mucoadhesion, the ability of a material to bind to the mucus lining of the GI tract, is regulated by the affinity of the drug delivery systems for the mucin glycoproteins of the mucus. Chitosan is very good mucoadhesives due to their non-toxic nature, and can be made to bind to mucins through either electrostatic or hydrophobic interactions. The amine and hydroxyl groups of it have been implicated in the chitosan's excellent mucoadhesive properties, leading to prolonged residence time in the gastrointestinal tract (Hejazi and Amiji, 2002).

Unlike the harsh environment of the gastrointestinal tract, low-molecular weight drugs can be administered by local transdermal drug delivery systems, which benefit from sustained drug release and easy therapy interruption by removal of the drug delivery system. Hydrogels offer attractive drug delivery system structures because of their high water content, providing a comfortable feeling on the patient's skin, leading to better compliance over the duration of the therapy.

Density determines the location of the matrix system in the stomach. Matrix with density lower than gastric contents can float to the surface, while high-density systems sink to bottom of the stomach. Gastric contents have a density close to water 1.004 g cm^{-3} (Bardonnet et al., 2006). A dense delivery system has been used for sinking of the matrix at the bottom of the stomach and so it significantly affects the gastric residence time. On the other hand, it floats when the density of the system is lower than of the stomach, and it remains buoyant in the stomach for a prolonged period of time, with a potential for continuous release of drug.

Among the drug delivery systems, the diffusion-controlled and the dissolution-controlled ones have been widely used for delivering the drug. Chitosan can be effectively used to develop such systems because of its biodegradability and hydrophilic characters.

5.3. Controlled-Release Mechanisms

There are three primary mechanisms by which active agents can be released from a delivery system: diffusion, swelling followed by diffusion, and degradation. Any or all of these mechanisms may occur in a release system.

Diffusion occurs when a drug or other active agent passes through the polymer that forms the controlled-release device. The diffusion can occur through pores in the polymer matrix or by passing between polymer chains. In Figure 5.1(a), a polymer and active agent have been mixed to form a homogeneous system, also referred to as a matrix system. Diffusion occurs when the drug passes from the polymer matrix into the external environment. As the release continues, its rate normally decreases with this type of system, since the active agent has a progressively longer distance to travel and therefore requires a longer diffusion time to release (Boateng et al., 2007). For the reservoir systems shown in Figures 5.1 (b) and (c), the drug delivery rate can remain fairly constant. In this design, a reservoir, solid drug, dilute solution, or highly concentrated drug solution within a polymer matrix, is surrounded by a film or membrane of a rate-controlling material. The only structure effectively limiting the release of the drug is the polymer layer surrounding the reservoir. Since this polymer coating is essentially uniform and constant thickness, the diffusion rate of the active agent can be kept fairly stable throughout the lifetime of the delivery system. In a transdermal drug delivery system, only one side of the device will actually be delivering the drug (Wang et al., 2005).

Since the drug delivery device is stable in the biological environment and does not change its size either through swelling or degradation in diffusion-controlled systems, the combinations of polymer matrices and bioactive agents chosen must allow for the drug to diffuse through the pores or macromolecular structure of the polymer (Sinha et al., 2004).

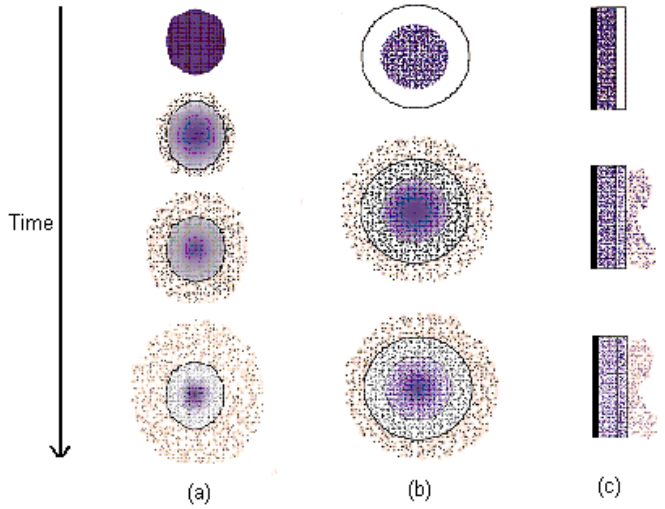


Figure 5.1. (a) Drug delivery from matrix drug delivery system; (b), (c) Drug delivery from reservoir devices: implantable or oral systems and transdermal systems, respectively.

Swelling-controlled release systems are initially dry and, when placed in the release media, body absorb water or other body fluids and swell. The swelling increases the aqueous solvent content within the formulation as well as the polymer mesh size, enabling the drug to diffuse through the swollen network into the external environment. Examples of reservoir and matrix systems are shown in Figures 5.2a and 5.2b, respectively. Most of the materials used in swelling-controlled release systems are based on hydrogels, which are polymers that swell without dissolving when placed in water or other biological fluids.

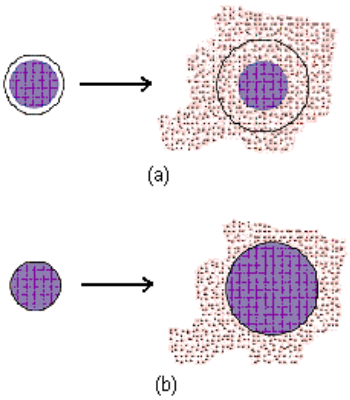


Figure 5.2. Drug delivery from (a) reservoir and (b) matrix swelling-controlled release systems.

Depending upon the polymer, the environmental change can involve pH, temperature, or ionic strength, and the system can either shrink or swell upon a change in any of these environmental factors. A number of these environmentally sensitive or "intelligent" hydrogel materials are listed in Table 5.2. For most of these polymers, the structural changes are reversible and repeatable upon additional changes in the external environment.

Table 5.2. Environmentally sensitive polymers for drug delivery
(Source: Kim, 1996)

Stimulus	**Hydrogel**	**Mechanism**
pH	Acidic or basic hydrogel	Change in pH Swelling-release of drug
Ionic strength	Ionic hydrogel	Change in ionic strength Change in concentration of ions inside gel Change in swelling-release of drug
Chemical species	Hydrogel containing electron-accepting groups	Electron-donating compounds Formation of charge/transfer complex Change in swelling-release of drug
Enzyme-substrate	Hydrogel containing immobilized enzymes	Substrate present Enzymatic conversion Product changes swelling of gel-release of drug
Magnetic	Magnetic particles dispersed in alginate microshperes	Applied magnetic field Change in pores in gel Change in swelling-release of drug
Thermal	Thermoresponsive hydrogel poly (N-isopro-pylacrylamide)	Change in temperature Change in polymer-polymer and water-polymer interactions Change in swelling-release of drug
Electrical	Polyelectrolyte hydrogel	Applied electric field Membrane charging Electrophoresis of charged drug Change in swelling-release of drug
Ultrasound irradiation	Ethylene-vinyl alcohol hydrogel	Ultrasound irradiation Temperature increase-release of drug

As an example, the pH sensitive materials are ideal for systems such as oral delivery, in which the drug is not released at low pH values in the stomach but rather at high pH values in the upper small intestine.

The third controlled drug delivery mechanism is achieved by using biodegradable polymers in the system. These materials degrade within the body, eliminating the need to remove a drug delivery system after release of the active agent has been completed. The most common formulation for these biodegradable materials is that of microparticles, which have been used in oral delivery systems and in subcutaneously injected delivery systems (Vasudev et al., 1997).

5.4. Theory and Mathematical Models of the Controlled Release

The concept of controlled release is to release a bioactive agent over a designated period of time at a particular rate. Controlled release devices most commonly utilize polymeric systems to control the release kinetics. Practical use of controlled drug release systems requires well-defined kinetic behavior that provides administration of a specific drug at an optimum level through release process.

Many controlled-release products are designed on the principle of embedding the drug in a porous matrix. Release of active ingredients from encapsulates can be induced or triggered by various methods including diffusion, mechanical rapture, melting, dissolution, hydration, enzyme attack, chemical reaction, hydrolysis, disintegration and do forth. It is possible to classify the controlled release systems as (Kim, 1996);

- **Diffusion Controlled Systems** – Diffusion of the drug through a polymer controls the release rate.
    - o Reservoir (Membrane) systems
    - o Monolithic (Matrix) systems
- **Swelling Controlled Systems** – Swelling of the polymer, and subsequent diffusion of the drug through the polymer controls the release rate.
- **Chemically Controlled Systems** – Hydrolytic or enzymatic cleavage results in polymer swelling, erosion, or release of covalently bonded drug by cleavage of a pendent chain.
- **Osmotically Controlled Systems** – Osmotic forces control the release of drug.

- **Dissolution Controlled Systems** – Dissolution of the polymeric carrier results in release of drug.
- **Externally Controlled Systems** – An external stimuli results in release of drug.
    - o Magnetically Controlled Systems
    - o Ultrasound Controlled Systems

Each system exhibits characteristic release properties depending on the physicochemical characteristics and structural conditions, which determine the resulting governing process.

A major challange in controlled release is to understand the complex interactions of various dynamic processes accompanying the mass transfer. Reservoir and matrix systems are commonly expected to be diffusion derived systems.

Fick's law of diffusion can often be used to describe the transport of a bioactive agent through a diffusion controlled polymer system. Fick's law in one dimension can be represented by the following equation;

$$J = -D\frac{\partial C_i}{\partial x} \tag{5.1}$$

Fick's law has been the basis for all theoretical and experimental studies on diffusion transport. It states that the steady state diffusion flux, J ($mol.m^{-2}.s^{-1}$), is proportional to the concentration gradient (dC_i/dx). D is the diffusivity of the bioactive agent.

Fick's first law presents a steady state diffusional release and Fick's second law is used for the description of transient phenomena where the concentration profile of the drug in the polymer is not constant during diffusion.

$$\frac{\partial C_i}{\partial t} = -D\left(\frac{\partial^2 C_i}{\partial x^2}\right) \tag{5.2}$$

Fick's models are adapted to passive diffusional systems where the diffusion coefficient (D) is assumed to be constant (no changes in the physicochemical properties of the polymer during the release). Such systems are called Fickian systems.

The considered geometry is important. For the spherical matrix, Fickian diffusion is commonly expressed in Equation 5.3.

$$\frac{\partial q}{\partial t} = \frac{D}{r^2}\frac{\partial}{\partial r}\left(r^2 \frac{\partial q}{\partial r}\right) \tag{5.3}$$

Equation 5.3 shows the change in amount of substance released from spherical particles, where q represents amount of substance transferred through particle, r is distance from the center of the sphere and D is diffusion coefficient. This equation assumes constant diffusion coefficient and the case is valid at low concentrations considering the following initial and boundary conditions (Crank, 1975):

$$\begin{gathered} \text{at } t=0,\ q=M \\ \text{for } t>0 \text{ and at } r=Rp,\ q=M_t \\ \text{for } t>0, \left(\frac{\partial q}{\partial r}\right)_{r=0} = 0 \end{gathered} \tag{5.4}$$

Integrating Equation 5.3 with a given boundary conditions;

$$\frac{M_t}{M_\infty} = 1 - \frac{6}{\pi^2}\sum_{n=1}^{\infty}\frac{1}{n^2}\exp\left(-\frac{n^2\pi^2 D_c t}{R_p{}^2}\right) \tag{5.5}$$

Equation 5.5 is known as Crank equation, which is valid for the homogeneous spherical encapsulates under infinite sink condition. M_t is the material released from the encapsulate, M_∞ is the initial loading, D is the effective diffusion coefficient of solute through the encapsulate matrix and R_p is the radius of the encapsulate.

Corresponding to early stage of release, the short term [$M_t / M_\infty < 0.3$], solution of the Equation 5.5 (Crank, 1975) is;

$$\frac{M_t}{M_\infty} = \frac{6}{\sqrt{\pi}}\left(\frac{D_c \cdot t}{R_p^2}\right)^{1/2} \tag{5.6}$$

For long term [$M_t / M_\infty > 0.7$], Equation 5.5 is written as follows:

$$1 - \frac{M_t}{M_\infty} \cong \frac{6}{\pi^2} \exp\left(- \frac{\pi^2 D_c t}{R_p^2} \right) \tag{5.7}$$

Equation 5.6 suggests a square root relation as shown in Equations 5.8 and 5.9;

$$\frac{M_t}{M_\infty} \alpha\, t^{0.5} \tag{5.8}$$

$$\frac{M_t}{M_\infty} = K\, t^{0.5} \tag{5.9}$$

K is a constant reflecting the design variables of the system. Thus, the fraction of drug released is proportional to the square root of time.

Equation 5.7 might also be described as;

$$\frac{M_t}{M_\infty} = 1 - \exp\left[- kt \right] \tag{5.10}$$

The term first order equation can be derived from the equation;

$$\frac{\partial \left(M_\infty - M_t \right)}{\partial t} = k (M_\infty - M_t)^n \tag{5.11}$$

where n is the reaction order. Solving Equation 5.11 for n=1 gives the first order release equation.

$$\frac{\partial \left(M_\infty - M_t \right)}{\partial t} = k (M_\infty - M_t) \tag{5.12}$$

which can also be integrated to obtain the following equation;

$$\frac{M_t}{M_\infty} = e^{-kt} \tag{5.13}$$

For zero-order equation, one assumes that the release is not dependent on its concentration and therefore n=0;

$$M_\infty - M_t = kt \tag{5.14}$$

It should be noted that zero-order kinetic provides constant release of bioactive compounds with time. When the active ingredient loaded to an encapsulate system is over saturated, the release rate of encapsulated solute to an infinite sink environment becomes constant.

In literature, Equation 5.9 is known as Higuchi equation. An important advantage of this equation is its simplicity. However, when applying it to controlled drug delivery systems, the assumptions should carefully be kept in mind: (i) the initial drug concentration in the system is much higher than the soluble amount of the drug at time t (which explained by the initial short time solution of the Crank equation), (ii) Mathematical anaysis is based on one-dimensional diffusion, (iii) Swelling or dissolution of the polymer carrier is negligible, (iv) The diffusivity of the drug is constant.

It is evident that these assumptions are not valid for most controlled drug delivery systems. Generally encapsulates exhibit more complex release kinetics due to the interactions with other rate limiting steps, such as hydration, structural breakdown and chemical/or metabolic reactions. However, due to the extreme simplicity of the equation, it is often used to analyze experimental drug release data to get a rough idea of the underlying release mechanism.

Figure 5.3 indicates the possible release profiles from the microcapsules. Burst release is typical for soluble or broken ones. Release might also be triggered after a certain period of time by, e.g. pH change or addition of enzymes. Zero-order release is obtained with over saturated amounts of active component in the core or with microcapsules where the thin shell is the rate limiting step. First or multiple order release is common for matrix type of encapsulates and release due to other interactions.

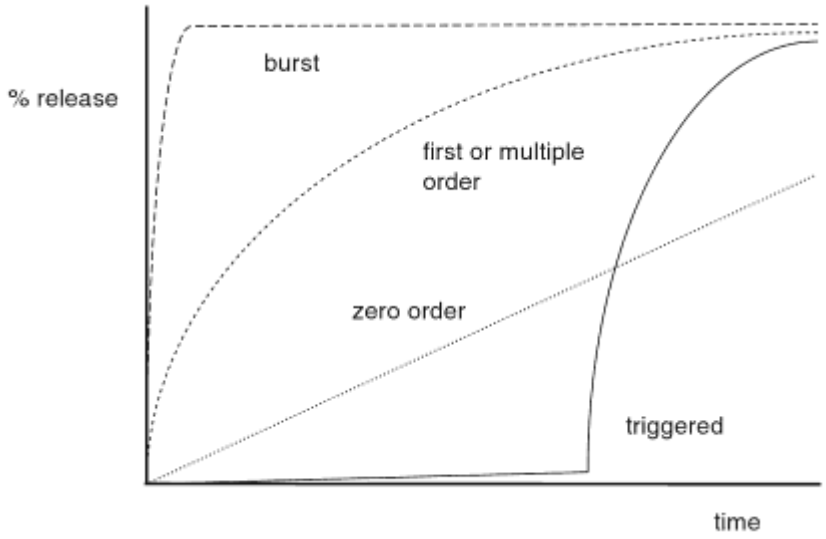


Figure 5.3. Release profiles from encapsulates.

A more comprehensive semiemprical equation to describe drug relase from polymeric systems is to so call power law:

$$\frac{M_t}{M_\infty} = kt^n \tag{5.15}$$

Here, M_t and M_∞ are the absolute cumulative amount of drug released at time t and infinite time, respectively; k is a constant incorporating structural and geometric characteristic of the device, and n is the release exponent, indicative of the mechanism of drug release (Siepmann and Peppas, 2001).

Equation 5.9 represents the special case of the power law where n=0.5. The power law or Peppas model can be seen as a generalization of the observation that superposition of two apparently independent mechanisms of drug transport, a Fickian diffusion and a case-II transport, describes in many cases dynamic swelling of and the type of coupling relaxation and diffusion. Case-II transport reflects the influence of polymer relaxation on molecules' movement in the matrix (Siepmann and Peppas, 2001).

Equation 5.15 has two special cases of n=0.5, which indicates diffusion controlled release; and n=1 (indicating swelling controlled drug release) for the release kinetics of bioactive compounds from the matrix having a slab geometry. For spheres and cylinders different values of n have been derived as listed in Table 5.3.

Table 5.3. Exponent n of the power law and drug release mechanism from polymeric controlled delivery systems of different geometry (Source: Siepmann and Peppas, 2001).

Exponent, n			Drug release mechanism
Thin film	Cylinder	Sphere	
0.5	0.45	0.43	Fickian diffusion
0.5<n<1	0.45<n<0.89	0.43<n<0.85	Anomalous transport
1	0.89	0.85	Case-II transport

Satojeni et al., (2010) developed a chitosan-albumin based microspheres including clarithromycin as a drug to provide prolonged contact time of drug delivery to treat stomach ulcer. Zero order, Higuchi and Peppas models were used to investigate the release kinetics. Both Higuchi and Peppas models were found to be best fitted in all dissolution profiles having a higher correlation coefficients and n value was determined around 0.5.

Table 5.4 indicates the controlled release of clarithromycin from different polymer/-crosslinker matrix including the kinetic analysis.

Table 5.4. Mathematical models applied to analyse kinetics of the release.

Polymer-crosslinker	Drug	Target	Models used	References
Chitosan-albumin	Clarithromycin	Stomach ulcers	Zero-order, Higuchi and Peppas	Satojeni et al., 2010.
Gellan gums	Clarithromycin	Stomach specific delivery	Zero-order, First-order, Higuchi	Rajinikanth and Mishra, 2009.
Chitosan-ethylcellulose-paraffin	Clarithromycin	*H. pylori*	Zero-order, First order, Higuchi and Peppas	Rajinikanth et al., 2008.
Chitosan-Carboxy-methylcellulose tablet	Clarithromycin	*H.pylori*	Zero-order, Peppas	Burgaz et al., 2008.
Hydroxypropyl methyl cellulose	Different drugs		Higuchi and Peppas	Siepmann and Peppas, 2001.

Rajinikanth and Mishra (2009) used gellan gums to obtain floating beads ontaining clarithromycin using iontotropic gelation method. They concluded that by kinetic treatment of the *in vitro* drug release data with different kinetic equations revealed matrix diffusion mechanism. In another study, chitosan and carboxymethylcellulose sodium interpolymer complexes were formed for controlled release of clarithromycin (Burgaz et al., 2008). They indicated that, release rate was explained by Fickian and dependent on pH and on polymer proportion. Siepmann and Peppas (2001) on the other hand, developed a mathematical models to predict the release rate kinetics of a drug from hydroxypropyl methylcellulose based pharmaceutical devices. Classical Higuchi equation and the so-called power law, as well as more complex mechanistic theories that consider diffusion, swelling and dissolution processes simultaneously are presented.

CHAPTER 6

MATERIALS AND METHODS

6.1. Materials

High molecular weight chitosan (Sigma-Aldrich Chemie, Germany, cat. # 419419, deacetylation degree %86) was used in the microsphere manufacturing. Clarithromycin was supplied by Ranbaxy Pharmaceuticals Inc. Cinnamon bark oil, clove oil, thyme oil, lemongrass oil and lemon oil were purchased from Sigma Chemical Co. Genipin was purchased from Challenge Bioproducts Co., Ltd. HPLC grade ethanol and methanol were used as a solvent to dissolve clarithromycin and essential oils. Acetic acid and acetonitrile was purchased from Merck, Darmstadt. Phosphate buffered saline (PBS) solution (pH=7.3) was obtained by dissolving PBS tablet in 100 ml deionized water. Simulated gastric fluid (SGF) was prepared by mixing pepsin (3 g/l) and NaCl (0.5 %) and it was adjusted to pH 3 and pH 5 with HCl. Sodium acetate and acetic acid were used in the preparation of buffer solutions with a pH range of 3-7. All buffer solutions were stored at 4 °C until use up to 7 days. Mueller Hinton Agar (Oxoid) and defibrinated sheep blood (Oxoid) were used in the antimicrobial susceptibility testing.

6.2. Methods

6.2.1. Minimum Inhibitory Concentration (MIC) Determination by Agar Dilution Method

The standard strain of *Helicobacter pylori* NCTC 11637 (cagA, vacA, oipA, babA (+)) was used in the study. *H. pylori* was identified according to colony morphology, Gram staining, microaerophilic growth (at 37°C), and the production of oxidase, catalase and urease. Antimicrobial susceptibility tests were performed according to performance standards for antimicrobial susceptibility testing of Clinical

and Laboratory Standards Institute (CLSI, 2007). Minimum inhibitory concentrations (MICs), the lowest concentration of an antimicrobial that inhibits the growth of a microorganism, were determined using the agar dilution method.

6.2.1.1. *H. pylori* Culture Media Preparation

%7 Horse Blood Supplemented H. pylori Selective Medium

19.5 gr Columbia Blood agar (Oxoid) was dissolved in 465 ml. distilled water and autoclaved at 121°C for 15 min. Then 35 ml of sterile Defibrinated Horse Blood (Oxoid) and 2 ml of *Helicobacter pylori* Selective Supplement (DENT, Oxoid) in sterile distilled water were added to the sterile agar solution at 50°C. 20 ml of culture medium was poured into each of 90 mm petri dishes. Agar plates were kept in incubator at 37°C for 24 h for contamination control.

H. pylori Stock Culture Medium with 20% Glycerol

Glycerol solution (40 %) (Baked Analyzed) and 40 ml of Brain Heart Infusion Broth (BHI) (Beckton Dickinson and Company) were autoclaved at 121°C for 15 min seperately. 10 ml of fresh human serum was added to medium kept at +4°C as 1 ml in sterile eppendorf tubes.

6.2.1.2. *H. pylori* Culture Protocol

Helicobacter pylori NCTC 11637 (*cagA*, *vacA*, *oipA*, *babA* (+)) standard strain was inoculated onto %7 Horse Blood Supplemented Columbia Blood agar (Oxoid) and incubated at 37°C for 3 days in microaerophilic environment in an anaerobic jar (Oxoid) with GasPak Campy Container System (Becton Dickinson and Company). *H. pylori* was identified according to colony morphology, Gram staining, microaerophilic growth (at 37°C), and the production of oxidase, catalase and urease. The subculture was obtained from the growing colonies on %7 Horse Blood Supplemented Columbia Blood agar. Colonies collected from subculture was kept at -80°C in a BHI Broth with %20 glycerol.

6.2.1.3. MIC Determination by Agar Dilution Method

Preparation of Stock Clarithromycin Solution

Clarithromycin solution was prepared by dissolving 25 mg clarithromycin in 25 ml methanol. 9 ml of PBS was added into 1 ml of clarithromycin solution and the stock solution was obtained as 100 µg/ml (w/v). The stock solution was sterilized with 0.22 µm sterile syringe filter and 2-fold serial dilutions were made in PBS resulting the clarithromycin concentration in the range of 50– 1.75 µg/ml. These concentrations were in the range of 1000 to 1 µg/ml when each of dilution was added into 50 ml growth medium.

Preparation of Stock Essential Oil Solutions

Lemongrass, cinnamon bark, clove leaf, lemon and thyme essential oils stock solutions were prepared by dissolving them in ethanol. 300 mg essential oil (Sigma) was dissolved in 3 ml ethanol. Stock solution as 100 mg/ml (w/v) was sterilized with 0.22 µm sterile syringe filter and 2-fold serial dilutions were made in PBS resulting the essential oil concentration in tubes in the range of 50 mg/ml–50 µg/ml. These concentrations were in the range of 1000 to 1 µg/ml when each of dilution was added into 50 ml growth medium.

Preparation of H. pylori Growth Medium

5**%** sheep blood supplemented Mueller Hinton Agar (Oxoid) was used as growth medium. 1.9 g Mueller Hinton agar was dissolved in 46.5 ml distilled water and autoclaved at 121°C for 15 min. For each dilution of antibiotic or essential oil from serial dilutions, 2.5 ml steril Defibrinated Sheep Blood (Oxoid) and 1 ml of that dilution were added into agar. Each of 50 ml growth medium was poured onto 90 mm steril petri dishes as two equal amounts (25 ml). 1 ml PBS was addded into growth medium instead of drug solution in control plates.

Preparation of Bacterial Suspension

H. pylori were harvested in 5 ml Brucella Broth and the number of bacteria was adjusted to McFarland 2 ($6x10^8$ CFU/ml). (Clinical and Laboratory Standards Institute, 2007). Turbidity of suspension was measured by Densimat (Biomerieux)

Inoculation of Bacterial Suspension

3 µl of bacterial suspension adjusted to McFarland 2 was dropped onto growth medium which was Mueller Hinton Agar supplemented with 5 % defibrinated sheep blood and containing 1 to 0.0037 µg/ml of clarithromycin and of essential oil. Inoculated plates were incubated in a microaerophilic atmosphere (85% N_2, 10% CO_2, 5% O_2) with a GasPak Campy Container System (Becton Dickinson and Company) at 37 °C for 96 h. After incubation the colony formation was visualized. The MIC was defined as the lowest concentration at which no visible growth was observed.

6.2.2. Determination of Composition and Antioxidant Activities of Essential Oils

The composition of essentil oils was determined by Gas Chromatography-Mass Spectrometry analysis. Chromatographic analysis was performed with an Agilent 6890 Network GC system equipped with a mass spectrometric detector (Agilent 5973 Network MSD). The separation was achieved on a HP-5MS (5% phenyl/methylsiloxane) (Capillary; 30 m x 0.25 mm x 0.25 µm) capillary column. Helium was used as carrier gas at a flow rate of 1 ml/min. 1 µl of samples were injected to a splitless injector that was maintained at 250 °C. The column temperature program was given in Table 1.

Table 6.1. GC-MS temperature program for determination of essential oil composition

Temperature (°C)	**Increase rate (°C/min)**	**Hold Time (min)**	**Total Time (min)**
40 °C	---	3	3
150 °C	2	---	58
230 °C	8	3	71

The antioxidant activities of essential oils were determined by TEAC (Trolox Equivalent Antioxidant Capacity) method (Altiok et al., 2007). In this method, ABTS was dissolved in water to a 7 mM concentration and reacted with 2.45 mM potassium persulfate solution to form $ABTS^{+}$. The mixture was kept in the dark at ambient temperature for 12-16 hours in order to complete the reaction. The absorbance of $ABTS^{+}$ solution was adjusted to 0.7 at 734 nm by diluting with ethanol. In the antioxidant capacity analysis, different amounts of essential oils were added into 3ml of $ABTS^{+}$ solution and the absorbance was measured at each 1 minute during 6 minutes at 734 nm by Perkin Elmer UV-Visible spectrophotometer. The antioxidant activities of the samples by using $ABTS^{+}$ were determined based on the antioxidant activity of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) solution and the results were given as the Trolox Equivalent Antioxidant Capacity (TEAC). The antioxidant capacity was calculated from the ratio of percentage inhibition (%) vs concentration graphs slopes of essential oil and trolox as ml essential oil / mmol trolox. This value was recorded as TEAC value (Altiok et al., 2007).

6.2.3. Production of Clarithromycin and Cinnamon Bark Oil Loaded Chitosan Microspheres

Chitosan microspheres were produced by spray dryer using Buchi® Mini Spray Dryer B-290 (Switzerland) with a 0.5 mm standard nozzle and a parallel flow. In the spray drying process, the filtered air is heated to a drying temperature and fed into the drying chamber. Feed solution is pumped to the atomizer. Drying air and sprayed feed solution move in a parallel flow. When the atomized droplets contact with hot air, evaporation occurs and dried products coming from cyclone are collected in the vessel.

1 % (w/v) chitosan solution was prepared by dissolving chitosan in 2 % acetic acid and control microspheres were obtained by drying this solution at 140 °C in a spray dryer. Clarithromycin and chitosan solutions prepared by dissolving them in separate beakers were mixed and stirred for 1 h. The final concentrations of clarithromycin and chitosan in this solution were 0.1% and 1% (w/v), respectively. Clarithromycin loaded chitosan microspheres were obtained by drying this solution in a spray dryer at an inlet air temperature of 180°C and a feed rate of 4 ml/min. In order to produce the cinnamon bark oil loded chitosan microspheres, 2.5 ml of cinnamon bark oil was added

dropwisely into 500 ml of 1% chitosan solution prepared by dissolving chitosan in 2% acetic acid and stirred for 1 h. This solution was pumped to the atomizer at 6 ml/min feed rate and dried at inlet air temperature of 195°C.

6.2.4. Optimization of the Production of Clarithromycin and Cinnamon Bark Oil Loaded Chitosan Microspheres

Optimization of the spray drying process could be very important phenomenon in terms of product quality and process efficiency in the development of clarithromycin and cinnamon bark oil loaded chitosan microspheres. Since the particle size distribution, shape and surface properties, water activity and the spray drying efficiency are governed by the inlet air temperature, feed rate and clarithromycin or cinnamon bark oil concentration of feed solution, the significance of these variables, as well as interactions between them, were examined using a central composite design and response surface methodology (RSM).

Water activity of the spray dried microspheres was measured by HygroLab 3 (Rotronic). The particle size distribution, shape and surface properties were examined by scanning electron microscope (Philips XL 30S FEG, FEI Company, Eindhoven, Netherlands). Spray drying efficiency (SE) was calculated to measure how much of microspheres were obtained after the manufacturing process as in Eqn (6.1);

$$SE(\%) = \frac{amount.of\ .spray.dried\ .microspheres}{amount.of\ .initial\ .polymer} \times 100 \qquad (6.1)$$

Three factors as process inputs were the drying air temperature, solution feed rate and clarithromycin or cinnamon bark oil concentration of solution. The response variables were the moisture content, particle size distribution and surface morphology and spray drying efficiency. The levels of the factors were coded as -1 and +1, corresponding to the low and high levels respectively. Alpha was taken as 1.68. Actual values are 170°C-190°C and 185°C-195°C for drying air temperature; 8-10 ml/min and 6-10 ml/min for solution feed rate and 0.2%-0.6% (w/v) and 0.7%-1.3% (w/v) for clarithromycin or cinnamon bark oil concentration of the solutions, respectively. All statistical analyses were performed by MINITAB Statistical Software, Release 15.

6.2.5. Crosslinking of Microspheres by Genipin

Microspheres were crosslinked by using genipin as a natural crosslinking agent for the clarithromycin and cinnamon bark oil loaded microspheres. The required amount of clarithromycin or cinnamon bark oil was added to the chitosan solution and stirred for 1h. 20 ml genipin solution was added to the 300 ml chitosan solution in different concentrations (1-5 mM) and the resulting mixture was incubated at 50 ºC with magnetic stirring during different time periods (1-18 h). The effects of genipin concentration and crosslinking time on release kinetics were investigated. Chitosan-genipin-drug solutions were spray-dried under optimum spray drying conditions. Briefly, spray drying conditions were inlet temperatures of 170 ºC for clarithromycin and 195 ºC for cinnamon bark oil and sample flow rate of 6 mL/min.

6.2.6. Characterization of Microspheres

The size and the surface morphology of spray dried particles were investigated by scanning electron microscope (Philips XL 30S FEG, FEI Company, Eindhoven, Netherlands) in Center for Materials Research in Izmir Institute of Technology, Izmir, Turkey. The powders were fixed on a brass stub using double-sided adhesive tape and then were made electrically conductive by coating, in a vacuum, with a thin layer of gold for 60 sec.

The zeta potential of clarithromycin, genipin and all formulations of microspheres was determined using a Malvern Zetasizer 3000 HSA (Malvern Instruments). All measurements were performed at 25°C in phosphate buffer of pH 5 and repeated in triplicate.

The thermal properties of microspheres were analyzed by Thermogravimetric Analysis (TGA) (Shimadzu TGA-51) under N_2 gas flow (40 ml/dak). In TGA analysis, the temperature was increased from 20°C to 400°C with heating rate of 10^oC /min. The thermal properties of cinnamon bark oil were analyzed by Differential Scanning Calorimeter (DSC) in the temperature range of -20°C -400°C.

The chemical structures of clarithromycin, chitosan and clarithromycin loaded microspheres were investigated by Nuclear Magnetic Resonance Spectrometry (Varian 400 MHz) analysis (^{1}H-NMR) by dissolving the samples in deuterium oxide (D_2O).

The swelling and degradation properties of microspheres in phosphate buffer of pH 5 were analysed by phase contrast microscope

6.2.7. Clarithromycin Stability in Different pH Solutions

Clarithromycin was dissolved in 2% acetic acid and incorporated into SGF with/without pepsin of pH 2.0 or 3.0 and phosphate buffer of pH 5.0 or 7.0. The degradation percentages of clarithromycin at different times in the medium were evaluated from the main peak area by HPLC assay. HPLC system working conditions and properties were given in Table 6.2.

Table 6.2. The properties and operating conditions of HPLC system for clarithromycin

Properties	**Specifications**
Column	Licrosphere C18 Reverse Phase Column
Column length	250 mm
Column diameter	4 mm
Particle size	5 μm
Mobile phase	A: acetonitrile ; B: phosphate Buffer (pH 4.4)
Flow rate	1.1 ml min^{-1}
Wavelength	205 nm
Temperature	40 ^{o}C
Detector	Diode Array Detector
Elution type	Gradient elution

6.2.8. Release of Clarithromycin and Cinnamon Bark Oil from Microspheres

The release of clarithromycin and cinnamon bark oil from microspheres in phosphate buffer solution at pH 5 was performed in a thermoshaker at 37°C and 150 rpm. 50 mg of microspheres were added into 20 ml buffer solution. 250 μl of sample was taken at different times and filtered from 0.45μm syringe filter. 250 μl of fresh

buffer solution was added to the release media after each sampling. Filtered sample was injected to HPLC. The calibration curve was obtained from different concentrations of clarithromycin solution.

Amount of cinnamon bark oil released from microspheres to phosphate buffer solution of pH 5 was determined by UV-Visible Spectrophotometer (Thermo Multiskan Spectrum). The most suitable wavelenght was 288 nm according to spectrum scanning. The calibration curve was obtained by different dilutions of cinnamon bark oil in ethanol.

6.2.9. The Effects of Clarithromycin and Cinnamon Bark Oil Released from Microspheres on *H. pylori*

In order to determine the antibacterial activity of clarithromycin and cinnamon bark oil released from microspheres, the microspheres were incorporated in *H. pylori* inoculated growth medium.

The growth medium was prepared by dissolving 1.8 gr of Brucella Broth (Beckton Dickinson, BD) in 95 ml of distilled water and test tubes each having 10 ml of this solution was autoclaved at 121°C for 15 min. When the Brucella Broth was cooled to 50°C, 0.5 ml of fetal bovine serum (FBS) (Biochrom AG), sterilized by 0.22 μm syringe filter (Sartorius Stedim), was added to each broth. Broths was incubated at 37°C for 24 h for contamination control and kept at +4 °C.

H. pylori NCTC 11637 standard strain cultured on 7% horse blood supplemented Columbia Blood Agar was inoculated into 40 ml broth having 5% FBS adjusted to McFarland 2 ($6x10^8$ CFU/ml).

In order to determine the antibacterial activity of control chitosan microspheres, 0.0022 g of control chitosan microspheres, sterilized under UV light for 30 min., were added into bacterial suspension of 40 ml 5% FBS incorporated Brucella Broth adjusted to McFarland 2. The suspension was incubated under magnetic stirring in an anaerobic jar (Oxoid) containing GasPak Campy Container System (Becton Dickinson and Company) at 37°C in a microaerophilic environment. 100 μl of sample was taken from the suspension at 0, 8, 24, 32 and 48 h of incubation and then 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} serial dilutions were prepared in steril eppendorf tubes having 900 μl of 5% FBS incorporated Brucella Broth. 100 μl sample taken from 10^{-4}, 10^{-5},

10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} dilutions was inoculated onto 7% horse blood supplemented Columbia Blood Agar in an anaerobic jar (Oxoid) containing GasPak Campy Container System (Becton Dickinson and Company) at 37°C in a microaerophilic environment for 3 days.

Similarly, 0.0022 g of clarithromycin loaded uncrosslinked and genipin crosslinked chitosan microspheres , sterilized under UV light for 30 min., were added into bacterial suspension of 40 ml 5% FBS incorporated Brucella Broth adjusted to McFarland 2 in order to determine the antibacterial activity of clarithromycin loaded chitosan microspheres (approximately 1µg/ml clarithromycin in solution after complete release). The remaining procedure was the same with that of control microspheres.

In order to determine the antibacterial activity of cinnamon bark oil loaded chitosan microspheres, 0.022 g of control chitosan microspheres, sterilized under UV light for 30 min., were added into bacterial suspension of 40 ml 5% FBS incorporated Brucella Broth adjusted to McFarland 2 (approximately 4.6 µg/ml cinnamon bark oil in solution after complete release). The remaining procedure was the same with that of control microspheres.

CHAPTER 7

RESULTS AND DISCUSSIONS

7.1. Minimum Inhibitory Concentration (MIC) Study

In the inhibition test by agar dilution method, the minimum clarithromycin concentration inhibiting *H. pylori* growth was observed as 0.125 μg/ml. This result was also proved by catalase, oxidase, urease test and Gram staining. Similarly, Vega et al. determined the MIC of clarithromycin on *H. pylori* NCTC 11638 standard strain as 0.125 μg/ml (Vega et al., 2009). The MIC of clarithromycin on *H. pylori* NCTC 11637 standard strain was determined as 0.12 μg/ml (Ustun et al., 2006).

The MIC values of lemongrass, cinnamon bark, clove leaf, lemon and thyme essential oils against *H. pylori* were determined as 62, 8, 125, 500 and 62 μg/ml, respectively. Bergonzelli et al. determined the MBC's (minimum bactericidal concentration) of cinnamon bark oil, lemongrass oil and thyme oil as 40 μg/ml and that of clove oil as 100 μg/ml. It was also expressed that the climate, soil type and age of plant affect the quality, amount and composition of essential oil resulting different antibacterial properties depending on the origin of the essential oil. So, determining the antibacterial effects of the components of essential oils could be more reliable and comparable (Bergonzelli et al., 2003).

As a result of the inhibition tests, cinnamon bark oil was the most effective essential oil on *H. pylori*. Therefore, in the following studies cinnamon bark oil loaded chitosan microspheres were only produced, characterized and the release experiments were carried out.

7.2. Composition and Antioxidant Activities of Essential Oils

The chemical and percentage composition of five essential oils used in this study was given in Table 3. The main components of lemongrass oil, cinnamon bark oil,

lemon oil, thyme oil and clove leaf oil were found as citral (58%), cinnamaldehyde (72%), limonen (65%), carvacrol (69%) and eugenol (82%), respectively.

Table 7.1. Chemical and percentage composition of essential oils

#	Chemical Compounds	Cinnamon Bark oil	Clove Leaf oil	Lemon oil	Lemongrass oil	Thyme oil
1	(-) Borneol					1.91
2	(-)-Camphor					0.20
3	(-)-Spathullenol					0.12
4	(+)-a-Terpineol					0.48
5	1,8-Cineole (Eucalyptol)	0.18				
6	1-Metoxy-4-methyl-2-(1-methylethyl)-benzene					0.57
7	4-Terpineol					1.47
8	4-Thujanol	0.10				0.45
9	a-bergamotene			0.36		
10	a-Caryophylene	0.82	1.60		0.13	0.18
11	a-citral				**58.39**	
12	a-lemonene		0.09	**65.42**		
13	a-Phellandrene	0.86		0.52		0.15
14	a-Pinene	0.66		2.39		0.30
15	a-Terpinene			0.25		
16	a-Terpineol	0.40		0.13		
17	a-Thujene	0.17				
18	b-Bisabolene			0.47		1.34
19	b-Caryophylene	4.16	14.73	0.21	0.88	1.35
20	b-citral			0.54	**37.75**	
21	Benzaldehyde	0.29				
22	Benzene propanal	0.23				
23	Benzyl benzoate	1.05				
24	b-Linalool	2.86				2.35
25	b-Myrcene			1.09		
26	b-Pinene	0.31		13.97		0.29
27	b-Thujene	2.36				0.05
28	Camphene	0.28		0.08		0.18
29	Carvacrol					**68.96**
30	Caryophylene oxide	0.76	0.31			0.19
31	Champore	0.12				
32	Cinnamyl acetate	3.72				
33	Cinnamyl alcohol	0.12				
34	Cis-Cinnamaldehyde	0.32				
35	cis-verbenol				0.88	
36	citral			0.91		

(Cont. on next page)

Table 7.1. (cont.)

#	Chemical Compounds	Cinnamon Bark oil	Clove Leaf oil	Lemon oil	Lemongrass oil	Thyme oil
37	Copaene	0.71	0.42			
38	Eucalyptol (cineole)					0.85
39	Eugenol	3.87	**82.41**			
40	Methylthymilether					0.15
41	Nerol				0.94	
42	nerol acetate			0.34	0.62	
43	o-Methoxy cinnamaldehyde	0.41				
44	p-a-Dimethylcityrene					0.42
45	p-Cymene	2.53		0.65		8.40
46	p-Terpinene					0.72
47	sabinene			1.57		
48	Safrene	0.16				
49	sigma-cadinene		0.29			0.18
50	Terpinene-4-ol	0.19				
51	Terpinolene	0.35		0.42		0.65
52	teta-terpinene			10.10		
53	Thymol					7.75
54	trans-Cinnamaldehyde	71.69				

The antioxidant activity of essential oils was calculated from the ratio of percentage inhibition (%) vs concentration graph slopes (Figure 7.1) of essential oil and trolox. This value was recorded as TEAC value and tabulated in Table 7.2.

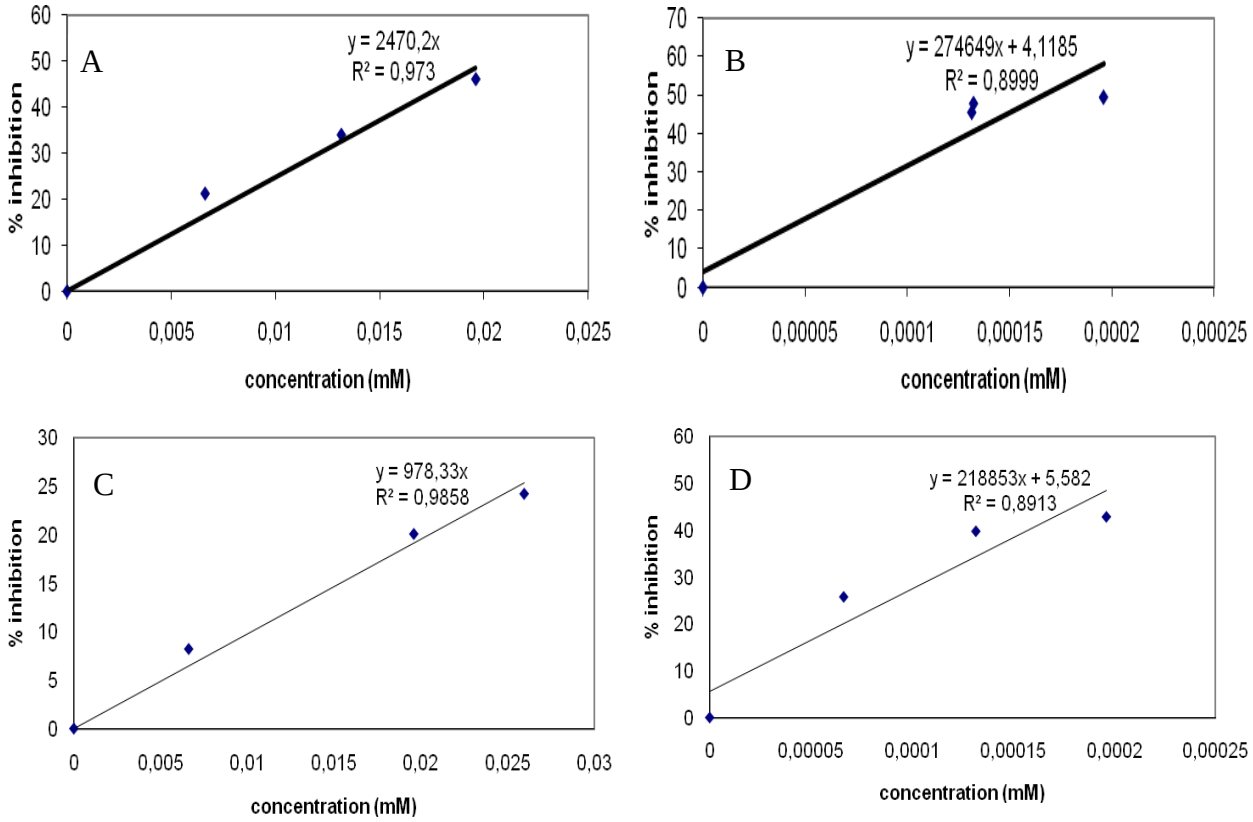


Figure 7.1. Lemongrass oil (a), cinnamon bark oil (b), lemon oil (c), thyme oil (d), clove leaf oil (e) and trolox (f) percentage inhibition graphs.

(cont. on next page)

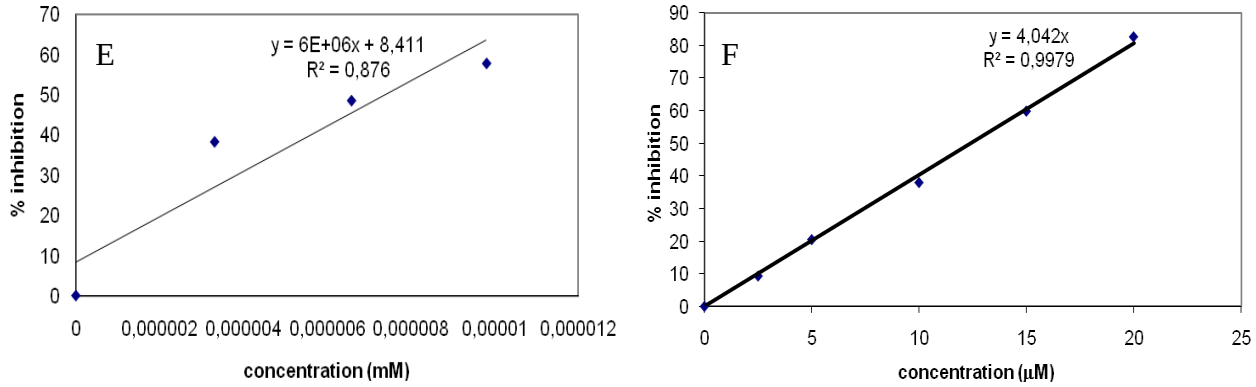


Figure 7.1. (cont.)

Table 7.2. The trolox equivalent antioxidant capacities (TEAC) of essential oils

Essential oil	**TEAC (ml essential oil/mmol trolox)**
lemongrass oil	0.61
cinnamon bark oil	67.95
lemon oil	0.24
thyme oil	54.14
clove leaf oil	1484.41

The highest antioxidant activity was observed in clove leaf oil. This activity may be due to its major component, eugenol. Moreover, cinnamon bark oil and thyme oil also have very high antioxidant activities probably due to cinnemaldehyde and carvacrol, respectively. These natural antioxidants may thus serve as novel therapeutic tools in alleviating *H. pylori*-induced oxidative damage.

7.3. Optimization of the Production of Clarithromycin and Cinnamon Bark Oil Loaded Chitosan Microspheres

Spray drying of chitosan solutions having 0.2-0.6% (w/v) and 0.7%-1.3% (w/v) clarithromycin and cinnamon bark oil concentrations, respectively, was carried out in the specified drying air temperatures and feeding rates. The synthesized particles were graded in four categories (Table 7.3) according to their shapes (wrinkled or smooth) and particle size distributions (monodisperse or polydisperse) which indicate the adequacy grade of the particles for the specific use. The surface properties, particle size and shape are very important for the practicability of the particles.The particle size in the range of 1-5 μm is expected to have good aerodynamic properties that brings also the tendency of small particles to agglomerate due to the Van der Waals forces. The stickness of a particle is related to the moisture content. Moisture content represents a measure of the quantity of water in a product. It provides information about yield, quantity and texture of product. A portion of the total water content present in a product is strongly bound to

specific sites on the chemicals that comprise the product. These sites may include the hydroxyl groups of polysaccharides, the carbonyl and amino groups of proteins, and other polar sites. Hydrogen bonds, ion-dipole bonds, other strong chemical bonds tightly bound water. Some water is bound less tightly, but is still not available. Water activity is a measure of how efficiently the water present can take part in a chemical reaction. It is defined as "free" or "unbound" water in a system and determined as the vapor pressure of water in a sample divided by the vapor pressure of pure water at the sample temperature (Beristain et al., 2002). Water activity affects the surface charge and stickiness of the particles. It provides valuable information about microbial spoilage, chemical stability and physical stability. Besides particle size, shape and moisture content, spray drying efficiency is also a important parameter in a spray drying process. Spray drying is a solvent evaporation process. The solvent in the droplets is removed very quickly due to heat energy provided in the spray dryer. The maximum spray drying efficiency can be achieved from a balance of the amount of heat energy input and the amount of heat energy used in the evaporation process which is related to the amount of the sample input.

Several factors were hypothesized to see the effects on the properties of drug loaded chitosan microspheres. The properties of the synthesized microspheres that were monitored were "water activity", "particle type" and "spray drying efficiency". Statistical analysis based on a central composite design was performed to examine the influence of the three variables (inlet air temperature, drug concentration and feed flow rate) on the final properties of clarithromycin and cinnamon bark oil loaded chitosan microspheres. These variables were examined at three levels: upper, medium and lower limits.

Table 7.3. Categorization of spray dried particles

Type 1	smooth, 1-5 micron, monodisperse
Type 2	smooth, 1-5 micron, polydisperse
Type 3	Wrinkled, 1-5 micron, polydisperse
Type 4	sticky, 1-5 micron, polydisperse

Table 7.4 lists the results of the experiments performed according to the experimental design for the production of clarithromycin loaded microspheres. The analysis was done using coded units.

Table 7.4. The results of experiments performed according to statistical design for the production of clarithromycin loaded microspheres.

Run Number	Inlet Air Temperature (A)	Feed Rate (B)	Clarithromycin Concenration (C)	Water Activity a_w	Particle Type	Efficiency (%)
1	-1	-1	-1	0.262	2	58.87
2	1	-1	-1	0.260	3	47.18
3	-1	1	-1	0.476	2	46.49
4	1	1	-1	0.922	4	34.59
5	-1	-1	1	0.483	2	65.40
6	1	-1	1	0.414	2	62.29
7	-1	1	1	0.524	4	47.84
8	1	1	1	0.675	4	61.92
9	-1.68	0	0	0.652	1	58.76
10	1.68	0	0	0.659	2	42.71
11	0	-1.68	0	0.320	1	52.54
12	0	1.68	0	0.281	4	14.19
13	0	0	-1.68	0.429	2	53.48
14	0	0	1.68	0.652	4	58.95
15	0	0	0	0.410	2	51.57
16	0	0	0	0.412	2	51.41
17	0	0	0	0.408	2	52.58

The estimated regression coefficients for water activity of clarithromycin loaded microspheres were given in Table 7.5.

Table 7.5. Estimated regression coefficients for water activity of clarithromycin loaded microspheres

Term	Coef	SE Coef	T	P	
Constant	25.0882	2.957	8.484	0.000	
A	1.4029	1.389	1.010	0.346	
B	4.8760	1.389	3.511	0.010	*
C	-1.5344	1.389	-1.105	0.306	
A*A	0.6684	1.529	0.437	0.675	
B*B	0.2265	1.529	0.148	0.886	
C*C	-1.1877	1.529	-0.777	0.463	
A*B	4.5000	1.814	2.480	0.042	*
A*C	-4.0000	1.814	-2.204	0.063	
B*C	-5.2500	1.814	-2.893	0.023	*

* The terms with P value less than or equal to 0.05 have been considered as significant.

The regression equation (Equation 7.1) for water activity was as follows, with a regression coefficient R^2 of 83.37 %:

$$\hat{y}_1 = 25.088 + 4.876x_1 + 4.5x_1x_2 - 5.25x_2x_3 \qquad (7.1)$$

Coefficient estimates and the p-values verified the significance of the main factor of inlet air temperature, second order interactions of inlet air temperature and feed rate and clarithromycin concentration and feed rate. The inlet air temperature was found to be the most effective parameter on water activity. Feed rate alone was not an effective parameter, but the interactions with inlet air temperature and concentration indicated significance.

Morphology of clarithromycin loaded microspheres was investigated by scanning electron microscopy to decide particle classifications according to Table 7.3. Mostly, all particles were smooth and uniformly distributed in the size range of 1-5 μm. (Figure 7.2). However, more sticky particles with low sphericity were also observed due to the insufficient drying. The micrographs depicted different particle shaped and sized microspheres, obtained at each run are given in Figure A.1 in Appendix A.

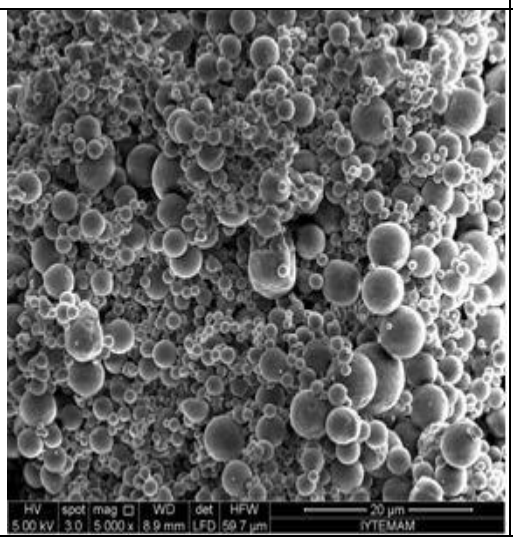
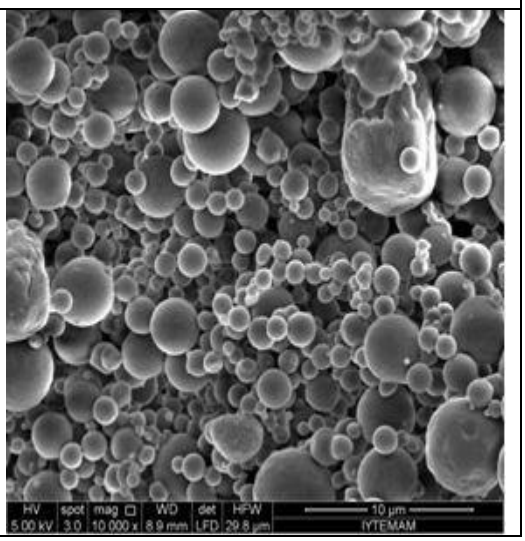
Run No	5000x	10000x
10		

Figure 7.2. SEM micrographs of clarithromycin loaded microspheres manufactured by spray drying according to central composite design; run number: 10.

The estimated regression coefficients for particle type of clarithromycin loaded microspheres were given in Table 7.6.

Table 7.6. Estimated regression coefficients for particle type of clarithromycin loaded microspheres

Term	Coef	SE Coef	T	P
Constant	1.96639	0.3063	6.419	0.000
A	0.34282	0.1439	2.383	0.049 *
B	0.73556	0.1439	5.113	0.001 *
C	0.31952	0.1439	2.221	0.062
A*A	-0.06101	0.1583	-0.385	0.711
B*B	0.29254	0.1583	1.848	0.107
C*C	0.46932	0.1583	2.964	0.021 *
A*B	0.12500	0.1880	0.665	0.527
A*C	-0.37500	0.1880	-1.995	0.086
B*C	0.37500	0.1880	1.995	0.086

* The terms with P value less than or equal to 0.05 have been considered as significant.

The regression equation (Equation 7.2) for particle type was as follows, with a regression coefficient R^2 of 89.15 %:

$$\hat{y}_2 = 1.966 + 0.343x_1 + 0.736x_2 + 0.469x_3^2 \qquad (7.2)$$

This equation revealed that the key parameters that had dominant effect on particle type of microspheres as inlet air temperature, feed rate, and pure quadratic term (Table 7.6).

In spray drying, it was observed that some of the liquid droplets were attached inside the wall of the main chamber. Once the inlet air temperature was set below 170°C, or the pump rate was chosen to be faster than 8 ml/min, the solvent in the droplets could not be fully evaporated and the spray drying efficiency was calculated below 50%.

The estimated regression coefficients for drying efficiency of clarithromycin loaded microspheres were given in Table 7.7.

Table 7.7. Estimated regression coefficients for the efficiency of clarithromycin loaded microspheres

Term	Coef	SE Coef	T	P
Constant	51.4277	4.382	11.737	0.000
A	-2.8988	2.058	-1.409	0.202
B	-7.8651	2.058	-3.822	0.007 *
C	4.3578	2.058	2.118	0.072
A*A	1.0723	2.265	0.473	0.650
B*B	-5.0694	2.265	-2.238	0.060
C*C	3.0083	2.265	1.328	0.226
A*B	2.1214	2.688	0.789	0.456
A*C	4.3198	2.688	1.607	0.152
B*C	0.8786	2.688	0.327	0.753

* The terms with P value less than or equal to 0.05 have been considered as significant.

The regression equation (Equation 7.3) for efficiency was as follows, with a regression coefficient R^2 of 83.04 %:

$$\hat{y}_3 = 51.428 - 7.865x_2 \quad (7.3)$$

The analysis designated that the only significant factor was feed rate for efficiency.

The optimum conditions for the manufacturing of clarithromycin loaded microspheres by spray drying were determined as 168°C for inlet air temperature, 4.64 ml/min for feed rate and 0.064 % (w/v) for clarithromycin concentration with desirability of 0.76.

Statistical design for the production of cinnamon bark oil loaded microspheres was also carried out. Water activity, particle type and spray drying efficiency results were given in Table 7.8 according to run order of the design.

Table 7.8. Results of experiments performed according to statistical design for the production of cinnamon bark oil loaded microspheres.

Run Number	Inlet Air Temperature (A)	Feed Rate (B)	Cinnamon bark oil Concenration (C)	Water Activity a_w	Particle Type	Efficiency (%)
1	-1	-1	-1	0.317	3	26.20
2	1	-1	-1	0.326	3	36.85
3	-1	1	-1	0.613	3	20.04
4	1	1	-1	0.450	3	23.92
5	-1	-1	1	0.414	3	18.63
6	1	-1	1	0.325	4	16.60
7	-1	1	1	0.902	4	5.83
8	1	1	1	0.509	3	10.07
9	-1.68	0	0	0.542	2	21.67
10	1.68	0	0	0.409	3	14.79
11	0	-1.68	0	0.350	2	18.99
12	0	1.68	0	0.890	4	6.60
13	0	0	-1.68	0.628	4	17.65
14	0	0	1.68	0.391	4	5.33
15	0	0	0	0.591	4	9.08
16	0	0	0	0.585	4	9.41
17	0	0	0	0.601	4	8.98

The estimated regression coefficients for moisture content of cinnamon bark oil loaded microspheres were given in Table 7.9.

Table 7.9. Estimated regression coefficients for water activity of cinnamon bark oil loaded microspheres

Term	Coef	SE Coef	T	P
Constant	0.595525	0.05758	10.342	0.000
A	-0.105866	0.04548	-2.328	0.053
B	0.246314	0.04548	5.416	0.001 *
C	0.005593	0.04548	0.123	0.906
A*A	-0.147930	0.08418	-1.757	0.122
B*B	-0.003430	0.08418	-0.041	0.969
C*C	-0.113930	0.08418	-1.353	0.218
A*B	-0.168291	0.09993	-1.684	0.136
A*C	-0.115966	0.09993	-1.160	0.284
B*C	0.089095	0.09993	0.892	0.402

* The terms with P value less than or equal to 0.05 have been considered as significant.

The regression equation (Equation 7.4) for water activity was as follows, with a regression coefficient R^2 of 86.30 %:

$$\hat{y}_1 = 0.595 + 0.246x_2 \tag{7.4}$$

The p-values verified the significance of the main factor of feed rate.

Morphology of cinnamon bark oil loaded microspheres was also investigated by scanning electron microscopy. The particles were mostly wrinkled and the particle size was in the range of 1-5 μm. Particles were stickier than the clarithromycin loaded particles due to the insufficient drying. Low sphericity was observed (Figure 7.3). The micrographs depicted different particle shapes and sizes obtained at each run of central composite design for manufacturing of cinnamon bark oil loaded microspheres are shown in Figure A.2 in Appendix A.

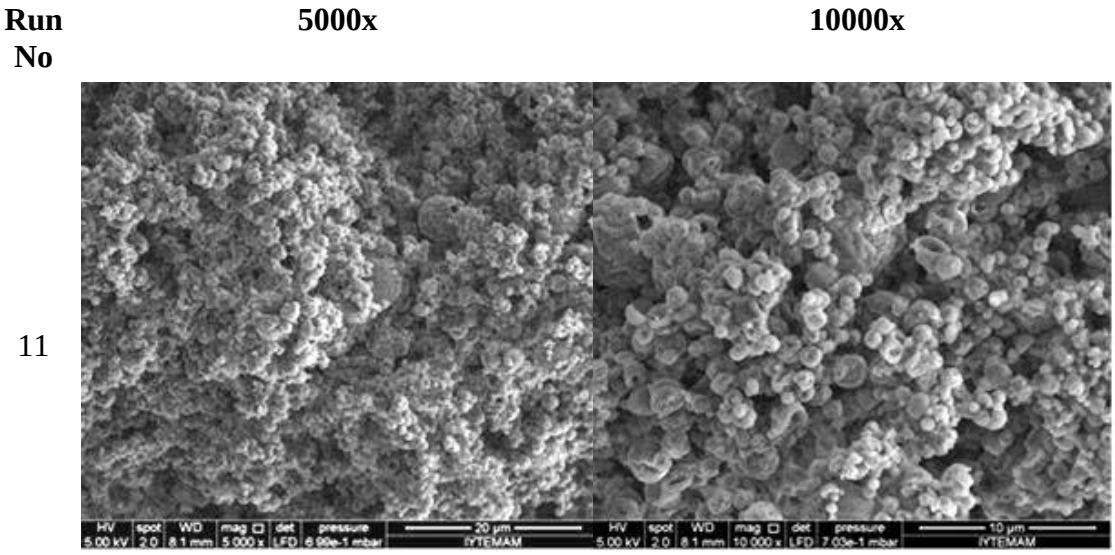


Figure 7.3. SEM micrographs of cinnamon bark oil loaded microspheres manufactured by spray drying according to central composite design run numbers.

The estimated regression coefficients for particle type of cinnamon bark oil loaded microspheres were given in Table 7.10.

Table 7.10. Estimated regression coefficients for particle type of cinnamon bark oil loaded microspheres

Term	Coef	SE Coef	T	P
Constant	3.99137	0.3251	12.276	0.000
A	0.20711	0.2568	0.807	0.446
B	0.41421	0.2568	1.613	0.151
C	0.24629	0.2568	0.959	0.369
A*A	-1.41593	0.4753	-2.979	0.021 *
B*B	-0.91593	0.4753	-1.927	0.095
C*C	0.08407	0.4753	0.177	0.865
A*B	-0.70711	0.5643	-1.253	0.250
A*C	0.00000	0.5643	0.000	1.000
B*C	0.00000	0.5643	0.000	1.000

* The terms with P value less than or equal to 0.05 have been considered as significant.

The regression equation (Equation 7.5) for particle type was as follows, with a regression coefficient R^2 of 71.73 %:

$$\hat{y}_2 = 3.991 - 1.416x_1^2 \quad (7.5)$$

This equation revealed that the main effect of pure quadratic term of inlet air temperature.

Table 7.11. Estimated regression coefficients for efficiency of cinnamon bark oil loaded microspheres

Term	Coef	SE Coef	T	P
Constant	8.8148	3.141	2.807	0.026
A	0.6398	2.480	0.258	0.804
B	-7.2972	2.480	-2.942	0.022 *
C	-9.4334	2.480	-3.803	0.007 *
A*A	12.3947	4.591	2.700	0.031 *
B*B	6.9639	4.591	1.517	0.173
C*C	5.6580	4.591	1.232	0.258
A*B	-0.1787	5.450	-0.033	0.975
A*C	-4.3549	5.450	-0.799	0.451
B*C	-0.0878	5.450	-0.016	0.988

* The terms with P value less than or equal to 0.05 have been considered as significant.

The regression equation (Equation 7.6) for spray drying efficiency was as follows, with a regression coefficient R^2 of 81.94 %:

$$\hat{y}_2 = 8.815 - 7.297x_2 - 9.433x_3 + 12.395x_1^2 \quad (7.6)$$

In the case of spray drying efficiency, coefficient estimates and the p-values verified the significance of the main factors of feed flow rate and cinnamon bark oil concentration. Inlet air temperature alone was not an effective parameter, but the pure quadratic term of inlet air temperature indicated significance.

The optimum conditions for the manufacturing of cinnamon bark oil loaded microspheres by spray drying were determined as 181°C for inlet air temperature. 4.64 ml/min for feed flow rate and 0.5 % (w/v) for clarithromycin concentration with desirability of 0.94.

7.4. Characterization of Microspheres

Particle Size and Surface Morphology

The surface morphology of uncrosslinked and genipin crosslinked clarithromycin and cinnamon bark oil loaded chitosan microspheres produced under optimum spray drying conditions was observed by scanning electron microscopy studies and SEM pictures were given in Figure 7.4. Surface morphology was affected by the drug concentration and crosslinking. The sphericity of all microspheres synthesized was generally very high. Particle size was generally in the range of 1-5 μm.

The surface of clarithromycin and cinnamon bark oil loaded microspheres was more wrinkled than that of control microspheres. This means that addition of clarithromycin or cinnamon bark oil decreaded the sphericity of the chitosan microspheres. Therefore, wrinkled structure was obtained. Moreover, the particle size of clarithromycin and cinnamon bark oil loaded microspheres was smaller than the control microspheres.

Particle size was significantly decreased by crosslinking due to the formation of more dense structure. Sphericity was very low in the case of genipin crosslinked cinnamon bark oil loaded microspheres.

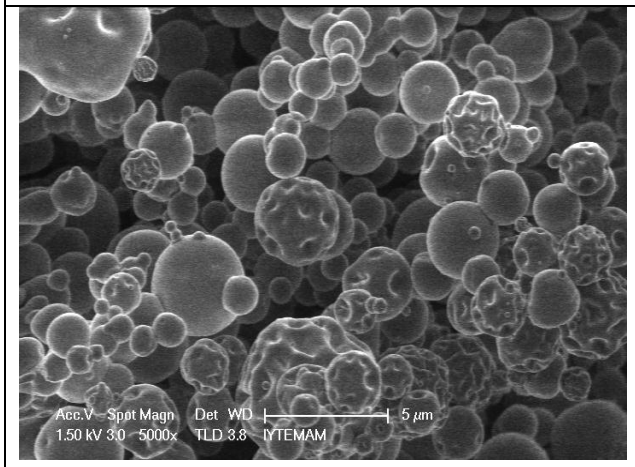
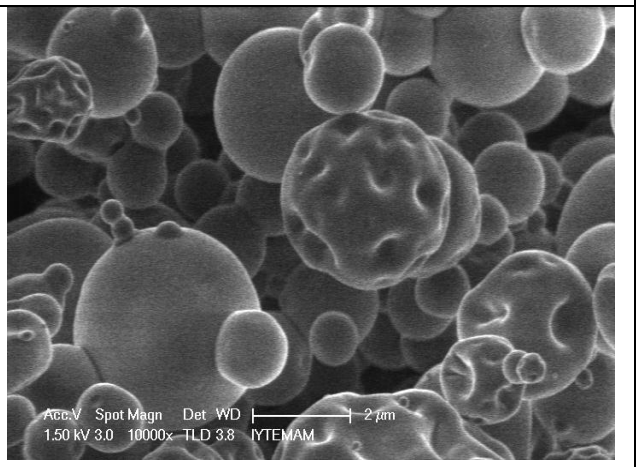
5000x	10000x
	
(a)	(b)

Figure 7.4. SEM micrographs: (a). (b) control chitosan microspheres; (c). (d) cinnamon bark oil loaded microspheres; (e). (f) clarithromycin loaded microspheres; (g). (h) 5mM genipin crosslinked clarithromycin microspheres for 18h crosslinking; (i). (j) 5mM genipin crosslinked cinnamon bark oil microspheres for 18h crosslinking.

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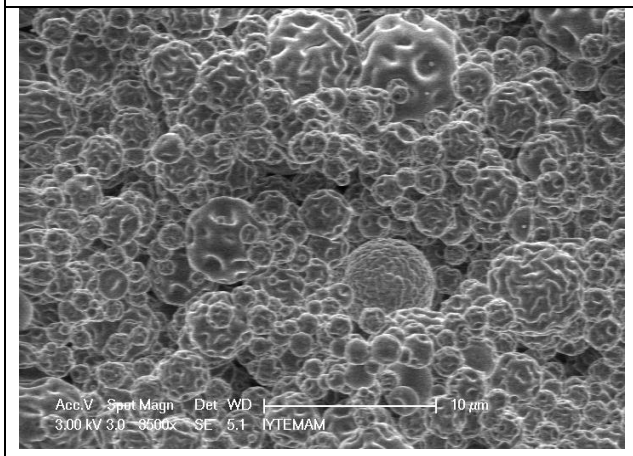
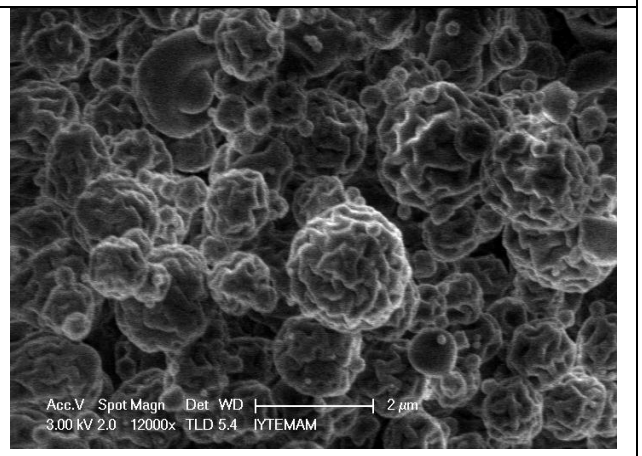
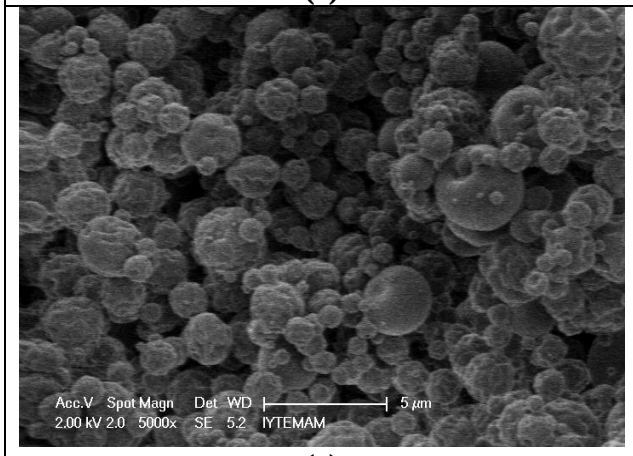
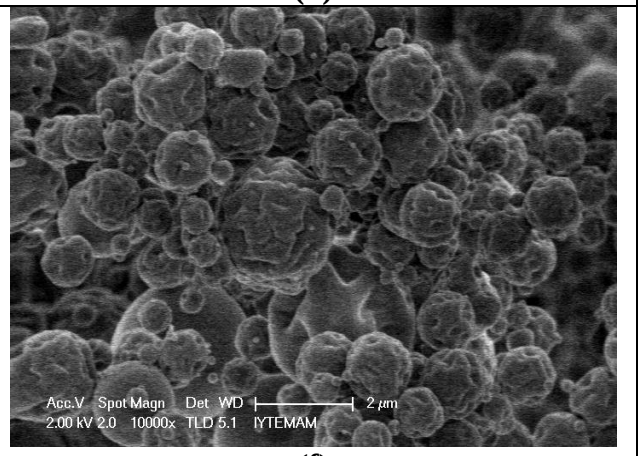
	
(c)	**(d)**
	
(e)	**(f)**

Figure 7.4. (cont.)

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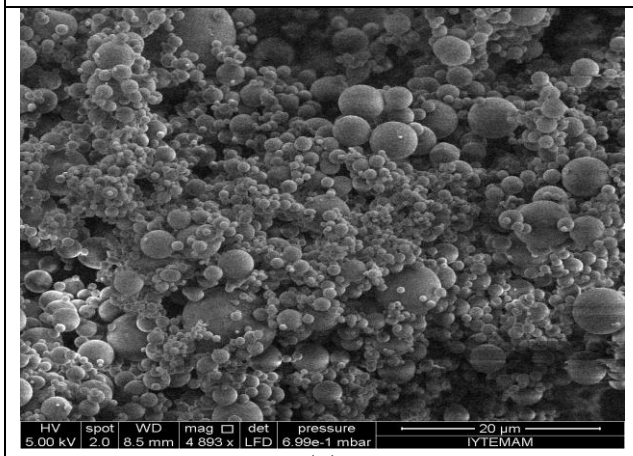
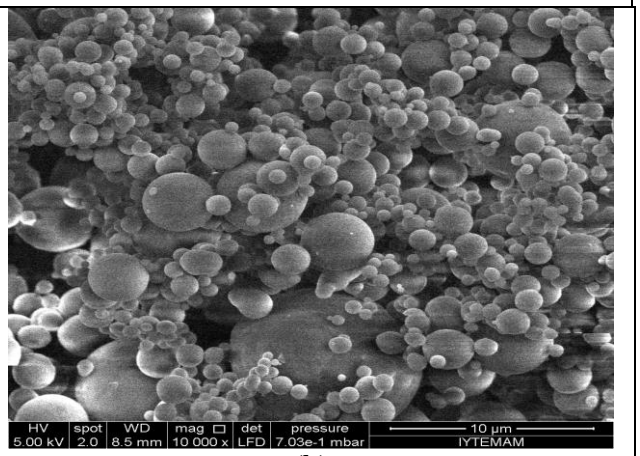
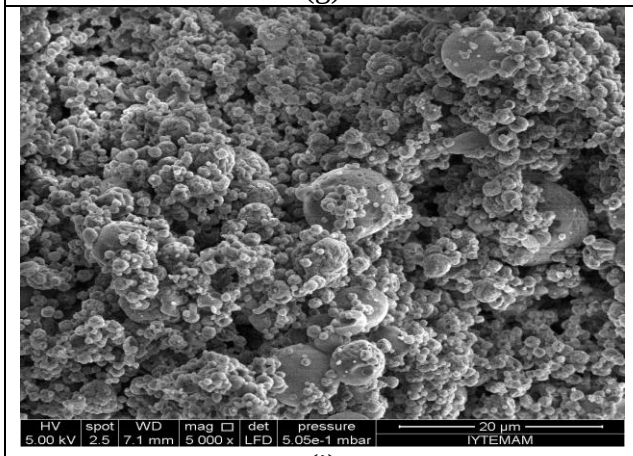
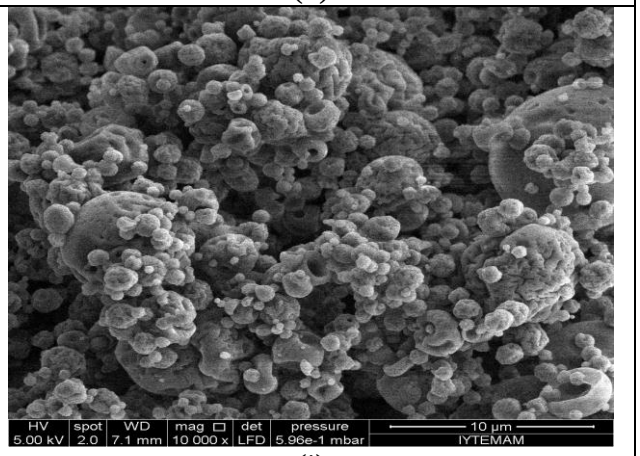
 (g)	 (h)
 (i)	 (j)

Figure 7.4. (cont.)

Zeta Potential

Zeta potential is representative of particle surface charge. Mucoadhesion is greatly dependent upon mucus and polymer structure including their charges. Chitosan is a cationic polysaccharide bearing primary amino and hydroxyl groups in each repeating unit. When protonated, primary amino groups carry a positive charge, which may facilitate electrostatic interactions with negatively charged mucin. When these amino groups are deprotonated, they can participate in hydrogen bonding with mucin along with the nonionic hydroxyl groups (Sogias et al., 2008). It was demonstrated that positive charge on the surface of chitosan could give rise to a strong electrostatic interaction with mucus or with a negatively charged mucosal surface (He et al., 2008; Dhawan et al., 2004). An insight into electrostatic interaction during mucoadhesion can be gained by measuring the zeta potential of chitosan microspheres. Table 7.12 shows the zeta potential values of clarithromycin, genipin and uncrosslinked/genipin crosslinked chitosan microspheres loaded with clarithromycin and cinnamon bark oil in phosphate buffer of pH 5. Our results showed that the zeta potential values of chitosan microspheres changed in the range of 21-30 mV.

The zeta potential of the microspheres remained positive after the crosslinking with genipin, however, it decreased when increasing genipin concentration. This indicates that the positive charge of free amino groups at the microsphere surface decreased possibly by the electrostatic interaction between negatively charged genipin and positively charged free amino groups of chitosan. The positive zeta potential value of clarithromycin and cinnamon bark oil loaded microspheres is of great importance since positively charged microspheres can interact with negatively charged mucins (mucus glycoprotein), exhibiting mucoadhesive properties.

Table 7.12. Zeta potentials of clarithromycin, genipin and uncrosslinked/genipin crosslinked chitosan microspheres loaded with clarithromycin and cinnamon bark oil

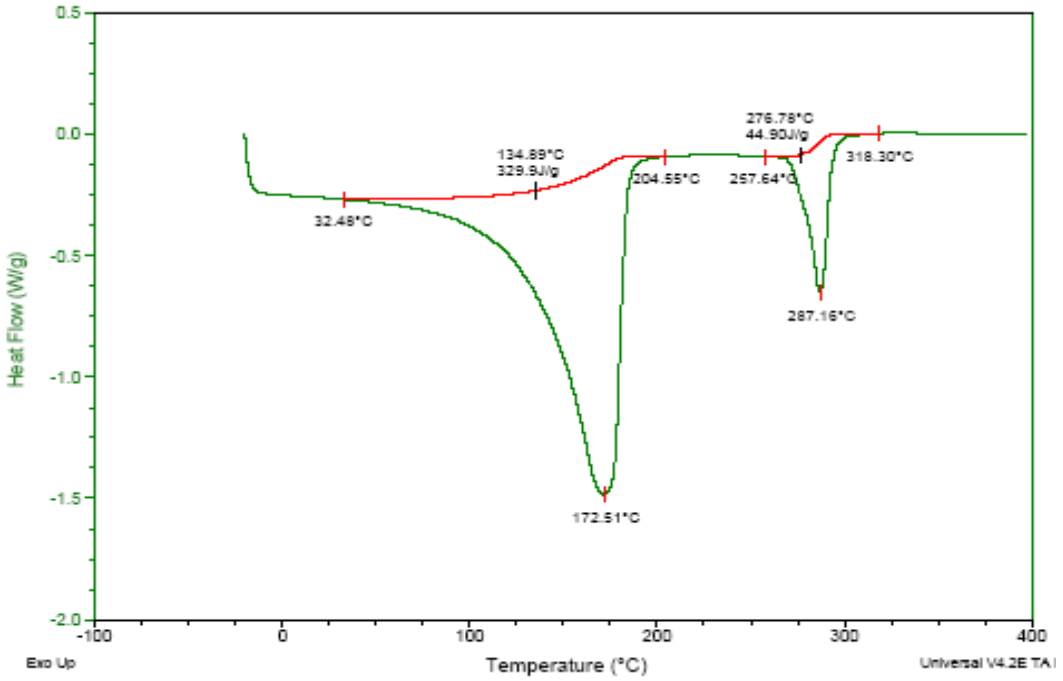
Formulation	**Zeta potential (mV)**
clarithromycin	-9.5
genipin	-12.4
chitosan	32.5
uncrosslinked chitosan-CLA microspheres	26.1
chitosan-CLA- microspheres 1mM genipin-1h crosslinked	29.2
chitosan-CLA- microspheres 5mM genipin-1h crosslinked	24.0
chitosan-CLA- microspheres 1mM genipin-18h crosslinked	28.6
chitosan-CLA- microspheres 5mM genipin-18h crosslinked	21.2
uncrosslinked chitosan-cinnamon bark oil microspheres	27.8
chitosan-cinnamon bark oil- microspheres 5mM genipin-1h crosslinked	25.4
chitosan-cinnamon bark oil- microspheres 5mM genipin-18h crosslinked	21.7

Thermal Analysis

Thermal properties of cinnamon bark oil was determined by DSC (Figure 7.5a) whereas thermal properties of clarithromycin, control microspheres, clarithromycin and cinnamon bark oil loaded microspheres were determined by TGA (Figure 7.5b-5e).

TGA thermograms of control microspheres were shown in Figure 7.5d. Three decomposition steps were observed in thermograms. The first weight loss occurred in the range of 40-90°C, which can be attributed to free water and acetic acid evaporation. The second step occurred in the range of 110-175°C due to the evaporation of bound water. The third step, the main decomposition, occurred in the range of 250-290 °C with approximately 50% weight loss that should be due to the degradation of the polysaccharide chains and degradation of deacetylated units of chitosan. The weight loss occured in 3 steps even loading clarithromycin to the chitosan microspheres (Figure 7.5e. g and h). The free water and acetic acid evaporation occured at lower

temperature around 65°C in clarithromycin and cinnamon bark oil loaded microspheres than that of control microspheres as 72°C (Figure 7.5f). The evaporation of bound water was around 150°C in all cases. A little increase in the main decomposition temperature of clarithromycin and cinnamon bark oil loaded microspheres shows the higher stability of these microspheres compared to control microspheres. As a result, there was no significant difference in the thermal stabilities of the microspheres with the addition of essential oil or clarithromycin.



(a)

Figure 7.5. (a) Cinnamon bark oil DSC analysis; TGA thermograms of (b) clarithromycin; (c) genipin; (d) control chitosan microspheres; (e) clarithromycin loaded microspheres; (f) cinnamon bark oil loaded microspheres; (g) 1h genipin crosslinked clarithromycin loaded microspheres; (h) 18h genipin crosslinked clarithromycin loaded microspheres.

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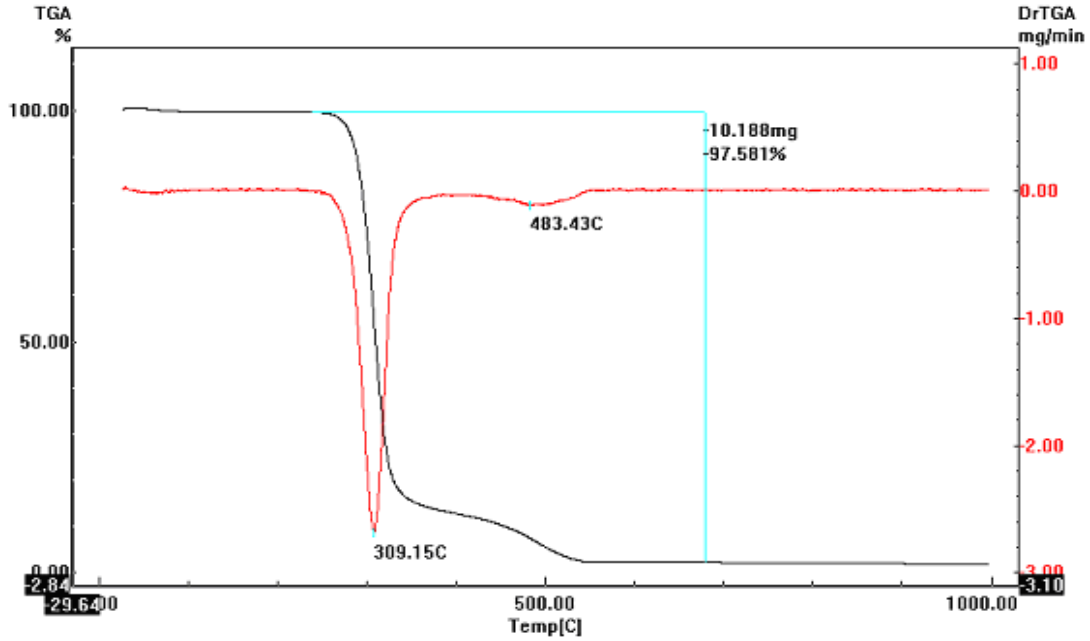
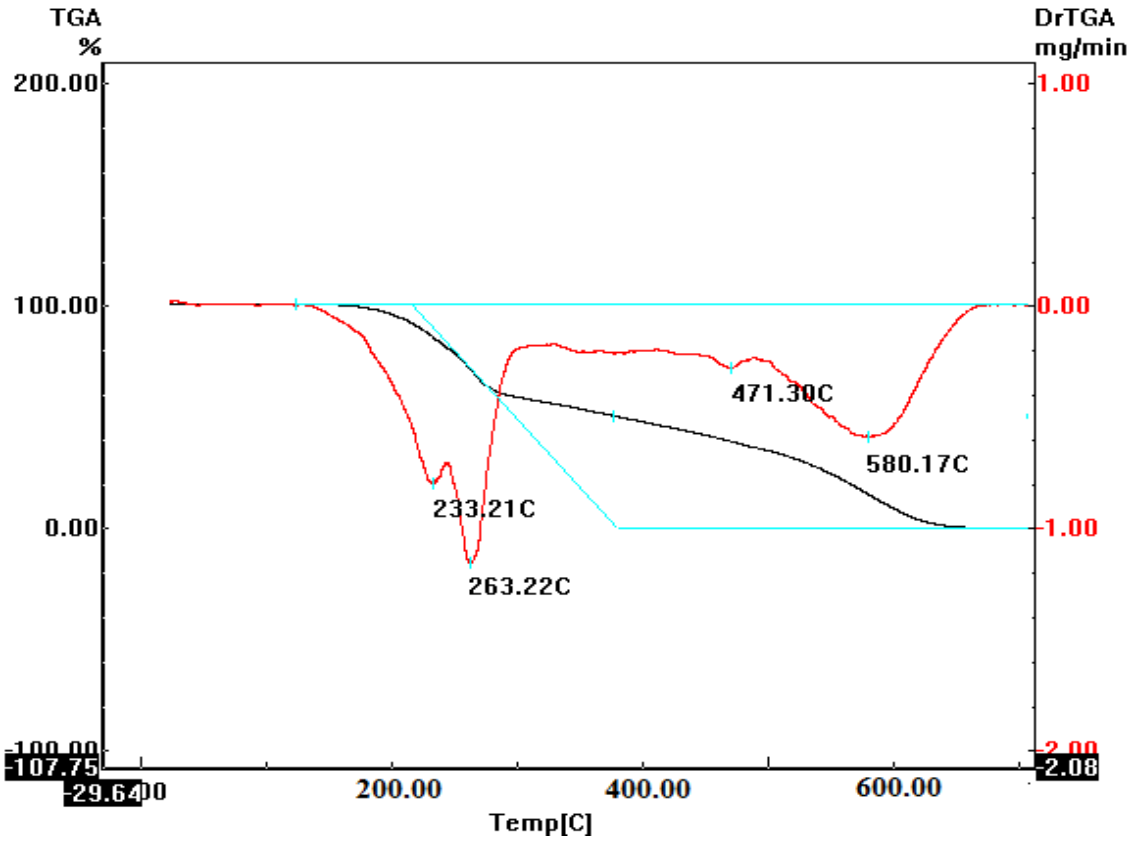


(b)



(c)

Figure 7.5. (cont.)

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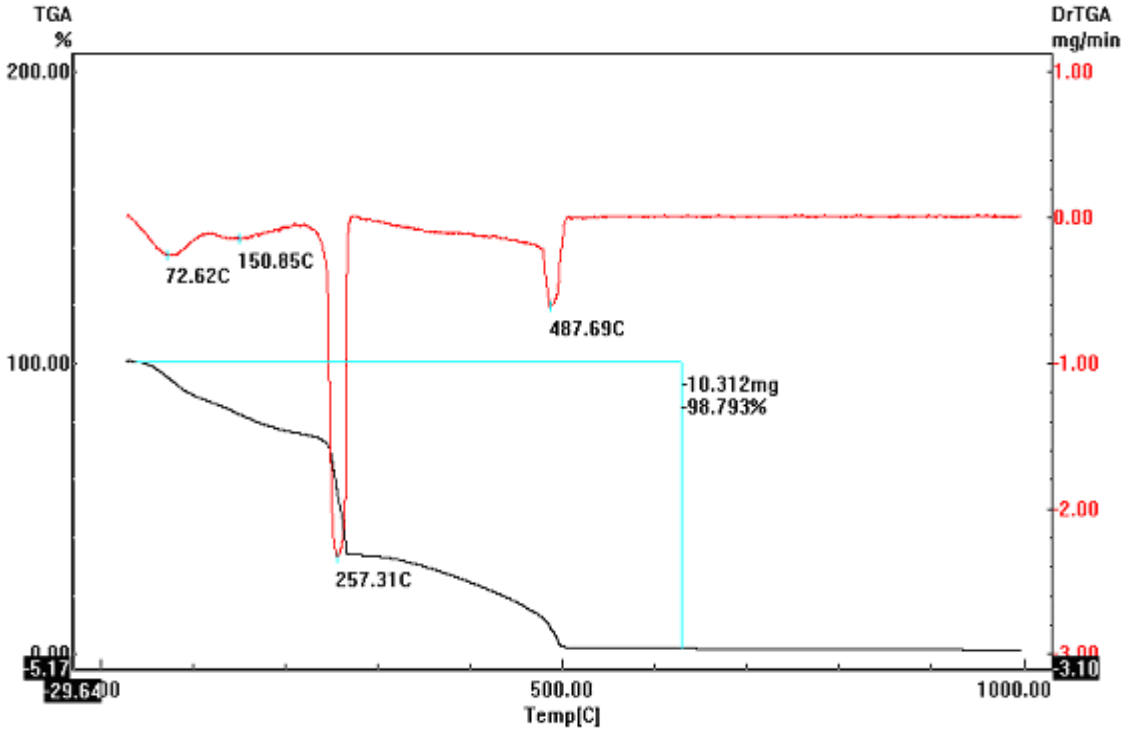
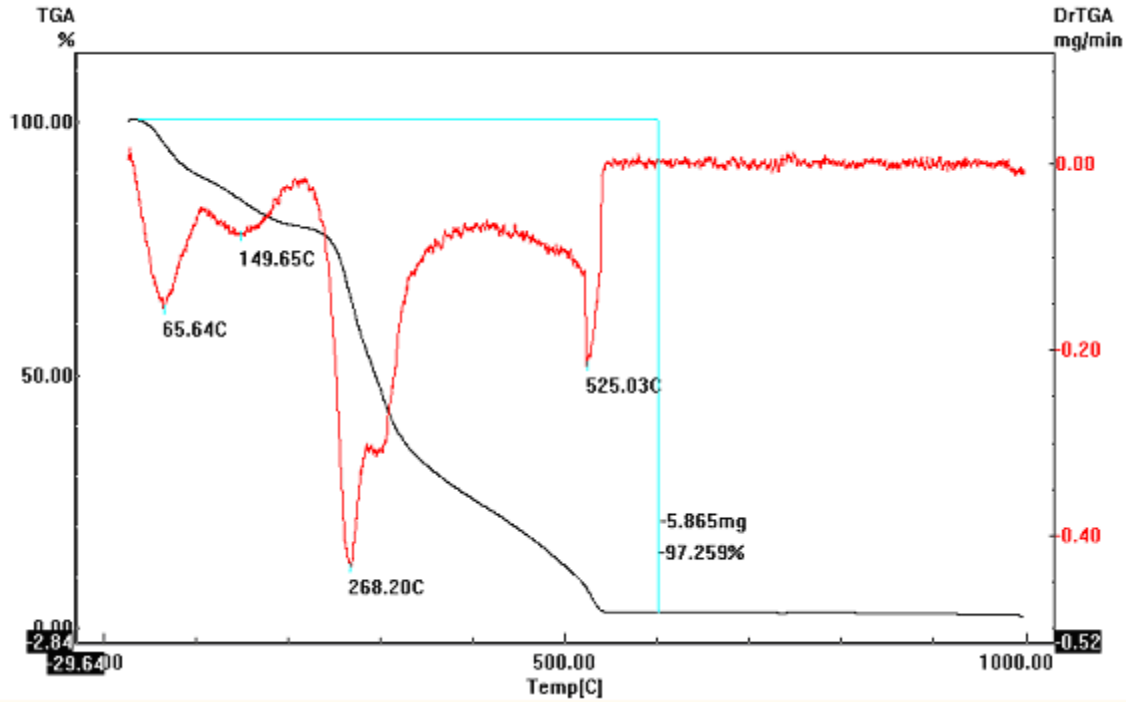


(d)



(e)

Figure 7.5. (cont.)

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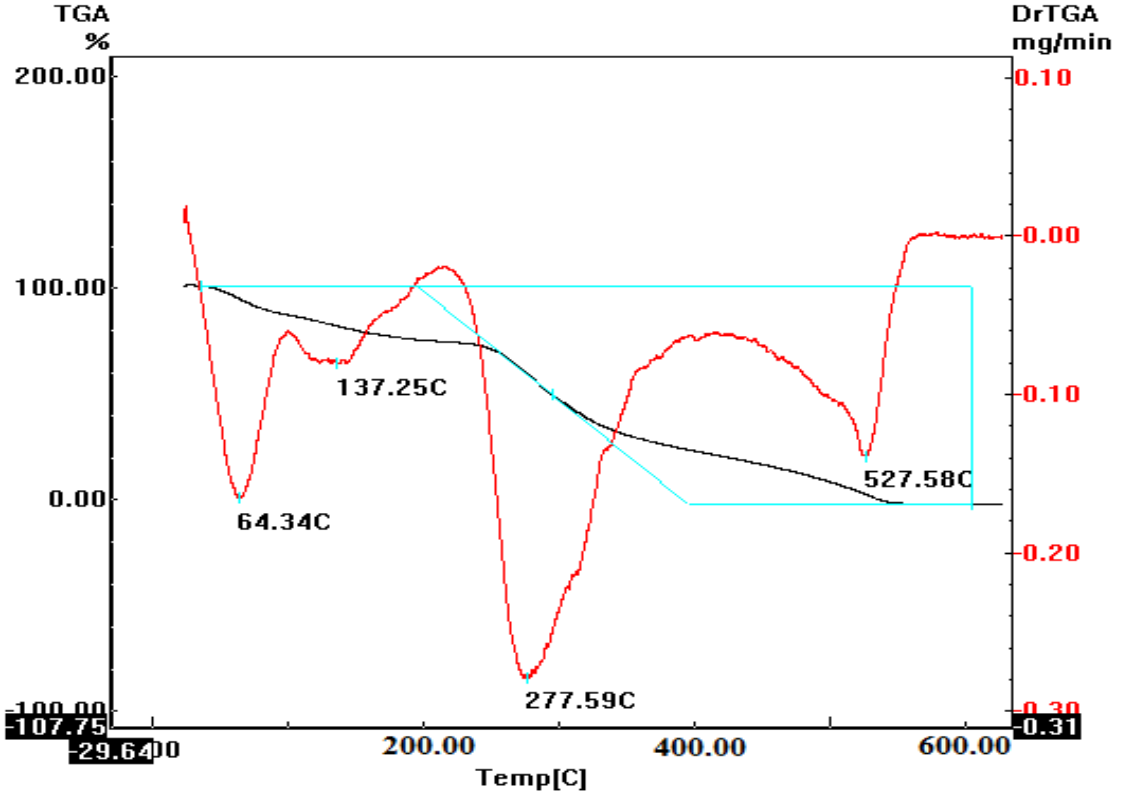
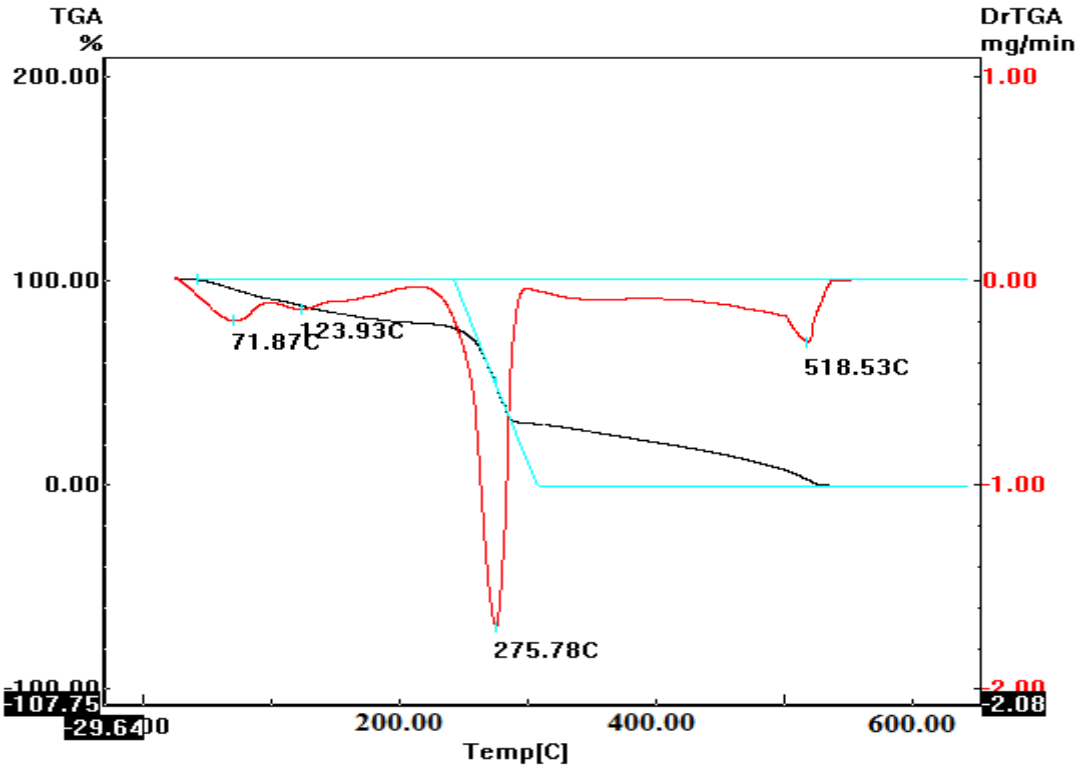
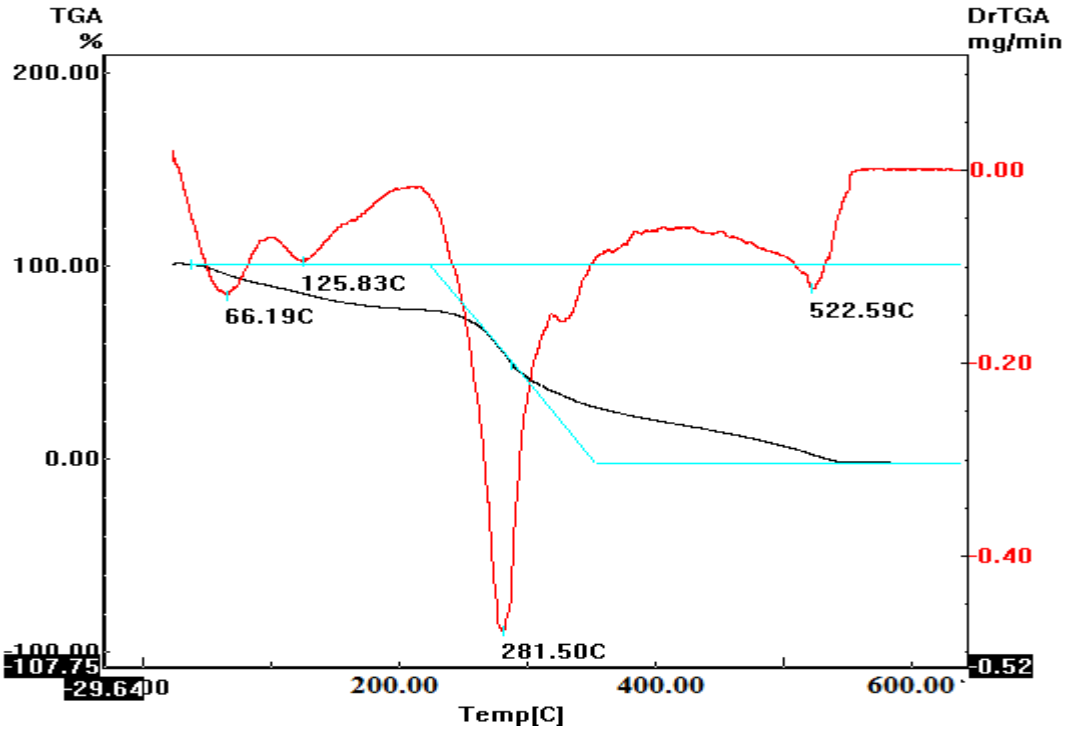


(f)



(g)

Figure 7.5. (cont.) **(cont. on next page)**



(h)

Figure 7.5. (cont.)

The decomposition temperature of chitosan microspheres was 257.3 ºC (Figure 7.6A), whereas that of the genipin crosslinked was 275 ºC (Figure 7.6B), which was between the decomposition temperatures of clarithromycin (at 309.1 ºC Figure 7.6C) and chitosan microspheres. Comparing the decomposition temperatures, it could be seen that the crosslinking of the polymer increased the stability of the microspheres with respect to the chitosan control microspheres.

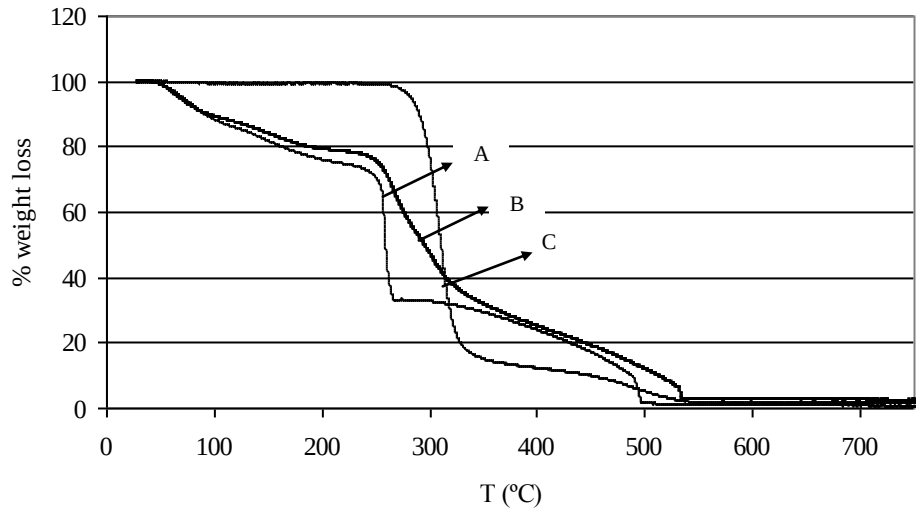


Figure 7.6. TGA thermograms of (A) chitosan microspheres; (B) clarithromycin loaded chitosan microspheres and (C) clarithromycin.

Effects of crosslinking and its time on the thermal stability of chitosan microspheres were investigated (Figure 7.7). It is evident that, stability of chitosan microspheres increased by increasing the crosslinking time. Because of the covalent bond between chitosan and genipin (i.e. amide (N-C=O) bonding), genipin crosslinked microspheres were considerably stable and had higher decomposition temperature (Figure 7.7A and B).

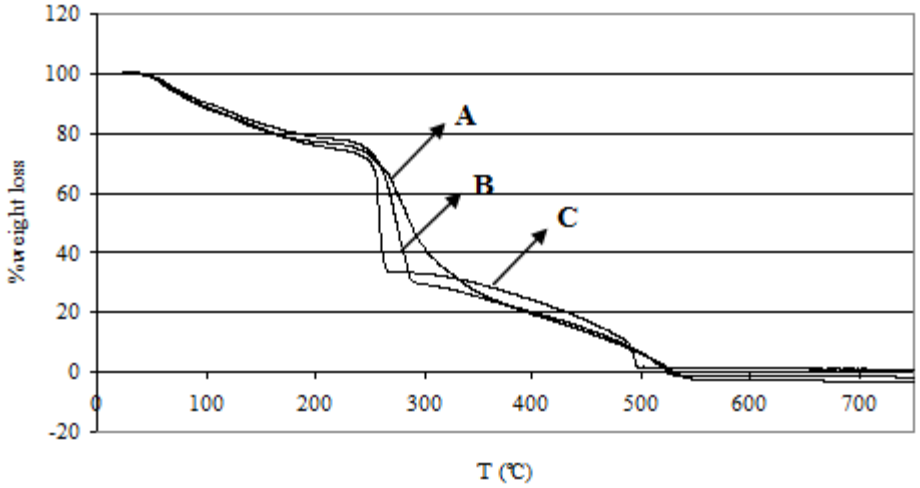


Figure 7.7. TG curves of clarithromycin loaded genipin crosslinked chitosan microspheres (A) 18 hour; (B) 1h and (C) microspheres without genipin.

Nucleer Magnetic Resonans Spectrometry Analysis (^{1}H-NMR)

The structure of clarithromycin, control and clarithromycin loaded microspheres was investigated by ^{1}H-NMR spectroscopy (Figure 7.8). In the range of 1.3-0.6 ppm, the characteristic signals of clarithromycin exist also in clarithromycin loaded chitosan microspheres meaning that clarithromycin was succesfully loaded into microspheres. However, the signal at 2.7 ppm belonging to (N-CH_3) group of clarithromycin could not be observed in the spectrum of clarithromycin loaded microspheres. This may be due to the loss of desozamine sugar bound to C_5 with β-glucosidic bond during the microsphere production. Clarithromycin is connected to the peptidil transferase zone in 50S ribosomal unit of *H. pylori* by hydrogen bonding with the cladinose sugar connected to C_3 with α-glicosidic bond of clarithromycin (Zuckerman, 2004; Gerrits et al., 2006). Since the cladinose sugar presents in clarithromycin loaded chitosan microspheres, the antimicrobial activity of clarithromycin in microspheres was maintained.

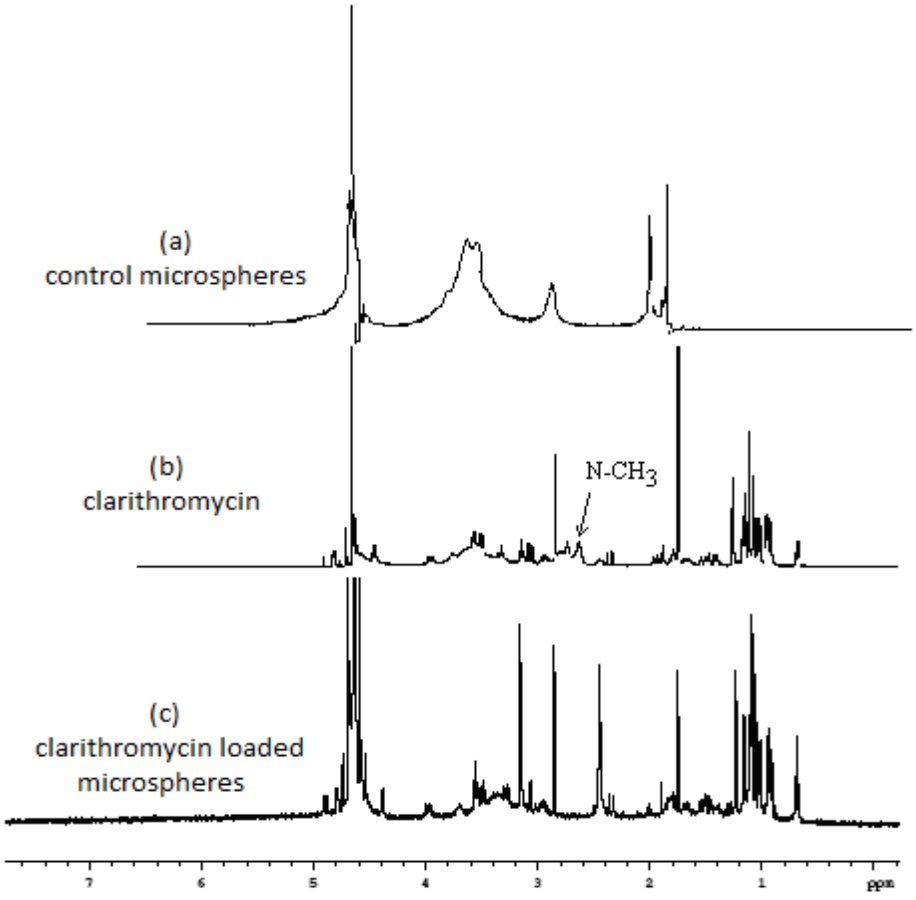
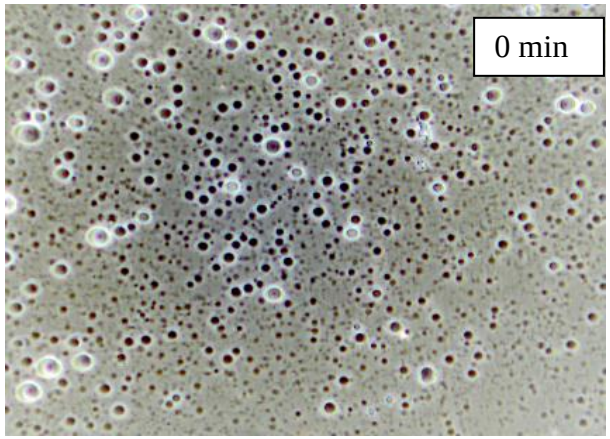


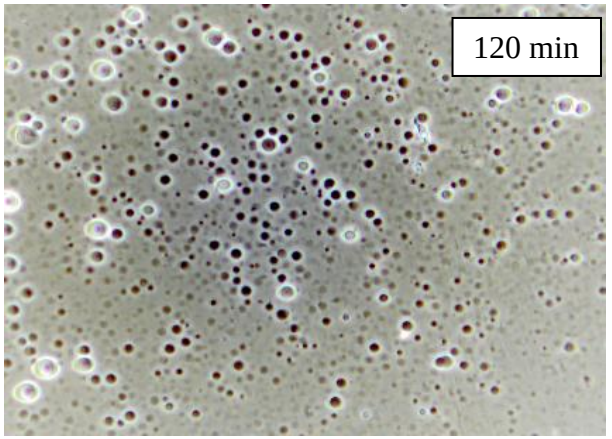
Figure 7.8. ^{1}H-NMR spectra: (a) control chitosan microspheres, (b) clarithromycin, (c) clarithromycin loaded microspheres.

Swelling and Degradation Properties of Microspheres

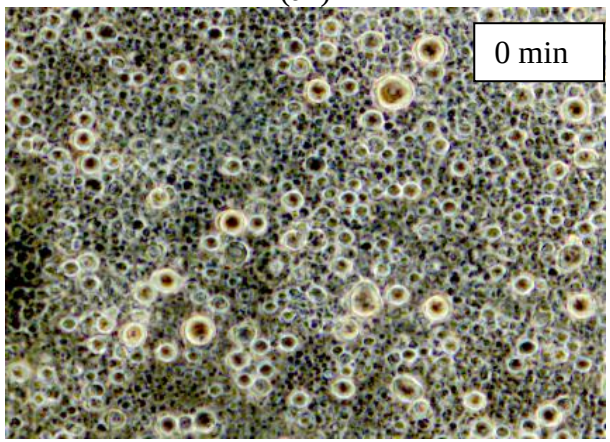
The swelling and degradation properties of uncrosslinked/genipin crosslinked clarithromycin and cinnamon bark oil loaded microspheres in phosphate buffer of pH 5 were examined by phase contrast microscope. After dropping buffer on microspheres, the photographs of microspheres were taken in specified time intervals. Swelling ratio was determined by the change of particle diameter with time. The photographs of clarithromycin and cinnamon bark oil loaded microspheres were given in Figure 7.9 and Figure 7.10, respectively.



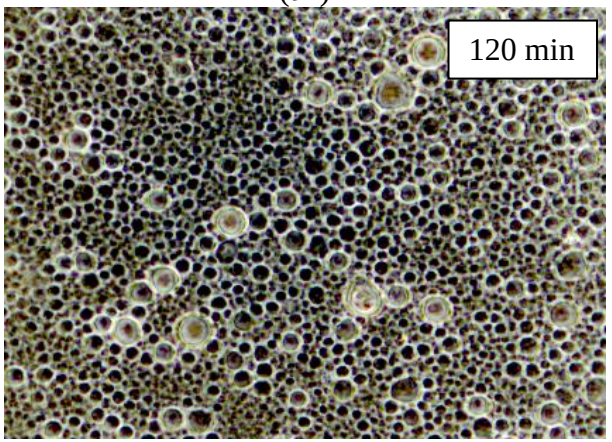
(a1)



(a2)



(b1)



(b2)

Figure 7.9. Phase contrast microscope photographs of clarithromycin loaded uncrosslinked (a1 and a2) and genipin crosslinked (b1 and b2) microspheres at t=0 min and t=120 min.

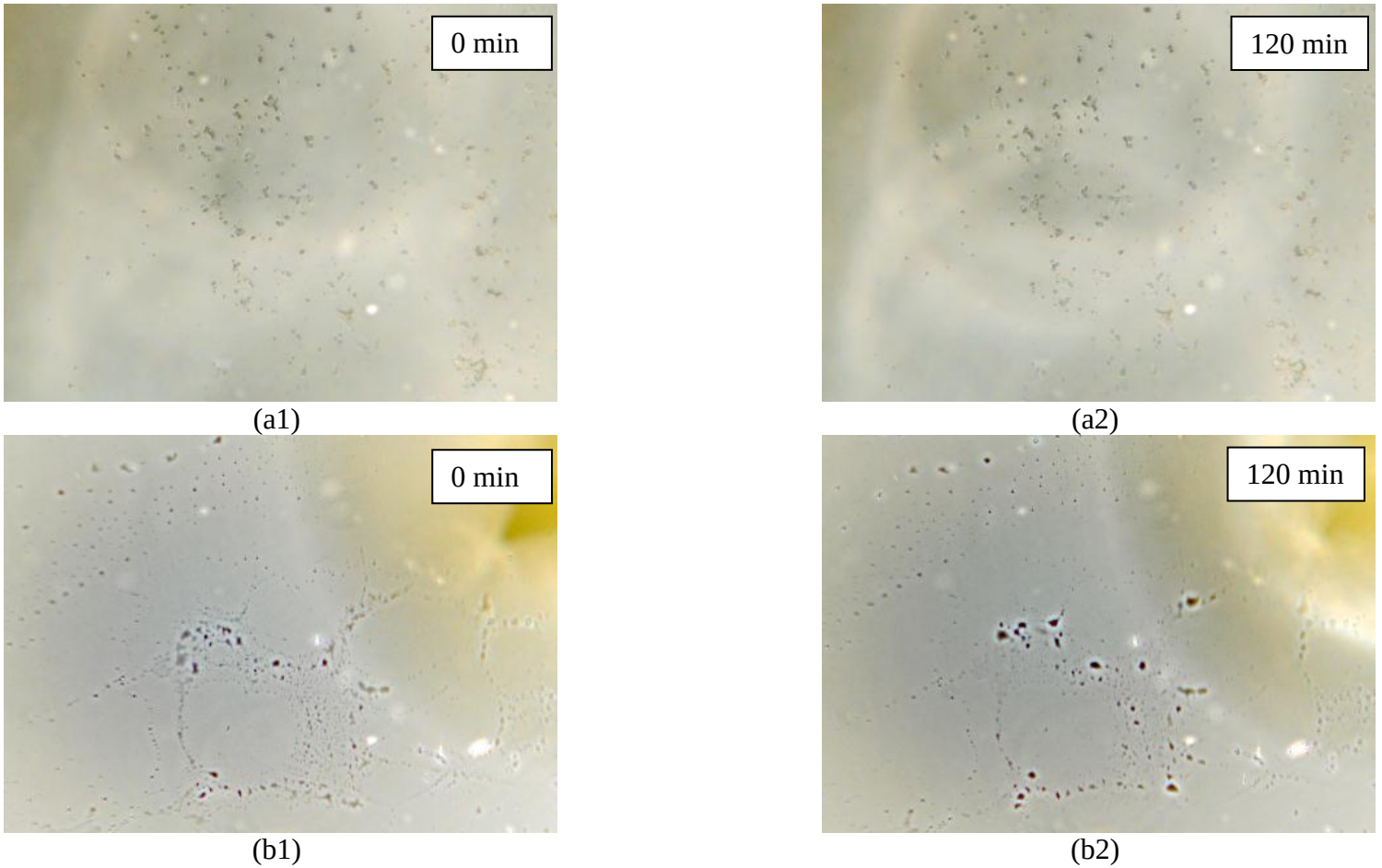
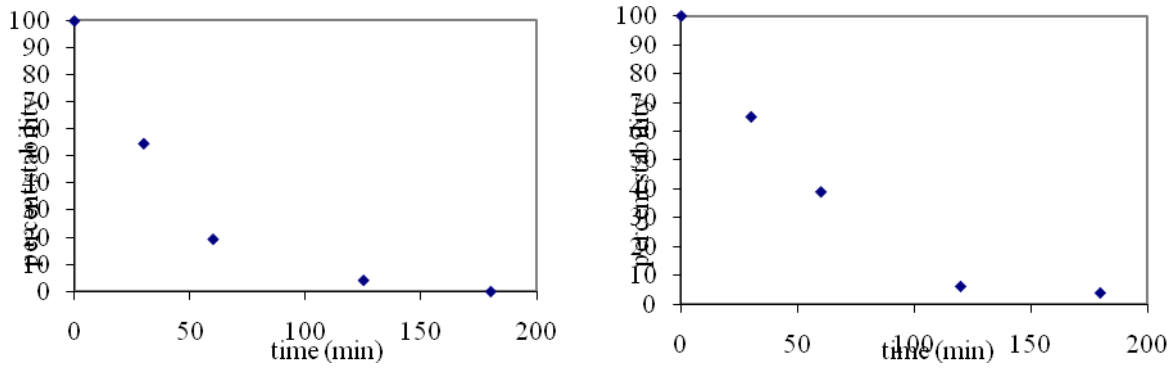


Figure 7.10. Phase contrast microscope photographs of cinnamon bark oil loaded uncrosslinked (a1 and a2) and genipin crosslinked (b1 and b2) microspheres at t=0 min and t=120 min.

Particle diameters of microspheres in all cases increased approximately 10% in buffer solution within 120 min. There was not a significant effect of genipin crosslinking on the swelling and degradation properties of microspheres. Particles remained stable for 120 min in buffer solution.

7.5. Clarithromycin Stability in Different pH Solutions

The stability of antibiotics in an acidic medium plays an important role in the eradication of *H. pylori*. In fact, lots of antibiotics have been reported with a strong in vitro *H. pylori* clearance effect but poor in vivo results. One reason for the failure is their instability in an acidic medium. It is crucial to maintain the stability of antibiotics during the prolongation of the gastric retarding time of a stomach-specific drug delivery system. As shown in Figure 7.11 and 7.12, the degradation profiles of clarithromycin in solutions at pHs 2.0. 3.0. 5.0 and 7.0 were determined. Furthermore the effect of pepsin addition to the gastric fluid on clarithromycin stability was investigated (Figure 7.11a and b).



(a) (b)

Figure 7.11. Stability of clarithromycin in simulated gastric fluid (pH 2.0) without pepsin (a); with pepsin (b).

At pH 2.0, the clarithromycin degradation was much faster in SGF than that of pepsin incorporated SGF, and about 80 % of the drug degraded within 60 min. Zhu et al., 2006 investigated the pepsin mediated proteolysis of *H.pylori* and addition of pepsin alone or in gastric juice causes a marked increase in bacterial susceptibility, suggesting an important role for proteolysis in the killing of bacteria (Zhu et al., 2006). Together

with the proteolysis effect of pepsin on *H. pylori* and to increase the stability of clarithromycin in gastric pH, presence of pepsin would be beneficial in the eradication of the bacteria.

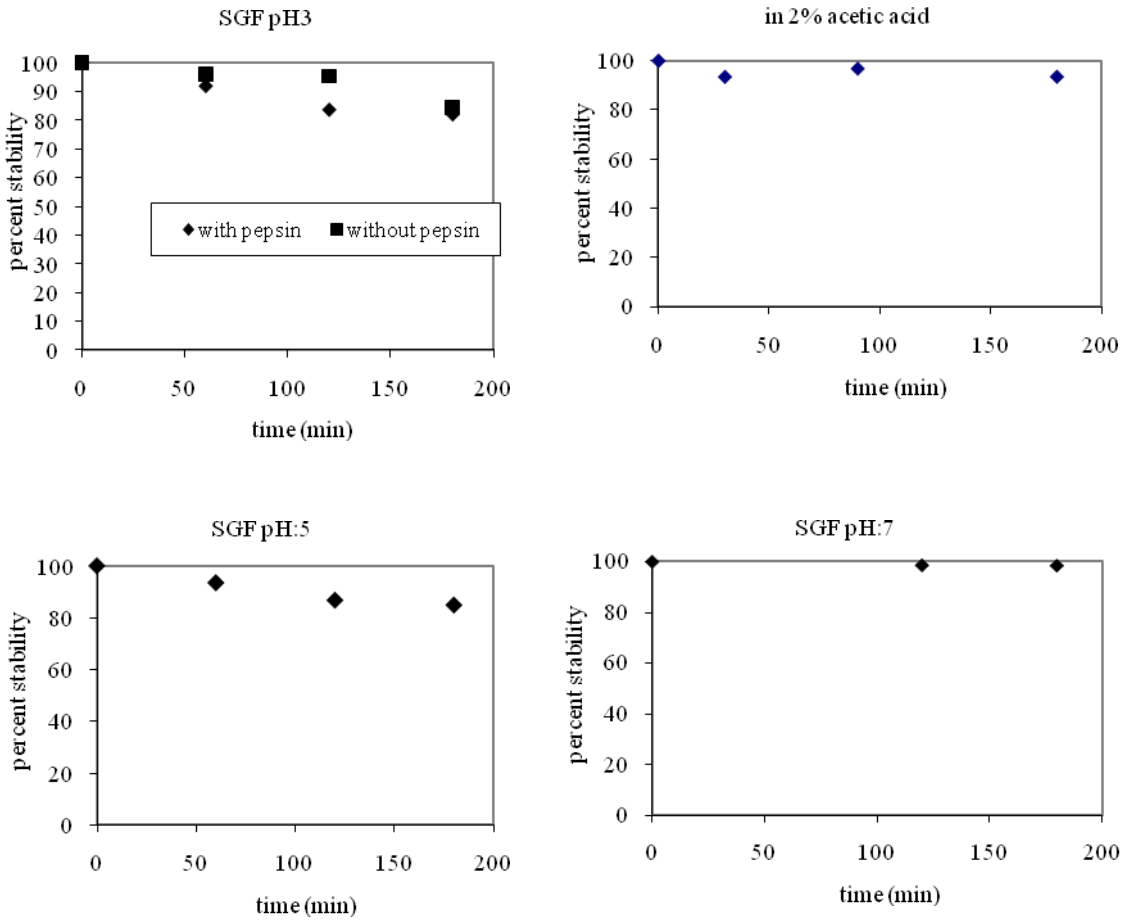


Figure 7.12. Stability of clarithromycin in different pH medium.

A different behavior was observed at pH's of 3.0 and 5.0. At pH 3.0 and 5.0, the clarithromycin degradation became slower, and about 15% of the drug degraded within 180 min. At pH 7.0, the drug degradation was almost negligible in the considered time ranges and clarithromycin was particularly stable (Figure 7.12).

From the data presented here it can be concluded that at gastric pH values of less than 2.0, which can readily occur in the stomach, clarithromycin will undergo rapid degradation. This is a factor which may significantly affect the antimicrobial activity of the drug since it lowers the drug concentrations at the target site to values below the MIC against *H. pylori*, leading to therapeutic failure (Erah et al., 1997). The use of proton-pump inhibitor is helpful for keeping the clarithromycin stability by increasing

the gastric pH. Incorporation of the drug into microspheres which yields the controlled release may be another way in maintaing the clarithromycin stability.

7.6. Release of Clarithromycin and Cinnamon Bark Oil from Microspheres

The in vitro release of clarithromycin and cinnamon bark oil from microspheres was performed at 37°C and 150 rpm in a thermoshaker in phosphate buffer (pH 5). 250 μl release medium was withdrawn at predetermined time intervals and equivalent fresh medium was replaced. Collected samples were then analyzed for drug content (claritromisin and cinnamon bark oil). The clarithromycin and cinnamon bark oil amounts in release medium were obtained from the calibration curves given in Figure 7.13 and 7.14. respectively. The clarithromycin amount in release medium was determined by HPLC method as explained in experimental section.

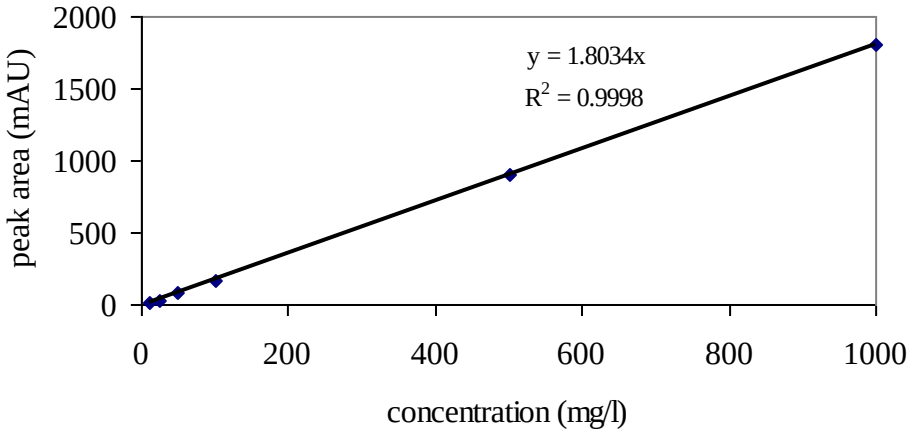


Figure 7.13. Calibration curve for clarithromycin.

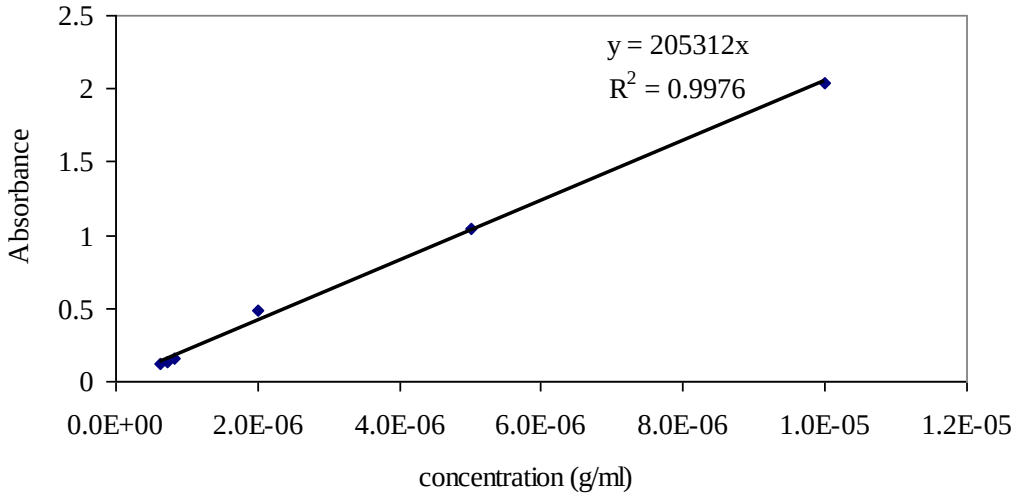


Figure 7.14. Calibration curve for cinnamon bark oil.

The clarithromycin amount in release medium was determined by HPLC method.

As shown in Figure 7.15, in vitro release of clarithromycin from the chitosan microspheres was evaluated under various conditions. As known, many parameters can affect the drug release behavior of chitosan microspheres. These parameters include chitosan concentration, crosslinker concentration, drug/polymer ratio, temperature, release medium, etc. In this study, at constant drug/polymer ratio, the effects of crosslinking concentration and its time on the drug release behavior were investigated. As stated in previous section, clarithromycin was confirmed to be stable for the considered time range of the release study at 37 ºC at pH 5.0 (Figure 7.12). The cumulative release of clarithromycin from chitosan microspheres showed a burst effect. In order to obtain prolonged release of clarithromycin from the microspheres, genipin was used as a crosslinking agent. Genipin addition significantly affected the release profile of the drug as shown in Figure 7.16. Clarithromycin release from crosslinked chitosan microspheres was characterized in two steps as an initial rapid release of drug (burst effect) followed by slow release. In general 45-55% of drug was released from microspheres within first few minutes (Figure 7.15). Most probably the drug was located at the surface of the microspheres during the particle formation, which resulted in the burst effect. It is evident that controlled slow release profile was observed thereafter (Figure 7.15).

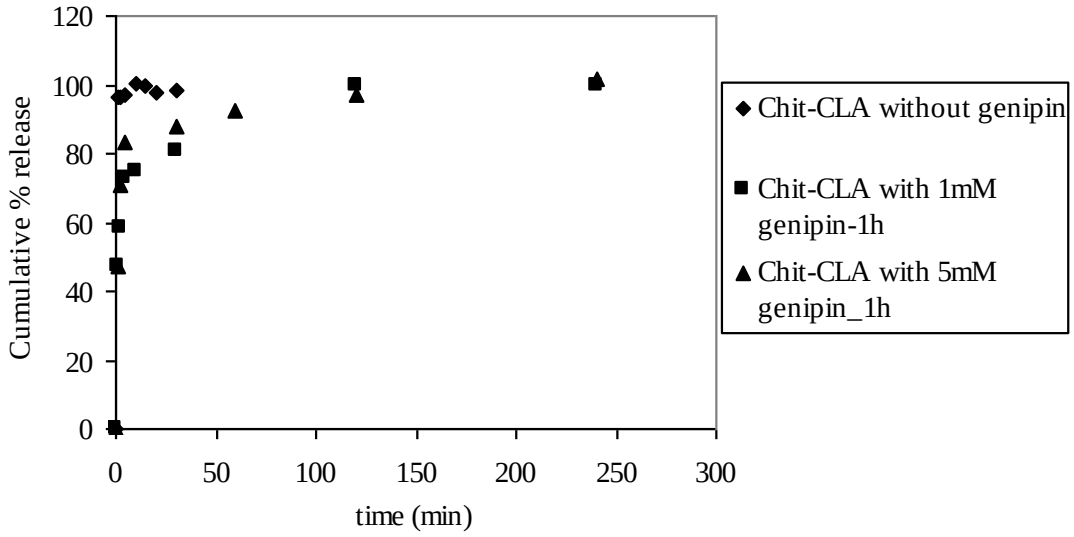


Figure 7.15. Clarithromycin release from chitosan microspheres to phosphate buffer of pH 5.0.

The effect of crosslinker concentration on the release profile was investigated by changing the genipin concentration from 1 mM to 5 mM by keeping crosslinking time constant as 1 hour. As the genipin concentration of the prepared microspheres increased, the release rate increased while extent of drug release decreased significantly. This could be attributed to increase in chitosan matrix density (because of the higher crosslinking profile), decrease in free volume and increase in diffusion path length which the drug molecules have to traverse.

In vitro release data were applied to various kinetics models; zero order (Equation 5.14), first order (Equation 5.13). Higuchi_square root of time analysis (Equation 5.9) to predict the drug release mechanism from the microspheres. The release kinetics of clarithromycin were analyzed by Ficks Law and applying square root of time analysis, which were shown in Figures 7.16 and 7.17.

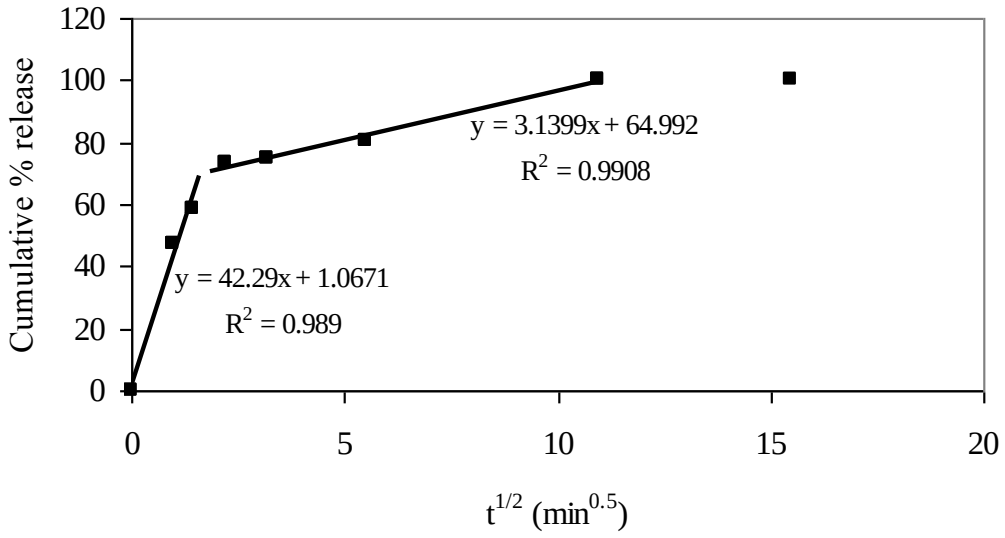


Figure 7.16. Fractional release of clarithromycin loaded microspheres crosslinked with 1 mM genipin for 1h.

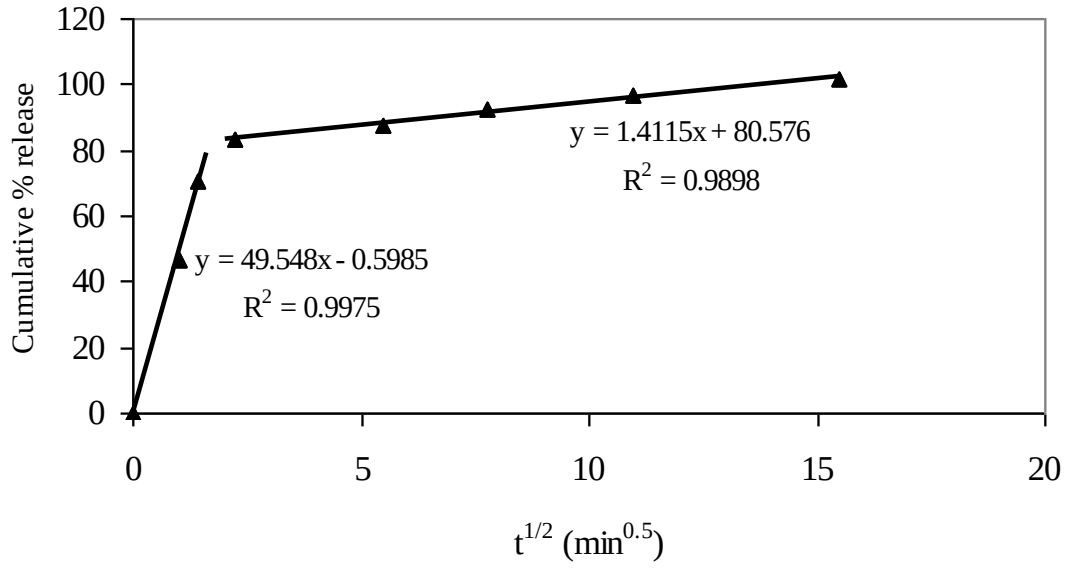


Figure 7.17. Fractional release of clarithromycin loaded microspheres crosslinked with 5 mM genipin for 1h.

According to linear patterns of both plots and the relevant R^2 values, square root time kinetic analysis suggested that release from microspheres was initially governed by drug dissolution and followingly diffusion through the swollen matrix. The slopes of the first linear portion of the plots indicated that about 75% of the drug was released within few minutes. This behaviour is important for the therapeutic activity of the

clarithromycin. Initial dose can be instanteneously achieved by administrating the microspheres. It is expected to achieve prolonged release of clarithromycin thereafter. Thus, second linear part in Figures 7.16 and 7.17 suggest that the controlled release of clarithromycin from genipin crosslinked chitosan microspheres.

It is important to notice that release rate decreased by increasing the genipin concentration, which is probably due to the higher crosslinking ratio of chitosan and accordingly higher resistance to clarithromycin to diffuse. The mucoadhesive property of chitosan is also very beneficial since after mucoadhesion of chitosan microspheres to the gastric surface, controlled release of clarithromycin will be continued. The drug dose was enough to achieve minimum inhibitory concentration to eradicate the *H. pylori*, 3.94 mg of clarithromycin remained in 50 mg of microspheres after dissolution of the clarithromycin, probably found in the outer surface of the microspheres. Minimum inhibitory concentration of the clarithromycin on the *H. pylori* was found as 0.125 μg/ml. The required MIC value is effectively achieved by encapsulated clarithromycin dose. The local delivery because of the mucoadhesive property of the chitosan will also be very beneficial to achieve MIC of clarithromycin at infected area.

The effect of crosslinking time on the release kinetic was also investigated by changing crosslinking time from 1hour to 18 hour. Crosslinking time directly affects the release profile of the clarithromycin (Figure 7.18).

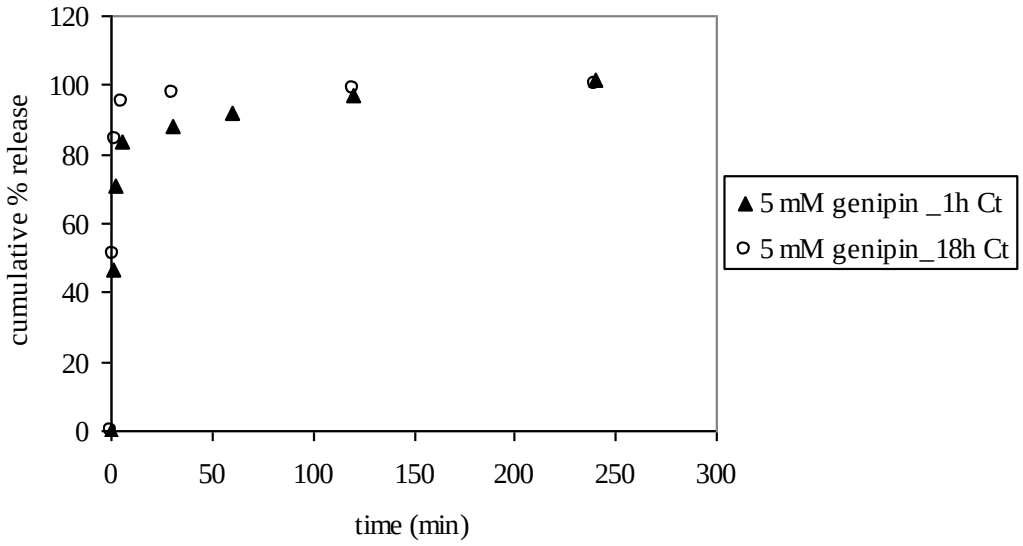


Figure 7.18. Effect of crosslinking time on the release profile of clarithromycin.

Increasing the crosslinking time yielded in burst effect. Square root of time kinetic analysis in Figure 7.19 indicates that about 90-95 % of the clarithromycin was released within few minutes.

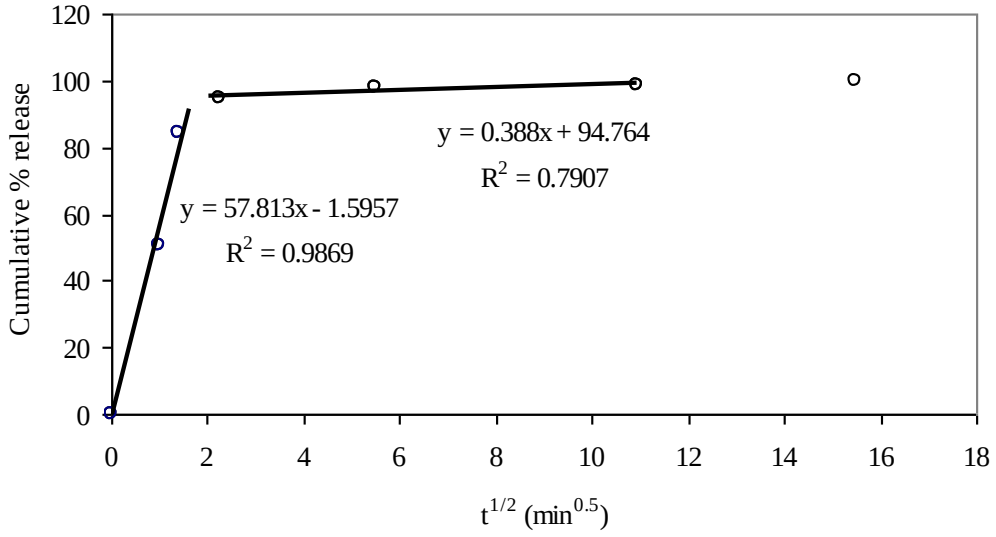


Figure 7.19. Square root of time analysis of clarithromycin release from clarithromycin loaded microspheres crosslinked with 5 mM genipin for 18h.

This inverse effect is due to the decrease in the free amino groups on chitosan surface, which also have role in drug-chitosan interaction. Electrostatic interactions between the negative surface charge of the clarithromycin (Bele et al., 2005), and the number of available positive chitosan charges could be the reason of prolonged release when the genipin crosslinking time is lower. This interaction resists clarithromycin to diffuse rapidly and decrease the diffusion rate. However, increasing the crosslinking time should decrease the positive charge on chitosan surface and accordingly to increase the diffusion rate.

Release profiles of cinnamon bark oil from chitosan microspheres are shown in Figure 7.20. The effect of crosslinking on the release profile is also included. 5 mM of genipin was used for crosslinking and the effect of crosslinking time was investigated by changing time from 1 h to 18h.

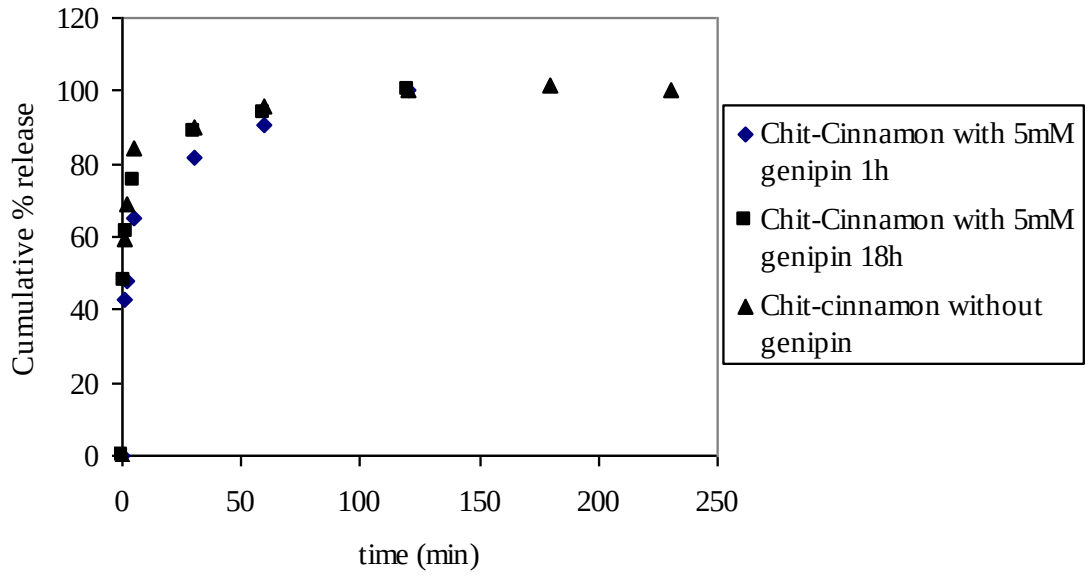


Figure 7.20. Cinnamon bark oil release profile from chitosan microspheres and effects of genipin and crosslinking time on release profile.

Rapid release of cinnamon oil was seen at the initial period of the release profiles. About 60% of cinnamon oil was released at that period for uncrosslinked microspheres. Interestingly, controlled release of the oil was achieved thereafter. Eugenol and trans-cinnamaldehyde are two very important terpenoids found in cinnamon oil, having –methoxy and =O groups, respectively, which are responsible in chemical reaction with the chitosan's amino groups. Similarly, genipin has active methoxy group, which reacts with chitosan for crosslinking. So, slow release profile of cinnamon oil from uncrosslinked chitosan microspheres can probably be explained by the chemical interaction.

Square root of time kinetic analysis in Figure 7.21 also indicates the controlled release profile of cinnamon oil from chitosan microspheres. Similarly, two linear sections were obtained; in the first linear section for burst release (at the initial period of release), and the other one for controlled release. The linearity of the line correponds to the diffusion controlled release of cinnamon oil.

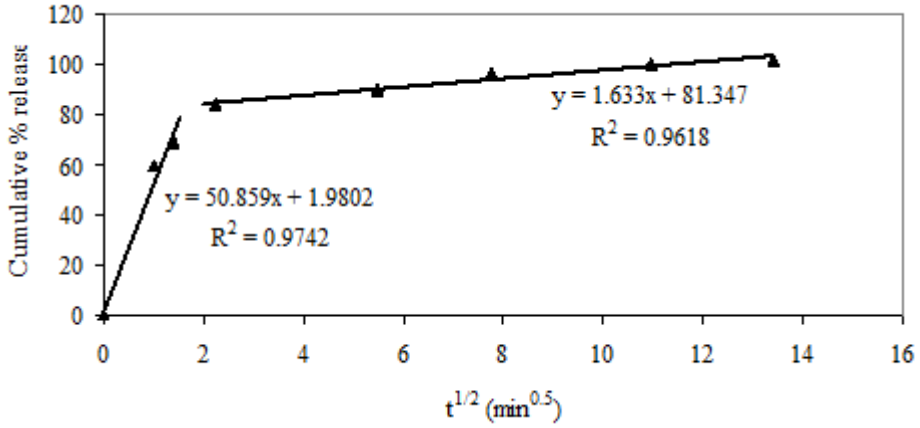


Figure 7.21. Square root of time analysis of cinnamon bark oil release from cinnamon bark oil loaded chitosan microspheres without crosslinking with genipin.

It significantly affected by the addition of 5 mM genipin as crosslinking agent. and about 45% of cinnamon oil was released within the initial period. Moreover, crosslinking time directly affected the rate of the cinnamon oil release at the second linear section (Figures 7.22 and 7.23). Release rate decreased by increasing the crosslinking time from 1h to 18h. However, slowest release profile was achieved in the case of the genipin free cinnamon oil-chitosan microspheres. This may probably because of the competition between the active groups of genipin and of cinnamon oil active compounds' (e.g. trans-cinnamaldehyde, eugenol etc.) for amino groups of chitosan.

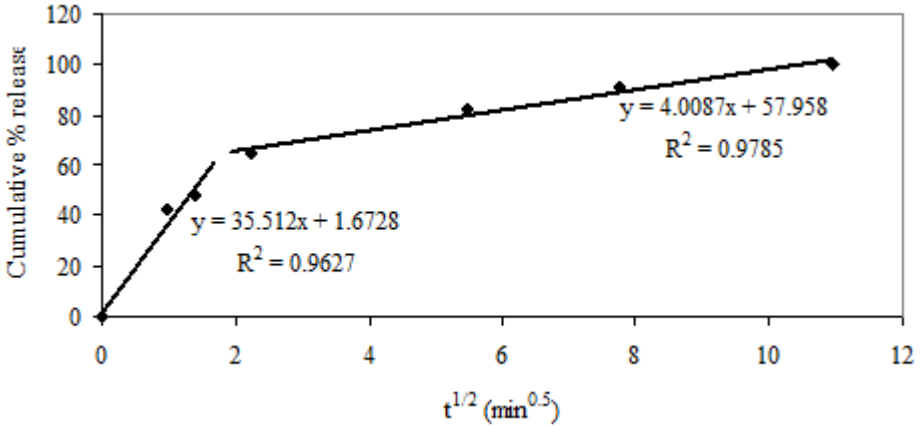


Figure 7.22. Square root of time analysis of cinnamon bark oil release from cinnamon bark oil loaded microspheres crosslinked with 5 mM genipin for 1h.

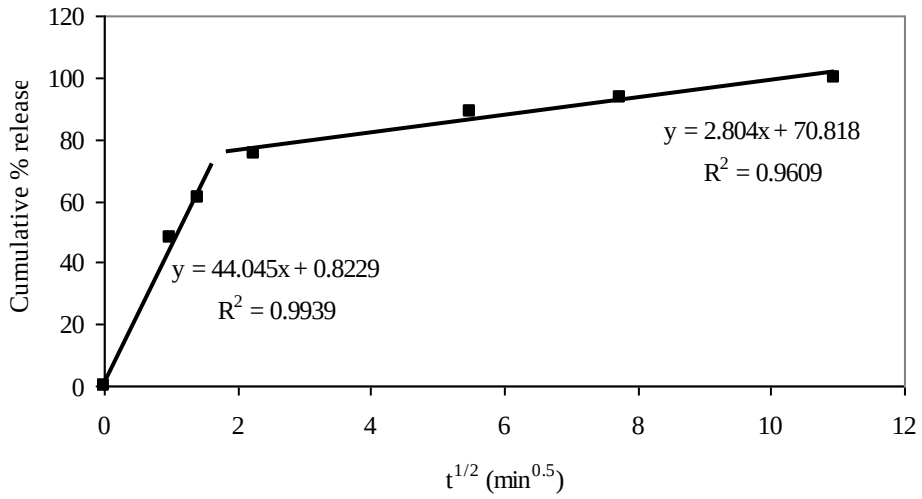


Figure 7.23. Square root of time analysis of cinnamon bark oil release from cinnamon bark oil loaded microspheres crosslinked with 5 mM genipin for 18h.

The effective diffusivities (D_c) were calculated via the long term [$M_t / M_\infty > 0.7$] solution of Crank Equation (Equation 5.7) and given in Appendix B (Table B.1). In clarithromycin loaded microspheres genipin crosslinking time did not affect the diffusivity significantly. However, increasing crosslinker concentration decreased the diffusivity due to the increase in chitosan matrix density and decrease in free volume. In cinnamon bark oil loaded microspheres, crosslinking did not change the diffusivity significantly.

7.7. The Effects of Clarithromycin and Cinnamon Bark Oil Released from Microspheres on *H. pylori*

Serial dilutions of samples taken at 0, 8, 24, 32 and 48 h of incubation from the control microsphere suspensions were cultivated in 7% horse blood supplemented Columbia Blood Agar in an anaerobic jar (Oxoid) containing GasPak Campy Container System (Becton Dickinson and Company) at 37°C in a microaerophilic environment for 3 days. At the end of three days, there was *H. pylori* growth in each dilution plates.

Viable colony counts in antimicrobial activity test of clarithromycin and cinnamon bark oil released from microspheres were given in Table 7.13. It was proved before the inhibition tests that *H. pylori* can grow in liquid growth medium (5% FBS incorporated Brucella Broth) for four days. In the case of incorporating the control

chitosan microspheres into the growth medium, *H. pylori* was able to grow in the presence of control microspheres. This means that control microspheres did not inhibit the *H. pylori* growth.

Table 7.13. Viable colony counts in antimicrobial activity test of clarithromycin and cinnamon bark oil released from microspheres

Microsphere	**Viable Colony Count, CFU x10^9**				
	t=0h	**t=8h**	**t=24h**	**t=32h**	**t=48h**
chitosan (control) microspheres	62	115	138	162	168
uncrosslinked chitosan-clarithromycin microspheres	89	0	0	0	0
uncrosslinked chitosan-cinnamon bark oil microspheres	40	0	0	0	0
genipin crosslinked chitosan-clarithromycin microspheres	77	0	0	0	0

In the case of incorporation of the clarithromycin loaded chitosan microspheres into the growth medium, although there was a growth in the samples of t=0 hour, no growth was observed in the samples of t=8, t=24, t=32 and t=48 hours. This result indicated that clarithromycin released from the microspheres had an antimicrobial activity and inhibited the *H. pylori* growth. Similarly, except the sample of t=0 hour, there was not any growth in the t=8, t=24, t=32 and t=48 hours samples taken from the cinnamon bark oil loaded microspheres suspended growth medium. Thus, it can be said that cinnamon bark oil released from the microspheres also inhibited the growth of *H. pylori*.

The effects of clarithromycin and cinnamon bark oil released from genipin crosslinked microspheres were also tested on *H. pylori* in a similar way. In both cases, there was not any growth in the t=8, t=24, t=32 and t=48 hours samples taken from the growth medium which means that crosslinking by genipin did not affect the antibacterial activity of clarithromycin and cinnamon bark oil.

CHAPTER 8

CONCLUSIONS

Helicobacter pylori is one of the most common pathogens, colonising an estimated half of all humans. It is associated with the development of serious gastroduodenal diseases including peptic ulcers, gastric lymphoma and chronic gastritis. Current recommended regimes are not wholly effective due to poor permeability of the antibiotics across the mucus layer, poor stability of the drug in the acidic pH of the stomach, side effects, bacterial resistance and antibiotic allergy of some people. Gastric mucoadhesive delivery systems potentially allow increased penetration of the mucus layer and therefore increased drug concentration at the site of action. Incorporating antibiotic into the chitosan microspheres has a potential to overcome these difficulties. To eradicate the antibiotic-resistant strains and prevent reinfection the development of new drug delivery systems carrying biologically active compounds from plants and other natural sources are considerably attractive.

The objective of this study was to produce clarithromycin and cinnamon bark oil loaded microspheres to achieve controlled release. Among five essential oils cinnamon bark oil, having MIC of 8 µg/ml, showed the strongest anti-*H. pylori* activity. MIC value is important to adjust the suitable drug formulations and dosage. The significance of inlet air temperature, drug concentration and feed flow rate on spray drying efficiency and properties of clarithromycin and cinnamon bark oil loaded microspheres and the optimum spray drying conditions were analyzed by Central Composite Design (CCD) and Response Surface Methodology (RSM). The optimum conditions for the manufacturing of clarithromycin and cinnamon bark oil loaded microspheres by spray drying were determined as 168°C and 181°C for inlet air temperature, 4.64 ml/min for feed rate and 0.064 % and 0.5 % (w/v) for drug concentration with desirability of 0.76 and 0.94, respectively. Uncrosslinked and genipin crosslinked clarithromycin and cinnamon bark oil loaded chitosan microspheres to be used in release studies were produced by spray drying according to the optimum conditions. The use of natural crosslinker, genipin, to achieve controlled release has been important because of its markedly lower cytotoxicity as compared with alternative crosslinker, glutaraldehyde.

Resulting microspheres were wrinkled and spherical with size of 1-5μm. The positive zeta potentials of microspheres showed the potential electrostatic interaction between the negatively charged mucus and microspheres.Crosslinker concentration and crosslinking time were changed to evaluate their effects on release kinetics. Clarithromycin and cinnamon bark oil release from microspheres were performed in phosphate buffer of pH 5. Kinetic models were used to determine the release mechanism. Release from microspheres was diffusion controlled following the instantaneous burst effect. Higuchi equation (square root of time analysis) described the release characteristic. As the genipin concentration increased, clarithromycin release rate decreased whereas extent of release increased. However, slowest cinnamon bark oil release rate was achieved in the case of the genipin free (uncrosslinked) chitosan microspheres. This may probably because of the competition between the active groups of genipin and cinnamon bark oil for amino groups of chitosan. There is not any distinct evidence of chemical crosslinking according to the experiments and characterization studies performed. In release tests, cinnamon bark oil and clarithromycin released from the microspheres inhibited the growth of *H. pylori* resulting that the antibacterial activity of cinnamon bark oil and clarithromycin was maintained during the microsphere manufacturing.

This dissertation describes a novel approach that is based on the development of chitosan microspheres for controlled release of essential oil besides clarithromycin to eradicate *Helicobacter pylori*. This work has demonstrated the potential of chitosan based microspheres as mucoadhesive drug carrier system for the controlled release of clarithromycin and cinnamon bark oil in the treatment of *H. pylori* infection.

In further studies, the effects of polymer/drug ratio and genipin crosslinking of chitosan microspheres should be investigated in more detailed for sustained drug release. Moreover, the mucoadhesion tests are required to evaluate the mucoeadhesion strength of resultant microspheres due to the particular importance of stomach specific drug release. Additionally, researches on the investigation of in vivo product stability and tissue response and the impact on bioavailability and release rates using appropriate animal models should be initiated.

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APPENDIX A

SEM MICROGRAPHS OF MICROSPHERES IN OPTIMIZATION STUDY

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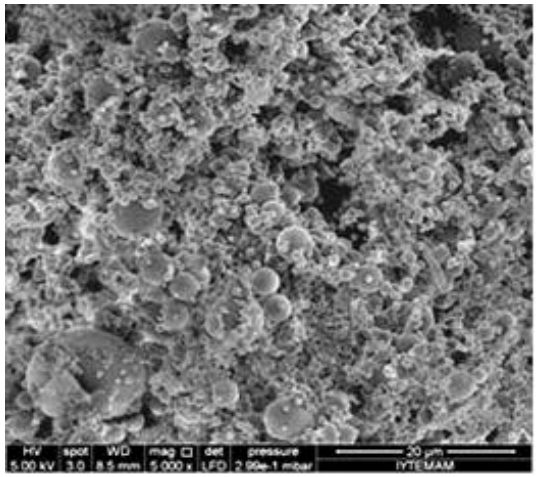
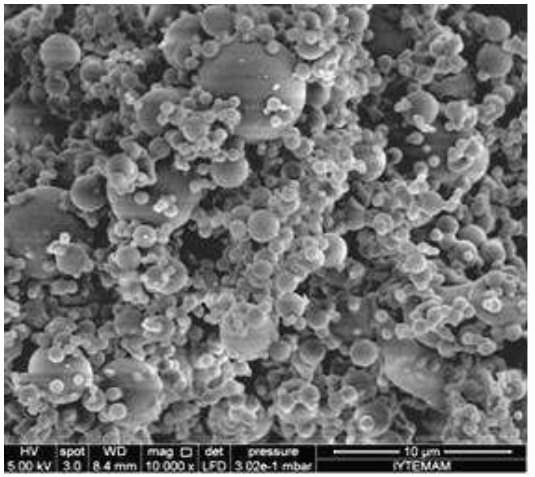
1		

Figure A.1. SEM micrographs of clarithromycin loaded microspheres manufactured by spray drying according to central composite design run numbers.

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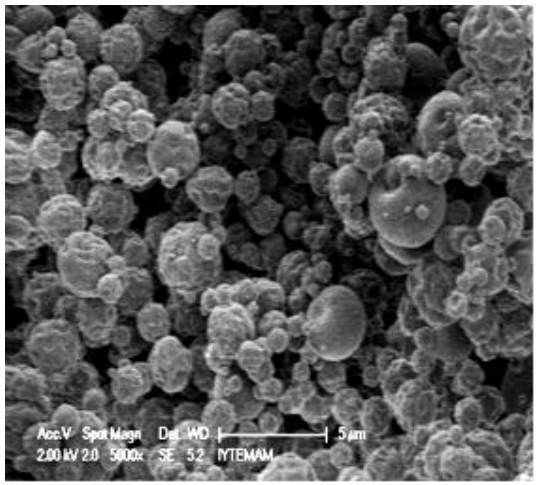
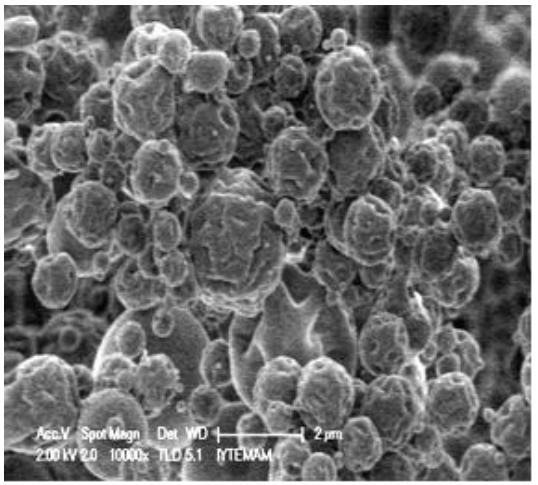
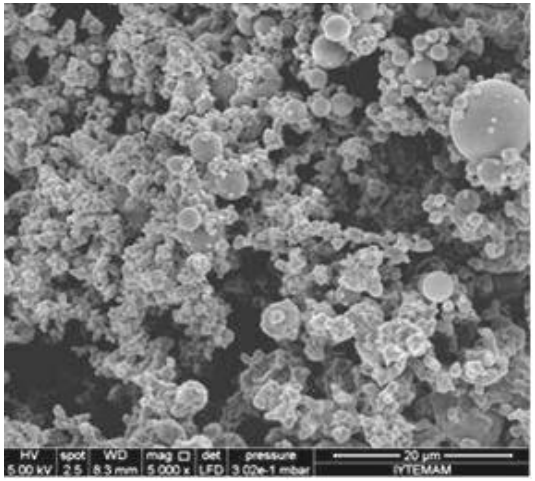
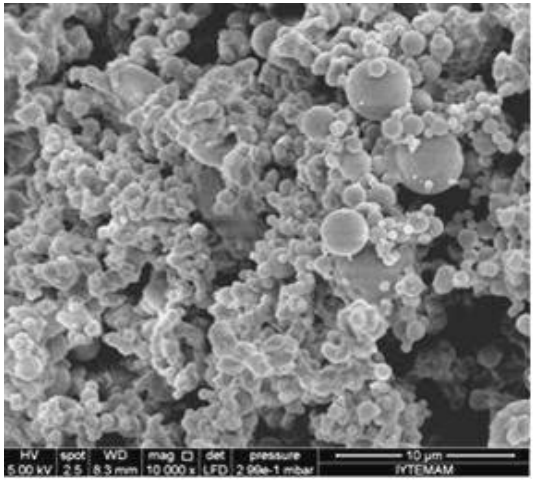
2		
3		

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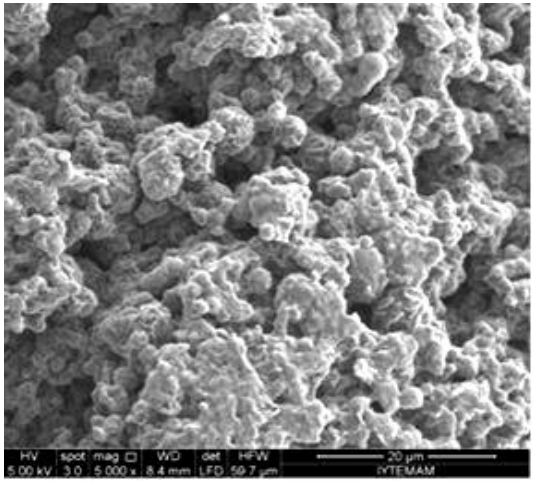
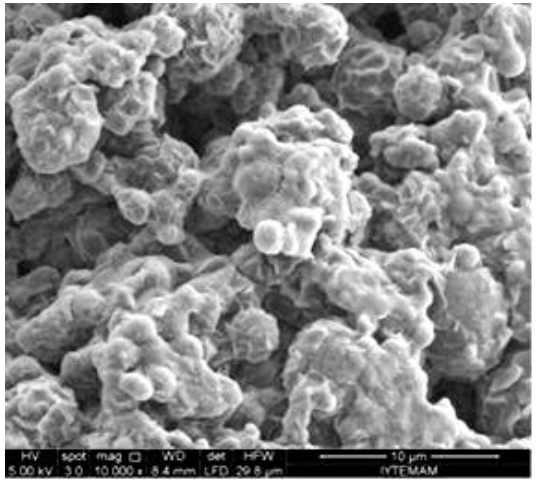
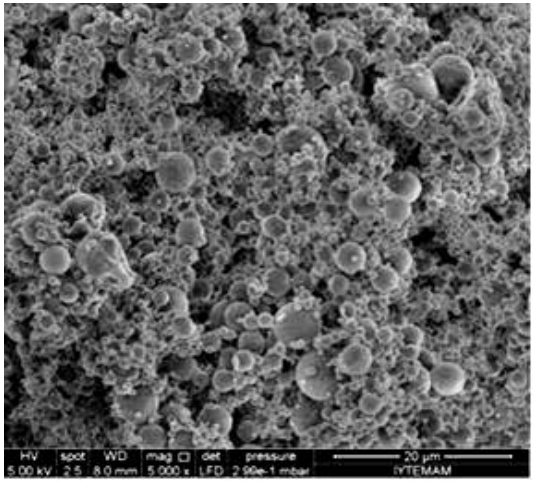
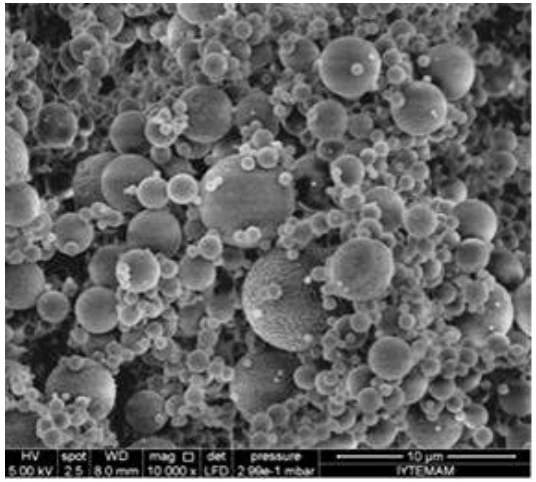
4		
5		

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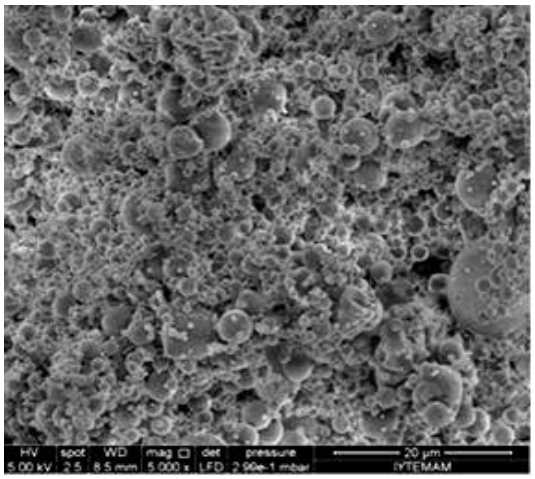
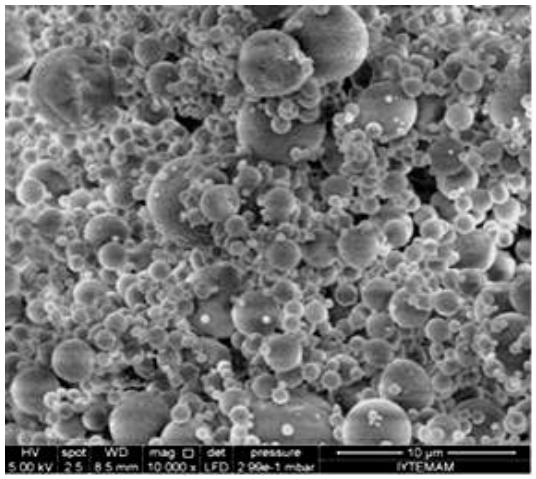
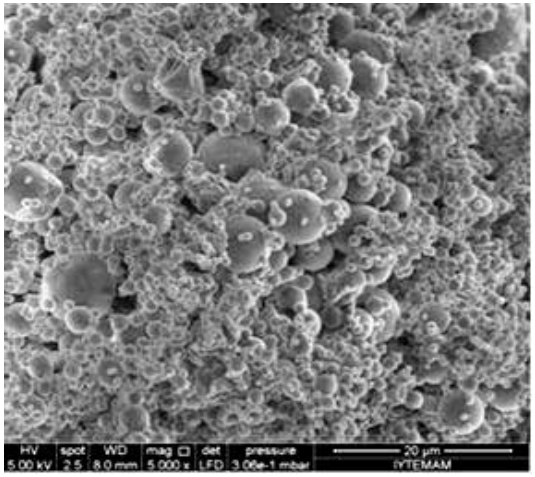
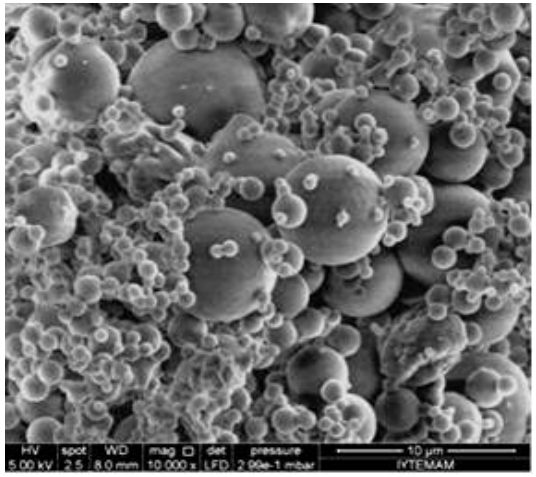
6		
7		

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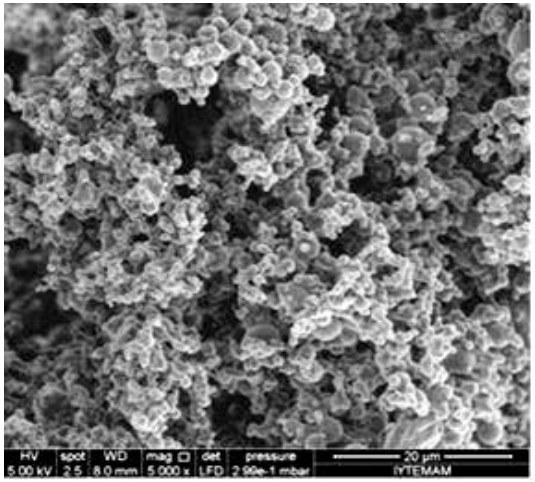
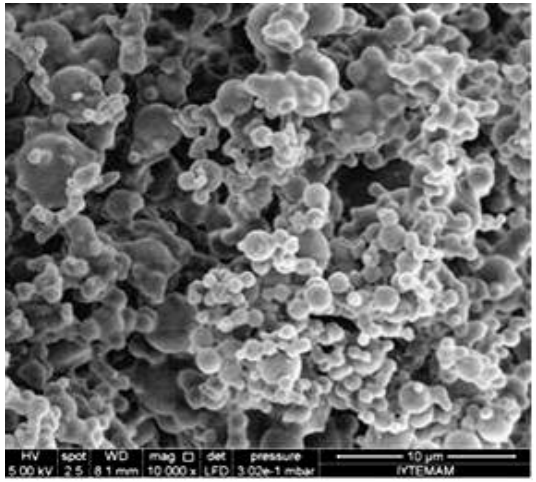
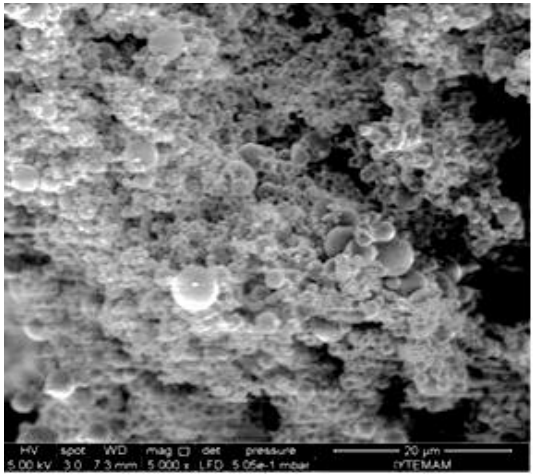
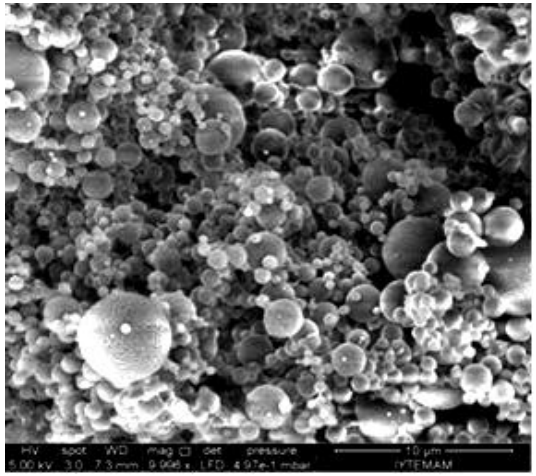
8		
9		

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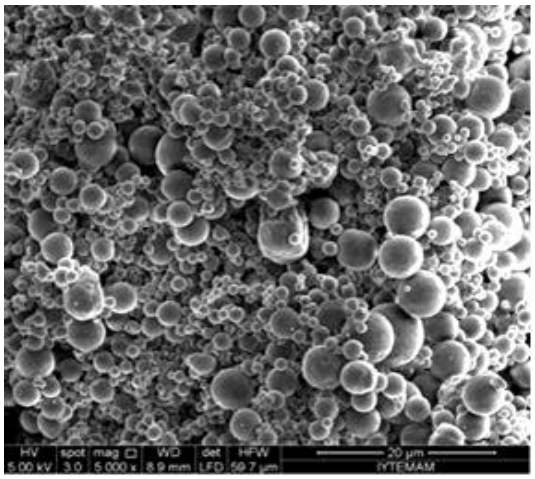
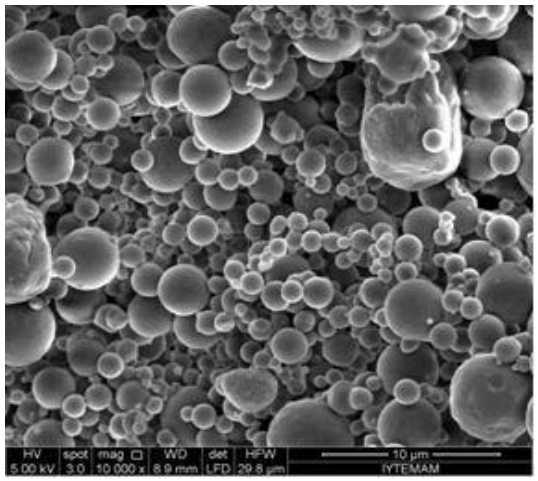
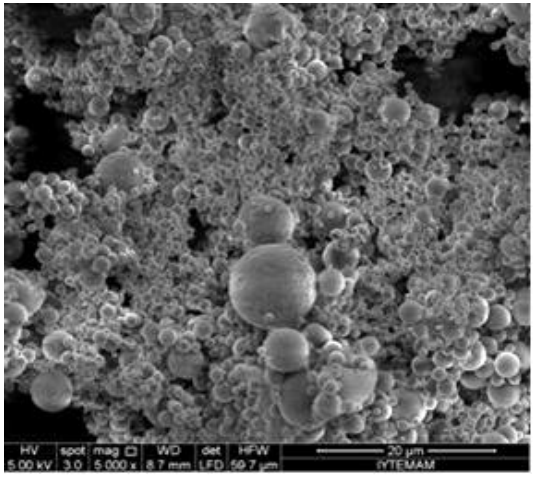
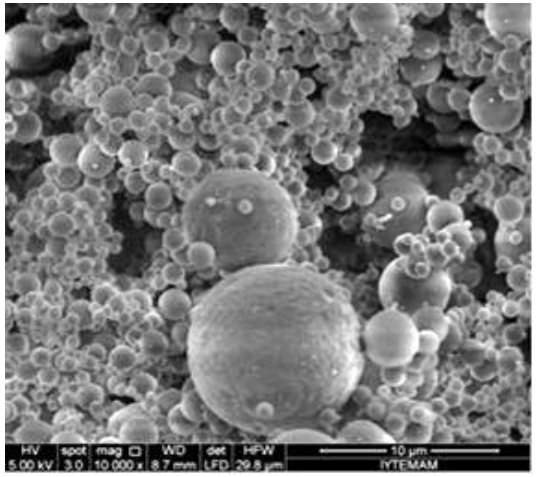
10		
11		

Figure A.1. (cont.)

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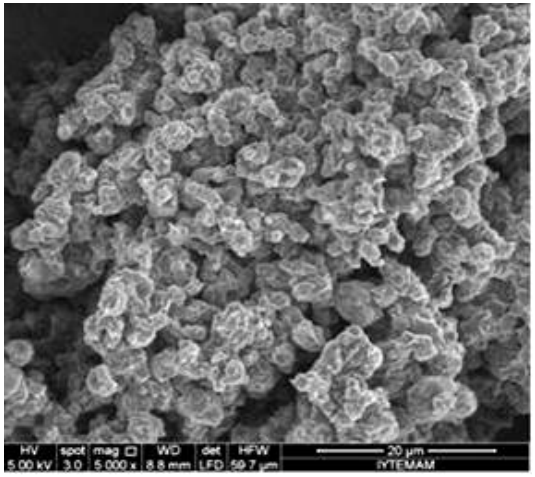
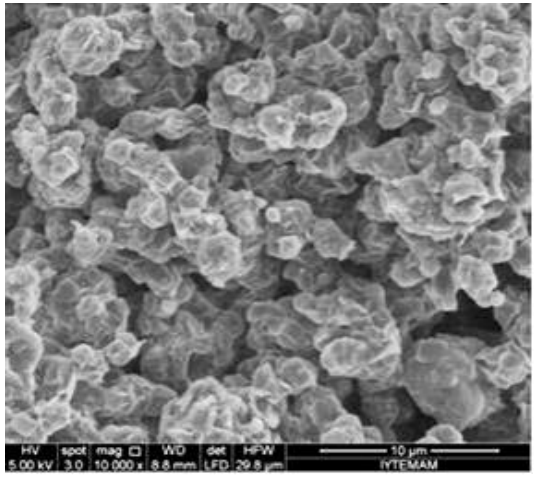
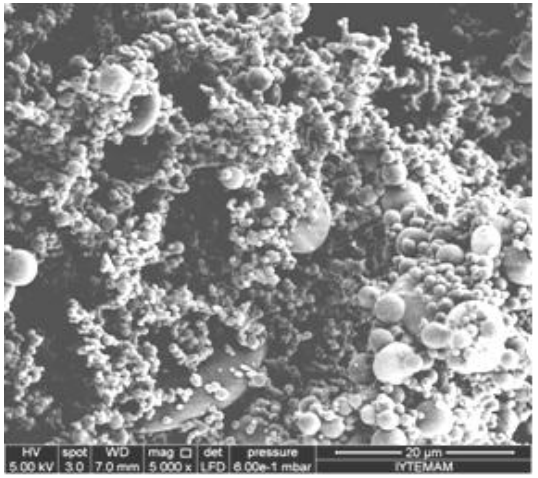
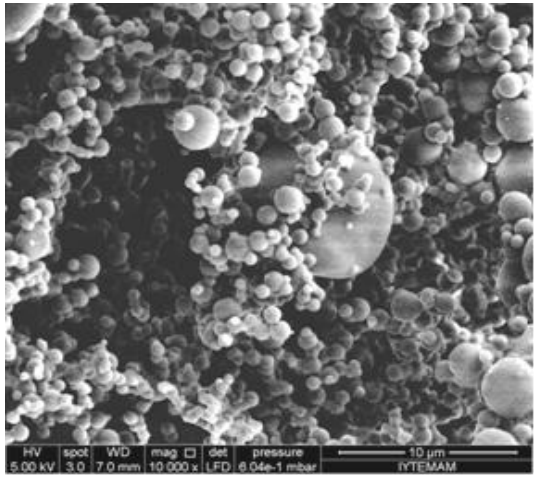
12		
13		

Figure A.1. (cont.)

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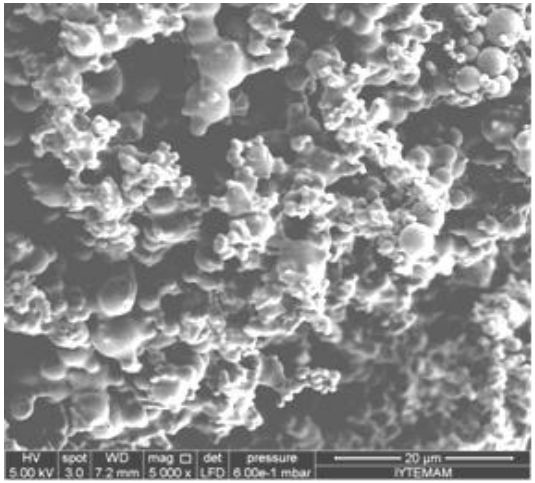
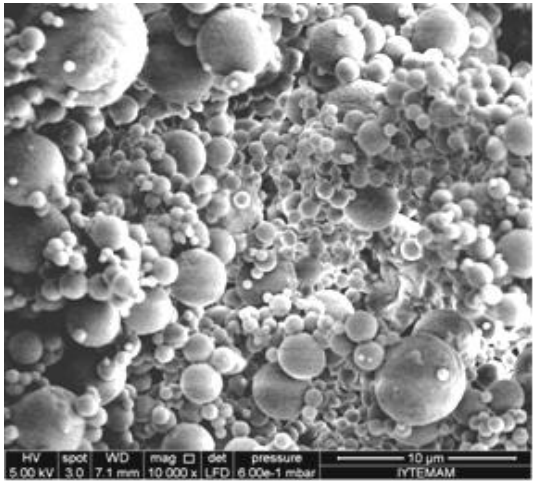
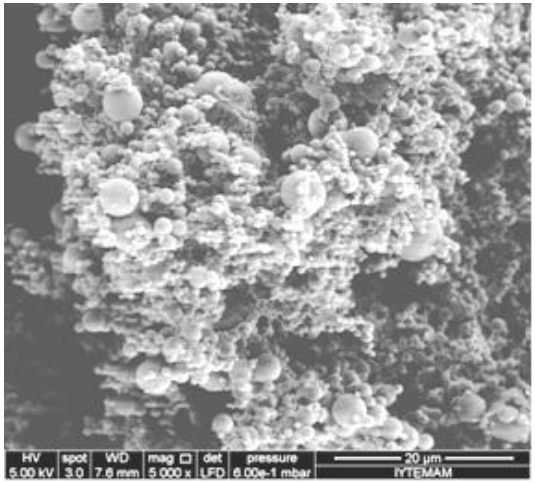
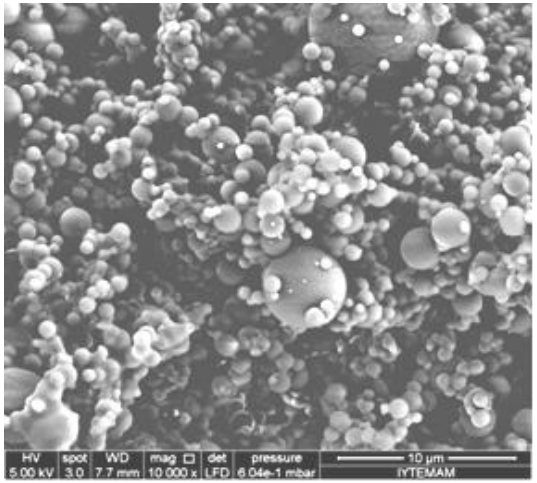
14		
15		

Figure A.1. (cont.)

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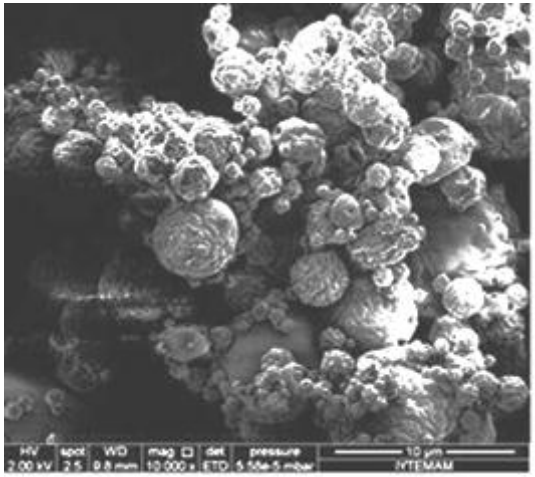
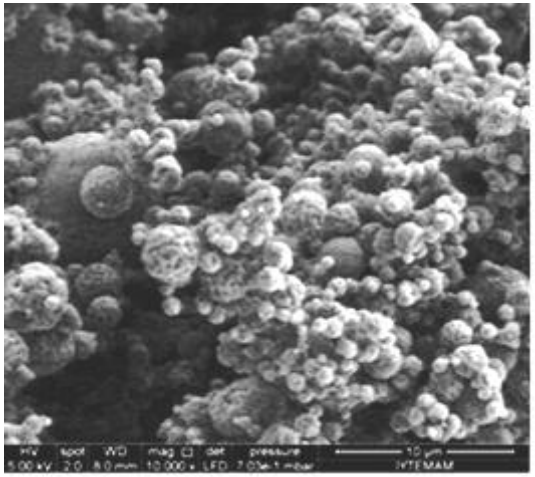
1		

Figure A.2. SEM micrographs of cinnamon bark oil loaded microspheres manufactured by spray drying according to central composite design run numbers.

(Cont. on next page)

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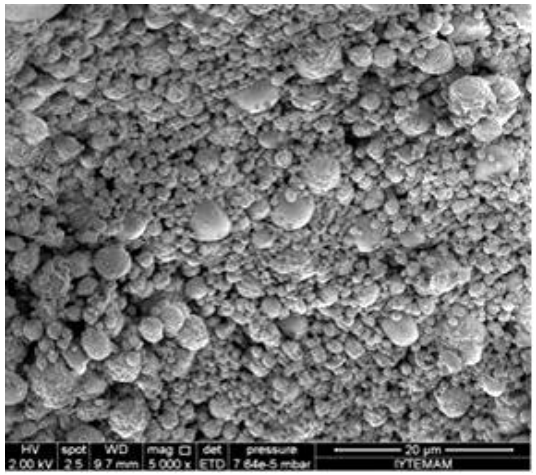
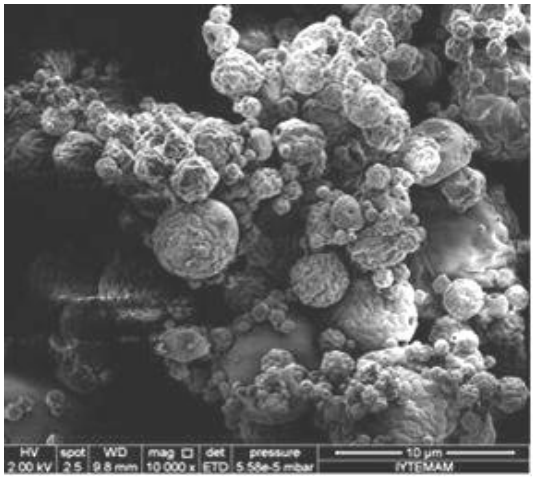
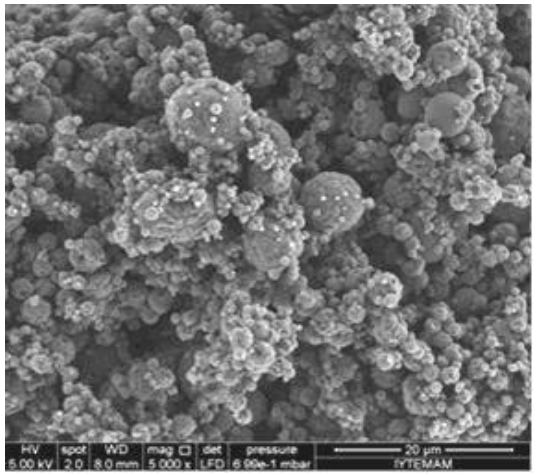
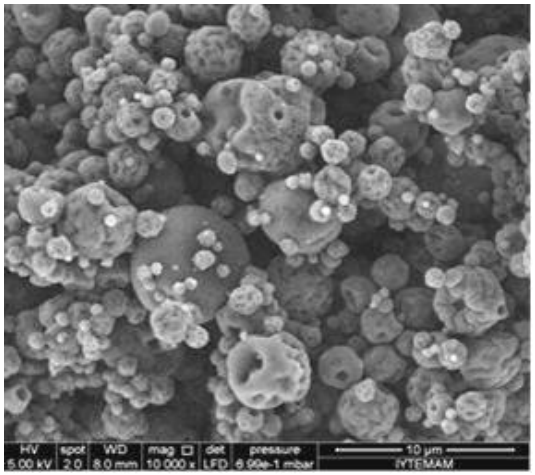
2		
3		

Figure A.2. (cont.)

(Cont. on next page)

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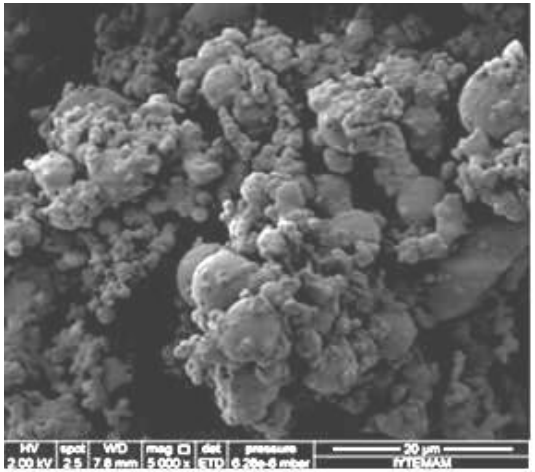
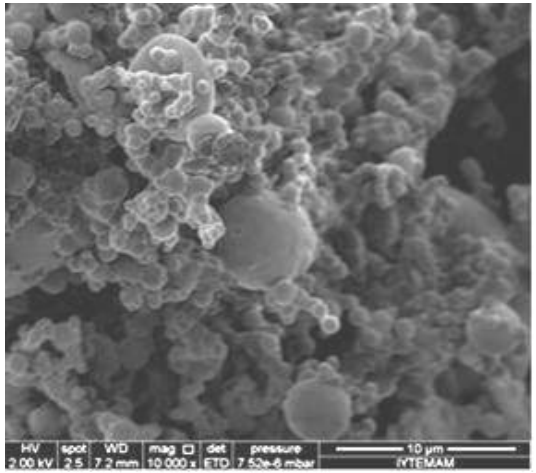
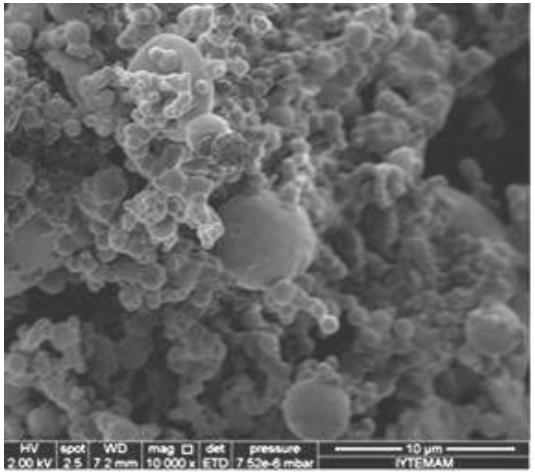
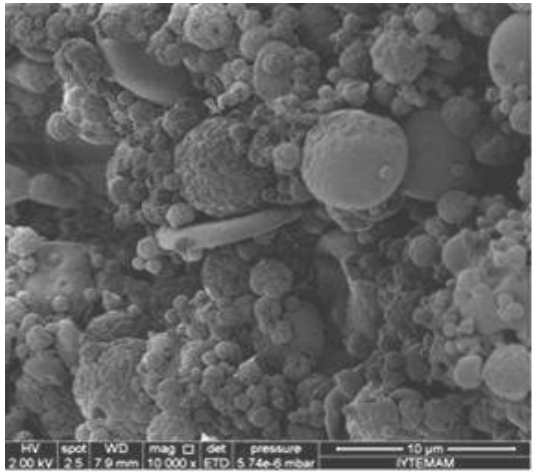
4		
5		

Figure A.2. (cont.)

(Cont. on next page)

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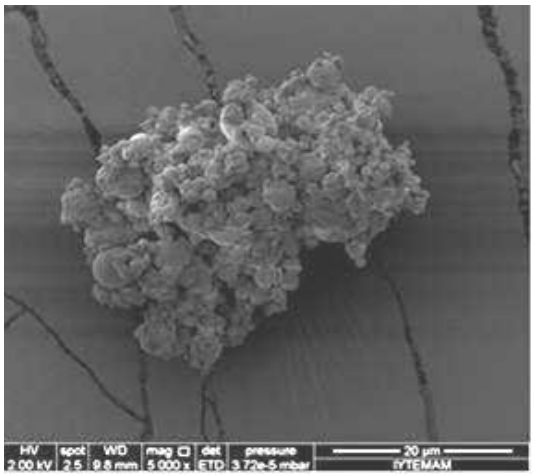
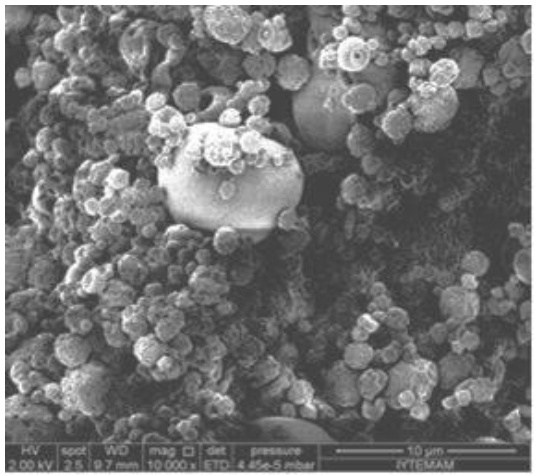
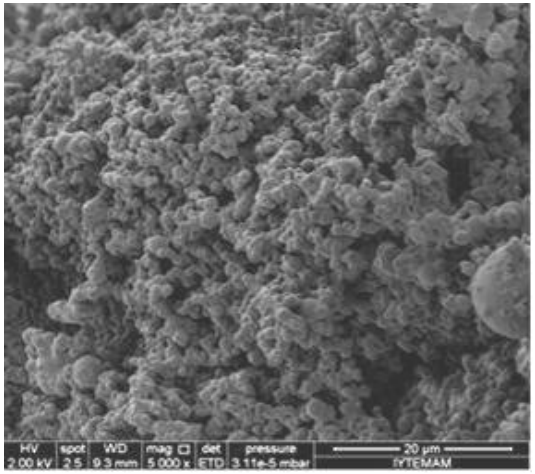
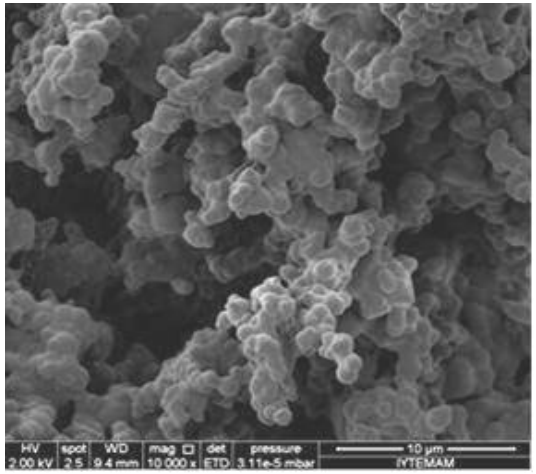
6		
7		

Figure A.2. (cont.)

(Cont. on next page)

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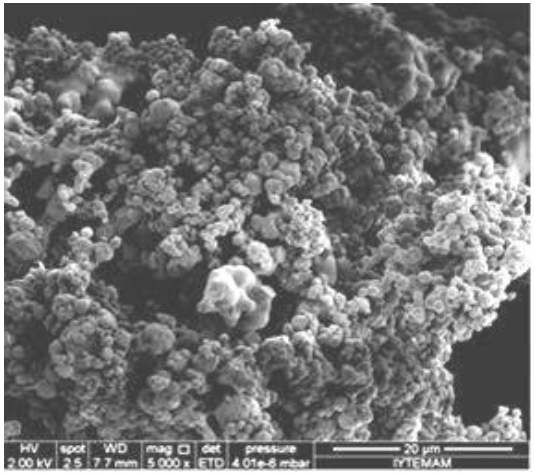
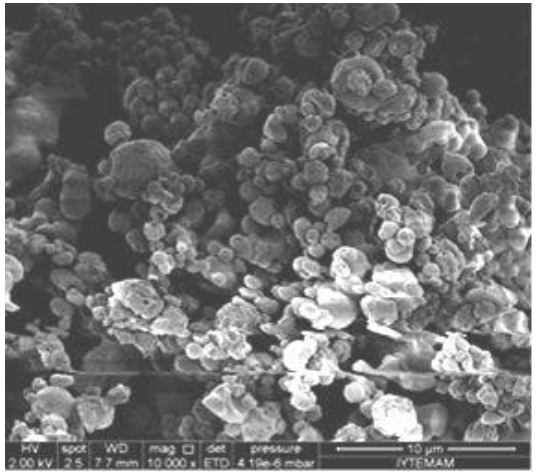
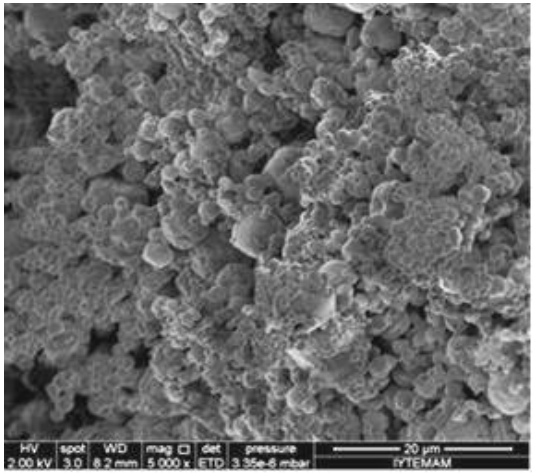
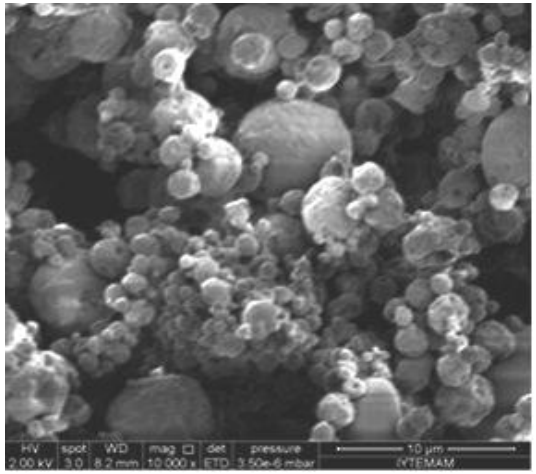
8		
9		

Figure A.2. (cont.)

(Cont. on next page)

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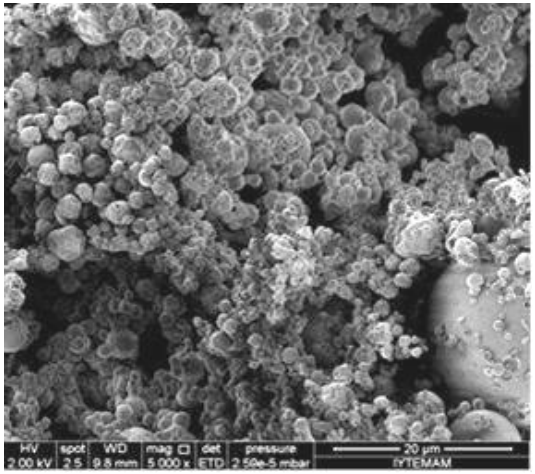
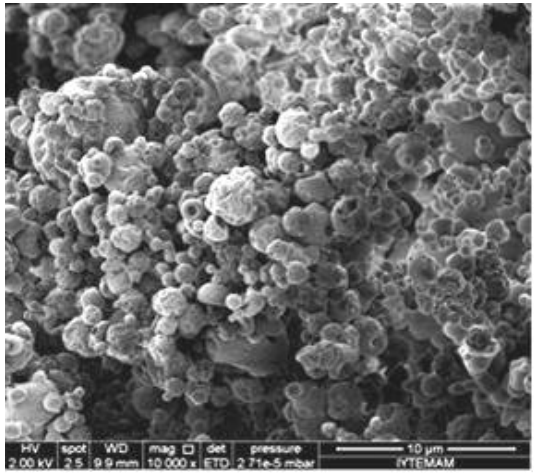
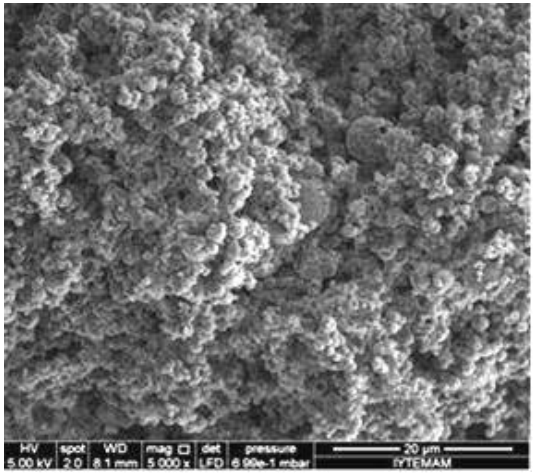
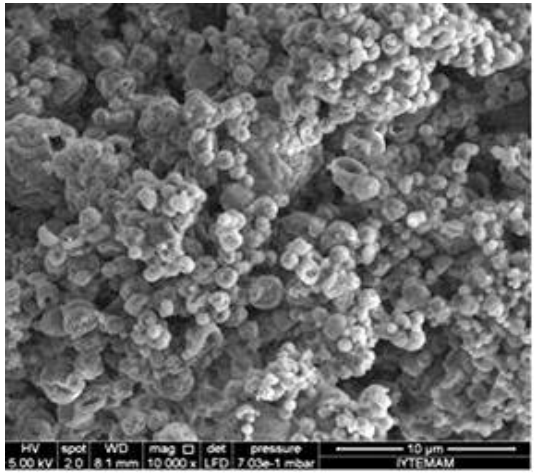
10		
11		

Figure A.2. (cont.)

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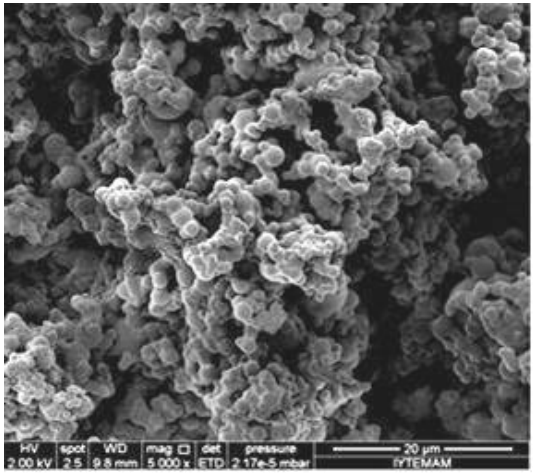
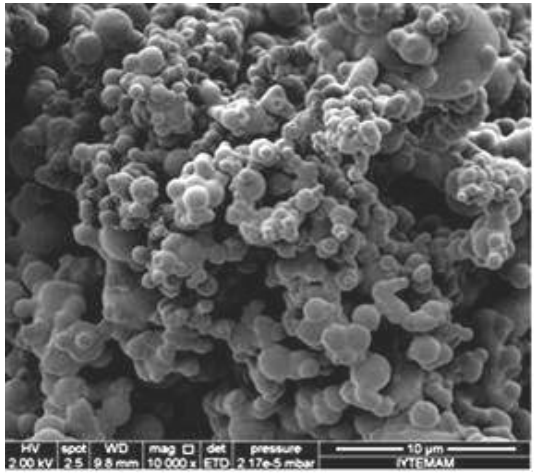
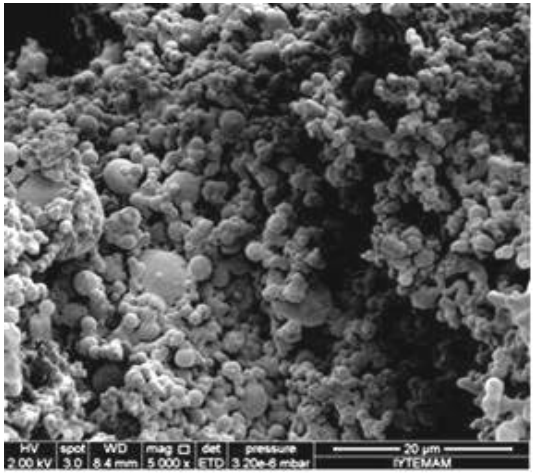
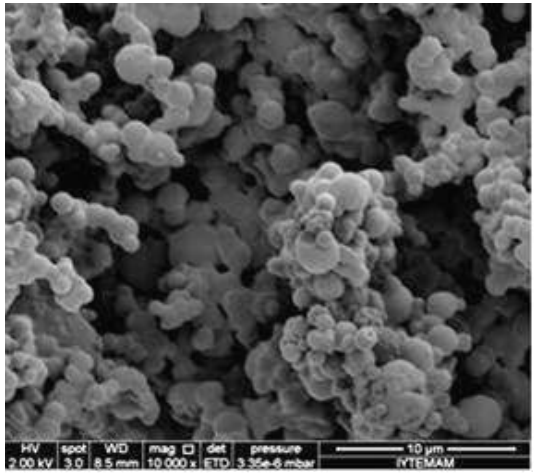
12		
13		

Figure A.2. (cont.)

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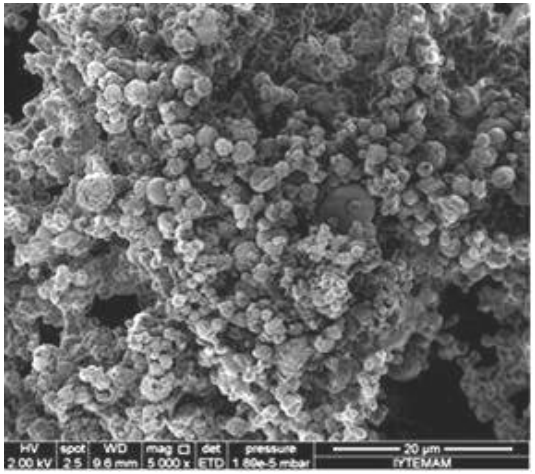
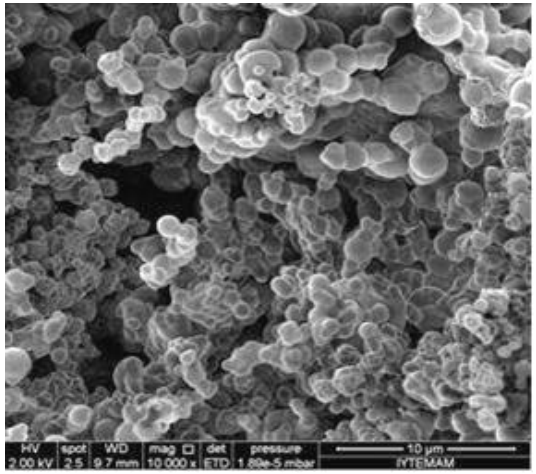
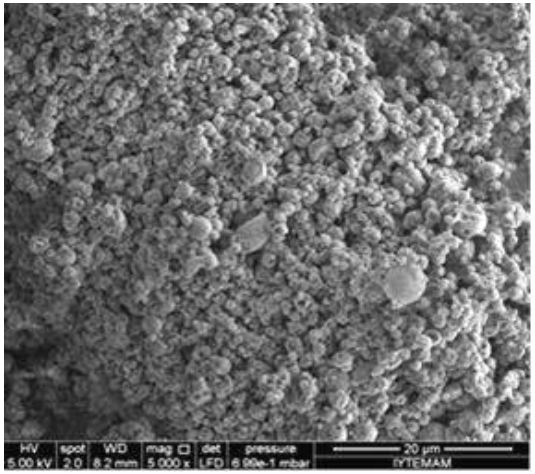
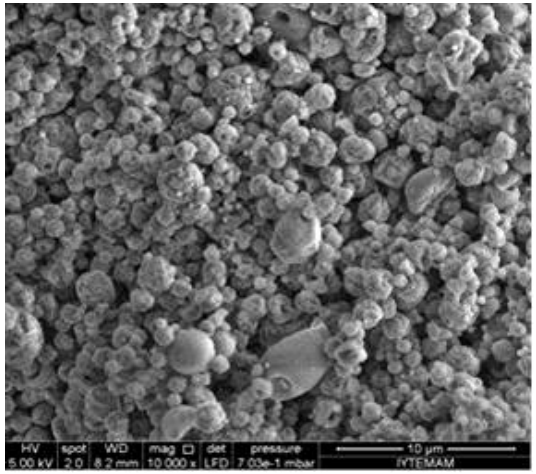
14		
15		

Figure A.2. (cont.)

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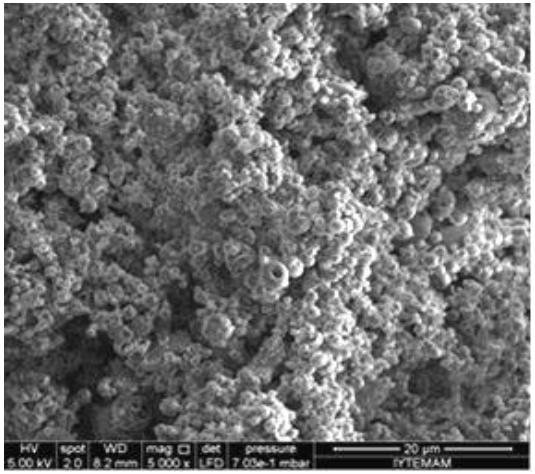
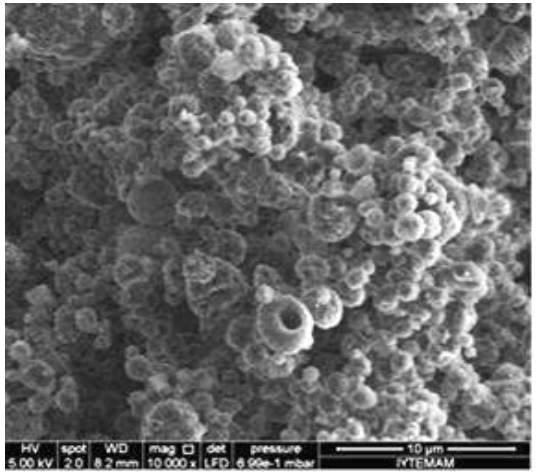
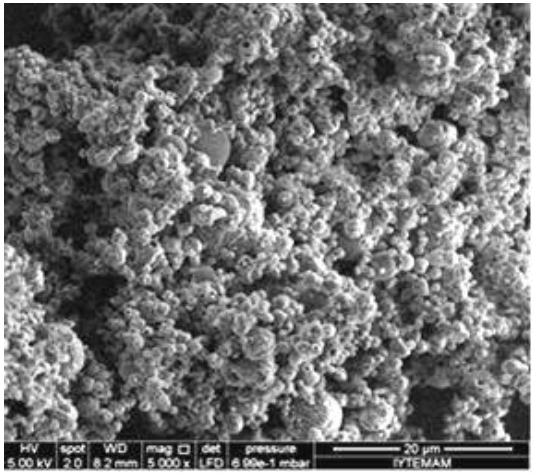
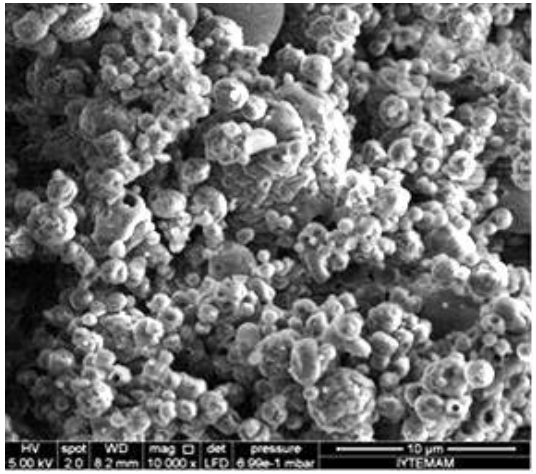
16		
17		

Figure A.2. (cont.)

APPENDIX B

DETERMINATION OF EFFECTIVE DIFFUSION COEFFICIENTS

The effective diffusivities (D_c) were calculated via the long term [$M_t/M_\infty > 0.7$] solution of Crank Equation (Equation 5.7) and given in Appendix B. By taking Ln of both sides;

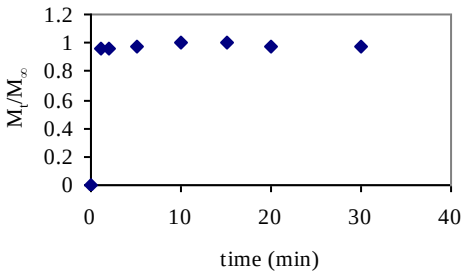
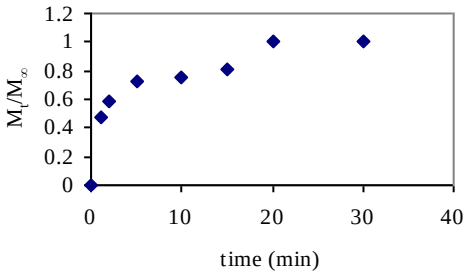
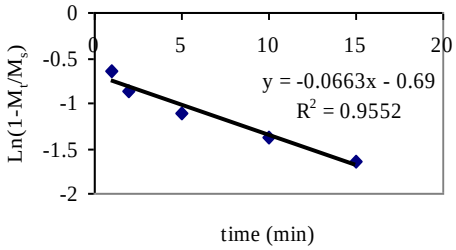
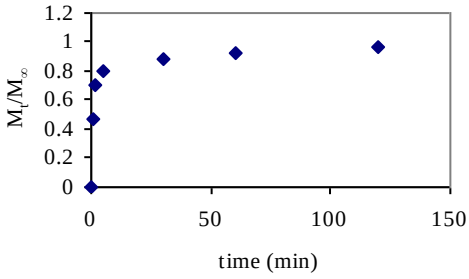
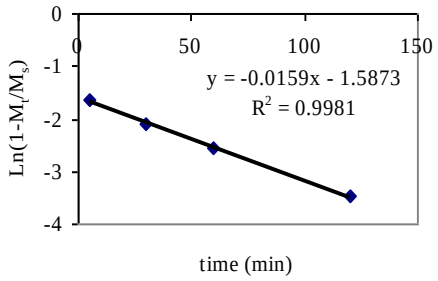
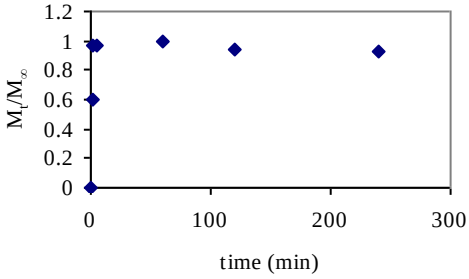
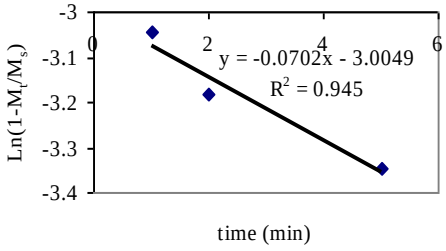
$$Ln\left(1-\frac{M_t}{M_\infty}\right)=Ln\left(\frac{6}{\Pi^2}\right)Ln\left[\exp(-\frac{\Pi^2 D_c t}{R_p^2})\right] \qquad \text{(B.1)}$$

Rearranging the Equation A.1;

$$Ln\left(1-\frac{M_t}{M_\infty}\right)=\left[Ln\left(\frac{6}{\Pi^2}\right)x\left(-\frac{\Pi^2 D_c}{R_p^2}\right)\right]t \qquad \text{(B.2)}$$

The plot between the Ln ($1-M_t/M_\infty$) versus t is plotted and D_c is found from the slope of the plot.

Table B.1. The plots used to calculate (D_p)

Sample code #	M_t/M_∞ versus time plot	Ln(1- m_t/m_∞) versus t plot for m_t/m_∞>0.7
1		Burst effect
2		
3		
4		

(Cont. on next page)

Table B.1. (cont.)

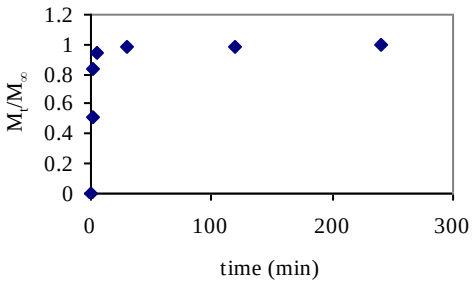
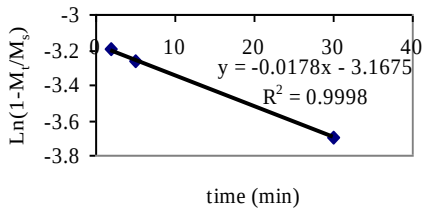
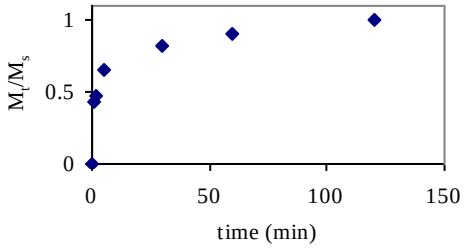
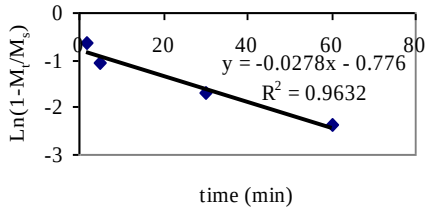
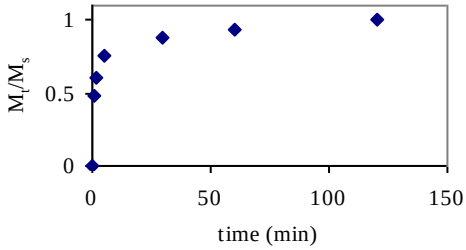
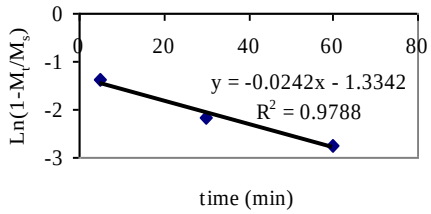
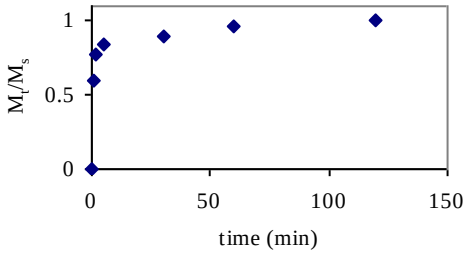
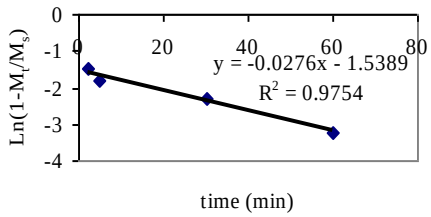
5		
6		
7		
8		

Table B.2. Effective Diffusion Coefficients

Sample Code and name	D_c (*10^{14}) (m^2/s)
#1. Clarithromycin loaded chitosan microspheres; without genipin.	-----
#2. 1mM genipin crosslinked clarithromycin loaded microspheres; 1h.	12.2
#3. 5mM genipin crosslinked clarithromycin loaded microspheres; 1h.	2.92
#4. 1mM genipin crosslinked clarithromycin loaded microspheres; 18h.	12.9
#5. 5mM genipin crosslinked clarithromycin loaded microspheres; 18h.	3.27
#6. Cinnamon bark oil loaded chitosan microspheres. with 5mM genipin.1h.	5.1
#7. Cinnamon bark oil loaded chitosan microspheres. with 5mM genipin.18h.	4.4
#8. Cinnamon bark oil loaded chitosan microspheres. without genipin.	5.1

VITA

DUYGU ALTIOK

Date of Birth : 01.04.1978
Place of Birth : Manisa/Turkey
Citizenship : Turkish

EDUCATION

[1996-2000] B.Sc. (Food Engineering)
Middle East Technical University
Department of Food Engineering, Ankara, Turkey

[2001-2004] M.Sc. (Food Engineering)
Izmir Institute of Technology
Department of Food Engineering, İzmir, Turkey
Dissertation: Kinetic Modelling of Lactic Acid Production from Whey.
Advisor: Prof. Dr. Şebnem HARSA
Co-advisor: Assoc. Prof. Dr. Figen TOKATLI

[2004-2011] Ph.D. (Chemical Engineering)
Izmir Institute of Technology
Department of Chemical Engineering, İzmir, Turkey
Dissertation: Preparation and Evaluation of Chitosan Microspheres for Eradication of *Helicobacter Pylori*.
Advisor: Prof. Dr. Funda TIHMINLIOĞLU

ACADEMIC POSITIONS HELD

Research Assistant Izmir Institute of Technologyi Faculty of Engineering, Department of Chemical Engineering (September 2004-July 2011).

Research Assistant Izmir Institute of Technologyi Faculty of Engineering, Department of Food Engineering (October 2001-September 2004).

PUBLICATIONS

ALTIOK, D.; Altıok, E.; Tıhmınlıoğlu, F. Physical, antibacterial and antioxidant properties of chitosan films incorporated with thyme oil for potential wound healing applications, **2010**, *Journal of Materials Science: Materials in Medicine*, 21, 2227-2236.

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ALTIOK, D.; Tokatlı, F.; Harsa, S. Kinetic modelling of lactic acid production from whey by *Lactobacillus casei* (NRRL B-441), **2006**, *Journal of Chemical Technology and Biotechnology*, 81, 190-1197.