REPUBLIC OF TURKEY
YILDIZ TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

MICROENCAPSULATION OF POLYPHENOLIC COMPOUNDS WITH CINNAMALDEHYDE MODIFIED CHITOSAN AND ITS ANTIFUNGAL ACTIVITY

Aşkıcan HACIOĞLU

MASTER OF SCIENCE THESIS
Department of Bioengineering
Bioengineering Program

Advisor
Assoc. Prof. Dr. Cem Bülent ÜSTÜNDAĞ

Co-Advisor
Prof. Dr. Soner ŞAHİN

February, 2021

REPUBLIC OF TURKEY

YILDIZ TECHNICAL UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

MICROENCAPSULATION OF POLYPHENOLIC COMPOUNDS WITH CINNAMALDEHYDE MODIFIED CHITOSAN AND ITS ANTIFUNGAL ACTIVITY

A thesis submitted by Aşkıcan HACIOĞLU in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE is approved by the committee on 07.02.2021 in Department of Bioengineering, Bioengineering Program.

Assoc. Prof. Dr. Cem Bülent ÜSTÜNDAĞ	Prof. Dr. Soner ŞAHİN
Yıldız Technical University	Bahçeşehir University
Advisor	Co-Advisor

Approved by the Examining Committee

Assoc. Prof. Dr. Cem Bülent ÜSTÜNDAĞ, Advisor

Yıldız Technical University _____

Prof. Dr. Musa TÜRKER, Member

Yıldız Technical University _____

Assoc. Prof. Dr. Oğuzhan GÜNDÜZ, Member

Marmara University _____

I hereby declare that I have obtained the required legal permissions during data collection and exploitation procedures, that I have made the in-text citations and cited the references properly, that I haven't falsified and/or fabricated research data and results of the study and that I have abided by the principles of the scientific research and ethics during my Thesis Study under the title of "Microencapsulation of Polyphenolic Compounds with Cinnamaldehyde Modified Chitosan and its Antifungal Activity" supervised by my supervisor, Assoc. Prof. Dr. Cem Bülent ÜSTÜNDAĞ. In the case of a discovery of false statement, I am to acknowledge any legal consequence.

Aşkıcan HACIOĞLU

Signature

Dedicated to my lovely family

and my best friends

ACKNOWLEDGEMENTS

In the first place, I would like to appreciate to my advisor Assoc. Prof. Dr. Cem Bülent Üstündağ for sharing his expertise, and his sincere and valuable guidance to me. I would like to acknowledge to Nanomik Biotechnology Company for supporting my studies and their infrastructural support to obtain my thesis. I also would like to appreciate Assoc. Prof. Dr. Oğuzhan Gündüz for providing the FTIR analysis. I would like to thank Mert Tözün who gave me the experimental support during the antifungal assays.

I am grateful to my family for their everlasting supports and encouragement, which made me the most luckiest person in the world. I am very thankful to my writing pals, Ecem Özden, Eren Can Sarıyarlıoğlu, and Şilan Gündoğdu. I will not forget their supports and our sleepless nights. I am very thankful to my dearest friends Yücem Özden, Taylan Baran Yeşil, Hakkı Batu Ordu, Arda Üstün, and Ali Yaşar. They have always been as my family. I wish to express my sincere thanks to my all lecturers and supervisors for their help and experience share, each of which are contributors to this labor.

Finally, I owe a debt of gratitude, to one and all, who directly or indirectly, have given their hand in this thesis.

Aşkıcan HACIOĞLU

TABLE OF CONTENTS

LIST OF ABBREVIATIONS vii

LIST OF FIGURES viii

LIST OF TABLES x

ABSTRACT xi

ÖZET xiii

1 INTRODUCTION 1

1.1 Literature Review 1

1.1.1 Chitosan and Modified Chitosan as an Antimicrobial Agent 1

1.1.2 Natural polyphenols as antifungal molecules 4

1.1.3 Curcumin 6

1.1.4 Quercetin 7

1.1.5 Antifungal Activity of Curcumin and Quercetin 8

1.1.6 Microencapsulation 11

1.2 Objective of the Thesis 14

1.3 Hypothesis 14

2 MATERIALS AND METHODS 16

2.1 Materials 16

2.2 Synthesis of Cinnamyl Chitosan 16

2.3 Microencapsulation of Curcumin and Quercetin with Chitosan and Cin-Ch 17

2.4 Characterization of Modified Chitosan and Microcapsules 17

2.4.1 Spectrometric Analysis 17

2.4.2 Determination of Free Amine Groups with TNBS Assay 17

2.4.3 Contact Angle Analysis 18

2.4.4 Determination of Encapsulation Efficiency and Loading Efficiency 18
2.4.5 DLS/Zeta Potential Analysis 18
2.4.6 Release Studies 19
2.5 Antifungal Activity Assays 19
2.5.1 Preparation of Culture Media 19
2.5.2 Antifungal Activity Assays 19

3 RESULTS AND DISCUSSION 22

3.1 Characterization of Cin-Ch 22
3.1.1 Spectrometric Analysis 22
3.1.2 Determination of Free Amine Groups with TNBS Assay 23
3.1.3 Contact Angle Analysis 24
3.2 Characterization of Microcapsules. 25
3.2.1 Optimization of Microencapsulation 25
3.2.2 Release Studies 26
3.2.3 Particle Size and Zeta Potential Analysis 27
3.2.4 FTIR Analysis of the Microcapsules 30
3.3 Antifungal Activity Assays 32
3.3.1 Antifungal Activity of Cinnamyl Chitosan 32
3.3.2 Antifungal Activity of Active Ingredients and Microcapsules . . . 33
3.4 Conclusion 35

REFERENCES 36

PUBLICATIONS FROM THESIS 47

LIST OF ABBREVIATIONS

Cin-Ch	Cinnamyl Chitosan
CinCh-Cur	Cinnamyl chitosan curcumin microcapsules
CinCh-Quer	Cinnamyl chitosan quercetin microcapsules
Ch	Chitosan
Ch-Cur	Chitosan curcumin microcapsules
Ch-Quer	Chitosan quercetin microcapsules
dH_2O	Distilled water
ddH_2O	Double distilled water
DLS	Dynamic Light Scattering
DMSO	Dimethyl sulfoxide
EtOH	Ethanol
FTIR	Fourier Transform Infrared Spectroscopy
MIC	Minimum Inhibitory Concentration
TNBS	Trinitrobenzene Sulfonic Acid
TPP	Sodium Tripolyphosphate

LIST OF FIGURES

Figure 1.1 Chemical structure of chitin and chitosan 2

Figure 1.2 Classification of plant-based non-flavonoid phenolic compounds 5

Figure 1.3 Classification of flavonoids 6

Figure 1.4 Chemical structures of curcumin and quercetin 7

Figure 1.5 Different microencapsulation techniques for producing biopolymer-based nanosized and microsized particles [118] 12

Figure 1.6 Ionic interaction between chitosan and tripolyphosphate in acidic media 13

Figure 1.7 Hypothetical strategy for obtaining microparticles with enhanced antifungal activity 15

Figure 2.1 Plate layout of MIC assay of chitosan and cinnamyl chitosan 20

Figure 2.2 Plate layout of MIC assay of curcumin and quercetin 21

Figure 2.3 Plate layout of MIC assay of microcapsules in SDA and YPD 21

Figure 3.1 Comparison between UV spectrum of chitosan and cinnamyl chitosan 22

Figure 3.2 FTIR Spectra of cinnamaldehyde (1), chitosan (2) and cinnamyl chitosan (3) 23

Figure 3.3 Contact angle of chitosan and cinnamyl chitosan on parafilm surface . 24

Figure 3.4 Calibration curves of curcumin and quercetin 25

Figure 3.5 Cumulative release profile of Cin-Ch microcapsules within 24 hours .. 26

Figure 3.6 DLS measurements of Ch-Cur and Ch-Quer 27

Figure 3.7 DLS measurements of CinCh-Cur and CinCh-Quer 28

Figure 3.8 Zeta potential analysis of microparticles 28

Figure 3.9 FTIR spectra of Ch-Cur (up) and CinCh-Cur (bottom) microcapsules... 30

Figure 3.10 FTIR spectra of Ch-Quer (up) and CinCh-Quer (bottom) microcapsules 31

Figure 3.11 Antifungal activity assay of chitosan (1,2) and cinnamyl chitosan (3,4) against *P. digitatum* (1,3) and *A. niger* (2,4) 32

Figure 3.12 Antifungal activity assay of curcumin (1) and quercetin (2) against *P. digitatum* and *A. niger* 33

Figure 3.13 Antifungal activity assays of Ch-Cur (1), Ch-Quer (2), Cin-Ch-Cur (3) and Cin-Ch-Quer (4) against *P. digitatum* and *A. niger* in SDA media . 33

Figure 3.14 Antifungal activity assays of Ch-Cur (1), Ch-Quer (2), Cin-Ch-Cur (3) and Cin-Ch-Quer (4) against *P. digitatum* and *A. niger* in YPD media . 34

LIST OF TABLES

Table 1.1 Antimicrobial activity of chitosan against various types of microorganisms 2

Table 1.1 Antimicrobial activity of chitosan against various types of microorganisms (continued) 3

Table 1.2 Antifungal studies of curcumin and quercetin 9

Table 1.2 Antifungal studies of curcumin and quercetin (continued) 10

Table 1.2 Antifungal studies of curcumin and quercetin (continued) 11

Table 3.1 TNBS assay results 24

Table 3.2 Microencapsulation trials and comparison 26

ABSTRACT

Microencapsulation of Polyphenolic Compounds with Cinnamaldehyde Modified Chitosan and its Antifungal Activity

Aşkıcan HACIOGLU

Department of Bioengineering

Master of Science Thesis

Advisor: Assoc. Prof. Dr. Cem Bülent ÜSTÜNDAĞ

Co-advisor: Prof. Dr. Soner ŞAHİN

Polyphenols are naturally derived molecules with diverse applications from medicine to the food industry. They are antimicrobially effective against a broad range of pathogen microorganisms. However, polyphenols have limited bioavailability and stability problems. Microencapsulation is one strategy for increasing antimicrobial molecule's bioavailability, with more beneficial properties of controlled release, targeted delivery, etc. Chitosan is a biopolymer which is widely used as a wall material of the microcapsules. It has antibacterial and antifungal properties, but its antifungal activity considered as poor.

In this thesis, cinnamaldehyde conjugated chitosan has been synthesized via reductive amination and it has been used as an encapsulation material for obtaining curcumin and quercetin microparticles. Antifungal activity assays have been performed and minimum inhibitory concentrations of chitosan, cinnamyl chitosan, curcumin, and quercetin against *Aspergillus niger* have been determined as >5000

ppm, 750 ppm, 2500 ppm, and 2500 ppm, respectively. Minimum inhibitory concentrations of chitosan, cinnamyl chitosan, curcumin, and quercetin against *Penicillium digitatum* have been determined as >5000 ppm, 250 ppm, 1666 ppm, and 1666 ppm, respectively. Ionic gelation of chitosan/tripolyphosphate and cinnamyl chitosan/tripolyphosphate have been performed for microencapsulation of curcumin and quercetin with high encapsulation efficiency. Higher antifungal activity against same pathogen microorganisms has been observed for cinnamyl chitosan/polyphenol microcapsules compared to the chitosan/polyphenol microcapsules.

Keywords: Polyphenol, chitosan, cinnamyl chitosan, microencapsulation, curcumin, quercetin.

ÖZET

Sinnamaldehit Modifiye Kitosan ile Polifenolik Bileşiklerin Mikroenkapsülasyonu ve Antifungal Aktivitesi

Aşkıcan HACIOĞLU

Biyomühendislik Bölümü

Yüksek Lisans Tezi

Danışman: Doç. Dr. Cem Bülent ÜSTÜNDAĞ

Eş-Danışman: Prof. Dr. Soner ŞAHİN

Polifenoller, tıptan gıda endüstrisine kadar çeşitli uygulamalara sahip doğal olarak elde edilen moleküllerdir. Çok çeşitli patojen mikroorganizmalara karşı antimikrobiyal olarak etkilidirler. Bununla birlikte, polifenollerin sınırlı biyoyararlanımı ve stabilite sorunları vardır. Mikrokapsülleme, kontrollü salım, hedefli dağıtım vb. hibi daha yararlı özelliklerle birlikte antimikrobiyal molekülün biyoyararlanımını artırmak için bir stratejidir. Kitosan, mikrokapsüllerin duvar malzemesi olarak yaygın olarak kullanılan bir biyopolimerdir. Antibakteriyel ve antifungal özelliklere sahiptir, ancak antifungal aktivitesi zayıf olarak kabul edilir.

Bu tezde sinnamaldehit konjuge kitosan, indirgeyici aminasyon yoluyla sentezlenmiş ve kurkumin ve kuersetin mikropartiküllerinin elde edilmesinde kapsülleme malzemesi olarak kullanılmıştır. Antifungal aktivite analizleri yapılmış ve *Aspergillus niger*'e karşı kitosan, sinnamil kitosan, kurkumin ve kuersetin minimum inhibisyon konsantrasyonları sırasıyla > 5000 ppm, 750 ppm, 2500 ppm

ve 2500 ppm olarak belirlenmiştir. *Penicillium digitatum*'a karşı minimum inhibisyon konsantrasyonları sırasıyla kitosan, sinnamil kitosan, kurkumin ve kuersetin için > 5000 ppm, 250 ppm, 1666 ppm ve 1666 ppm olarak belirlenmiştir. Kurkumin ve kuersetinin mikroenkapsülasyonu kitosan/tripolifosfat ve sinnamil kitosan/tripolifosfat ile iyonik jelasyon metoduyla, yüksek enkapsülasyon verimiyle gerçekleştirilmiştir. Kitosan/polifenol mikrokapsüllerine kıyasla sinnamil kitosan/polifenol mikrokapsülleri için aynı patojen mikroorganizmalara karşı daha yüksek antifungal aktivite gözlenmiştir.

Anahtar Kelimeler: Polifenol, kitosan, sinnamil kitosan, mikrokapsülleme, kurkumin, kuersetin.

1

INTRODUCTION

1.1 Literature Review

1.1.1 Chitosan and Modified Chitosan as an Antimicrobial Agent

Chitosan (Ch) is a marine origin biopolymer derived via deacetylation of chitin. Chitin is mainly abundant in the exoskeletons of insects and crustaceans like shrimps, crabs, lobsters and consists of β-(1-4)-N-acetyl-D-glucosamine units [1]. Besides arthropods, chitin is also can be synthesized by yeasts and fungi as well as higher organisms like nematodes, protozoa, algae, and mollusks [2].

Deacetylation of a chitin polymer backbone results in an acidic aqueous media soluble polymer, chitosan. Chitosan is the only cationic natural polymer which makes it attractive in a wide range of applications such as medical, agricultural, food and environmental applications. It can be used to form emulsions, gels, films and fibers with the advantages of being non-toxic, biodegradable and biocompatible [3-6]. Chitosan is formed of glucosamine units (Figure 1.1). When the acetyl groups remove from the chitin backbone via deacetylation, polysaccharide becomes a polyelectrolyte and solubility occurs in aqueous media with the protonation of remaining amine groups of the chitosan. This feature allows chitosan a great carrier for drug delivery applications, a potential scaffold material in tissue engineering applications, dye or metal removal applications and antimicrobial agent in food and agriculture industry [7-12] .

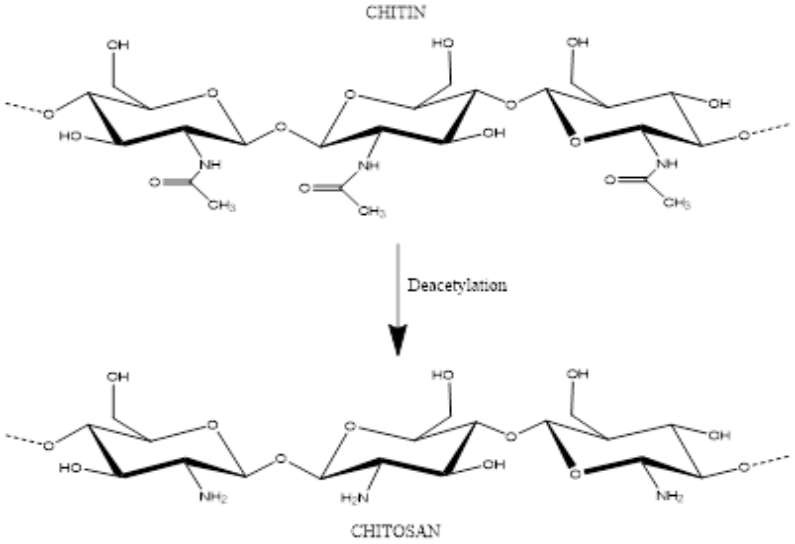


Figure 1.1 Chemical structure of chitin and chitosan

The usage of chitosan as an antimicrobial agent has been studied in literature. It shows antimicrobial activity against a wide range of microorganisms including filamentous fungi, yeasts and bacteria. It has been observed that the antimicrobial activity of chitosan depends on the pH of the growth medium, molecular weight of the chitosan, degree of deacetylation, concentration of the chitosan and type of microorganisms [13-15]. The antimicrobial mode of action of chitosan is still a mystery due to the factors that influence the activity of chitosan [16]. A brief list of the microorganisms inhibited by chitosan is shown in Table 1.1.

Table 1.1 Antimicrobial activity of chitosan against various types of microorganisms

MICROORGANISM	Minimum Inhibitory Concentration (ppm)	REFERENCE
Gram negative bacteria		
Escherichia coli	1000	[17]
	650	[18]
	100	[19]
	20	[20]
Samonella tiphymurium	2000	[21]
	1500	[19]
	>1000	[17]
Pseudomonas aeruginosa	1700	[21]
	>200	[19]
Pseudomonas fluorescens	1000	[17]

Table 1.1 Antimicrobial activity of chitosan against various types of microorganisms (continued)

Pseudomonas fluorescens	500	[20]
Vibrio parahaemolyticus	1000	[17]
	150	[19]
Shigella dysenteriae	>200	[19]
Vibrio cholerae	200	[19]
Aeromonas hydrophila	1000	[19]
Salmonella enterica	3000	[22]
Xanthomonas campestris	500	[23]
Gram positive bacteria		
Bacillus cereus	1000	[20]
	<1000	[17]
Bacillus megaterium	800	[24]
Staphylococcus aureus	>1250	[18]
	>800	[24]
	700	[21]
	20	[20]
Listeria monocytogenes	800	[17]
	150	[19]
Lactobacillus brevis	1000	[17]
Lactobacillus plantarum	<1000	[24]
Lactobacillus bulgaricus	>1000	[17]
Fungi		
Aspergillus parasiticus	>2000	[19]
Aspergillus fumigatus	>2000	[19]
Botrytis cinerea	10	[20]
Fusarium oxysporum	100	[20]
Candida albicans	>1250	[18]
	600	[21]
	500	[19]
Microsporum canis	1100	[21]
Trichophyton mentagrophytes	2200	[21]
Drechstera sorokiana	10	[20]

As be seen in Table 1.1, chitosan is a broad-range antimicrobial material. However, inconsistencies occur between the MIC doses different studies against similar microorganisms [25]. This is possible because of the factors stated above the Table 1.1. Chitosan's insolubility in water, high viscosity and coagulation tendency to proteins restrict the usage of chitosan. These disadvantages derived the effort of improving the solubility of chitosan in an aqueous medium as well as improving its

antimicrobial activity via chemical modifications which can be observed in many studies [10, 26-31].

Glucosamine groups contain three nucleophilic functional groups; a primary amine group, a primary hydroxyl group and a secondary hydroxyl group. This allows chitosan to be chemically modified either at the amino group or at the hydroxyl groups to produce its derivatives [32-37]. In literature, N-substitution reactions are widely used to modify chitosan. N,N,N-Trimethyl chitosan chloride is an important chitosan derivative due to its solubility, absorption and antibacterial properties [38]. N-alkyl and N-aryl substituents can be obtained via Schiff base intermediated reductive amination reactions using sodium borohydride ($NaBH_4$) and sodium cyanoborohydride ($NaCNBH_3$) [32, 39-41]. Another N-alkyl derivative of chitosan is N-carboxymethyl chitosan which can be obtained via alkylation reaction with sodium bicarbonate ($NaHCO_3$) in aqueous conditions [42]. Besides these, N-phosphomethylation, graft reactions with ionic liquids, Steglich esterification has been performed in the literature to modify amino groups of chitosan [37, 43-45]. O-substitutions of chitosan generally interfered with the higher reactivity of the primary amine group. A protective group should be used in this type of substitution reactions of chitosan, which makes N-phthaloylation as a key intermediate for O-substitution reaction of chitosan [46]. After reactions, the phthaloyl group can be easily removed via adding an electron withdrawing group results in deprotection of the amino group [47]. Additionally, N,O-substitution can be performed together to adjust the hydrophilicity of chitosan. These include oxidation reactions, which can be performed via chemically or enzymatically [48-50].

1.1.2 Natural polyphenols as antifungal molecules

Polyphenols are secondary metabolites of plants, responsible for the protection of the plants from UV radiation, pathogens, and they are important for the plant's growth and reproduction. Their chemical structure consists of at least one benzene ring and hydroxyl groups bonded directly to the aromatic carbon [51]. They are usually classified as flavonoid and non-flavonoid compounds. Non-flavonoid compounds can be classified as simple phenols, phenolic acids, diarylheptanoids, quinones, tannins, coumarins, terpenes, terpenoids, alkaloids, lectins and

polypeptides [52]. Some examples of non-flavonoid phenolic compounds have been shown in Figure 1.2.

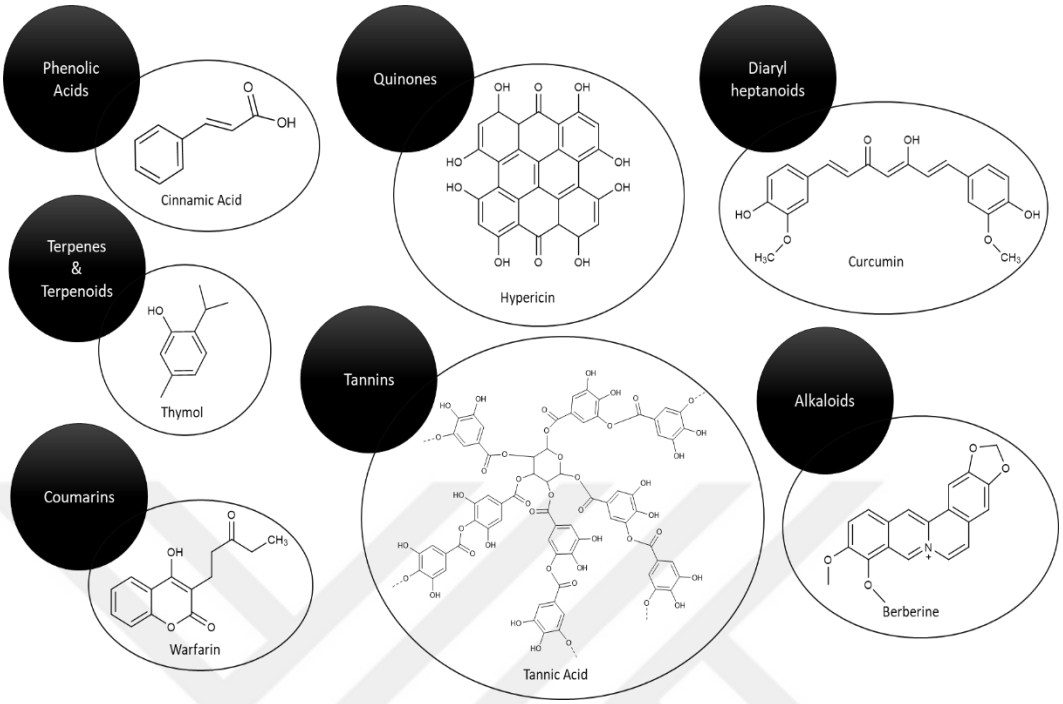


Figure 1.2 Classification of plant-based non-flavonoid phenolic compounds

Flavonoids are naturally occurring molecules and considered as the largest group of polyphenols [53]. These plant-based molecules are derived from multi-step biochemical processes of phenylalanine, generally synthesized via the shikimate pathway [54]. Their skeleton consisted of 15 carbon molecules and sub-grouped as flavanols, flavone, flavanone, flavan-3-ol, isoflavone, and anthocyanidin [55].

The best-defined feature of almost every flavonoid group, as well as polyphenols, is their antioxidant properties against reactive oxygen species [51, 56, 57]. There are lots of literature studies about the medical and industrial use of natural phenolic compounds which includes their anticarcinogenic [58], antidiabetic [59], anti-inflammatory [60], neuroprotective [61], cardioprotective [62], liver protective [63], antibacterial, antiviral and antifungal activities [56, 64-67]. In this study, the focal point besides the modification of chitosan is the antifungal activity of curcumin and quercetin.

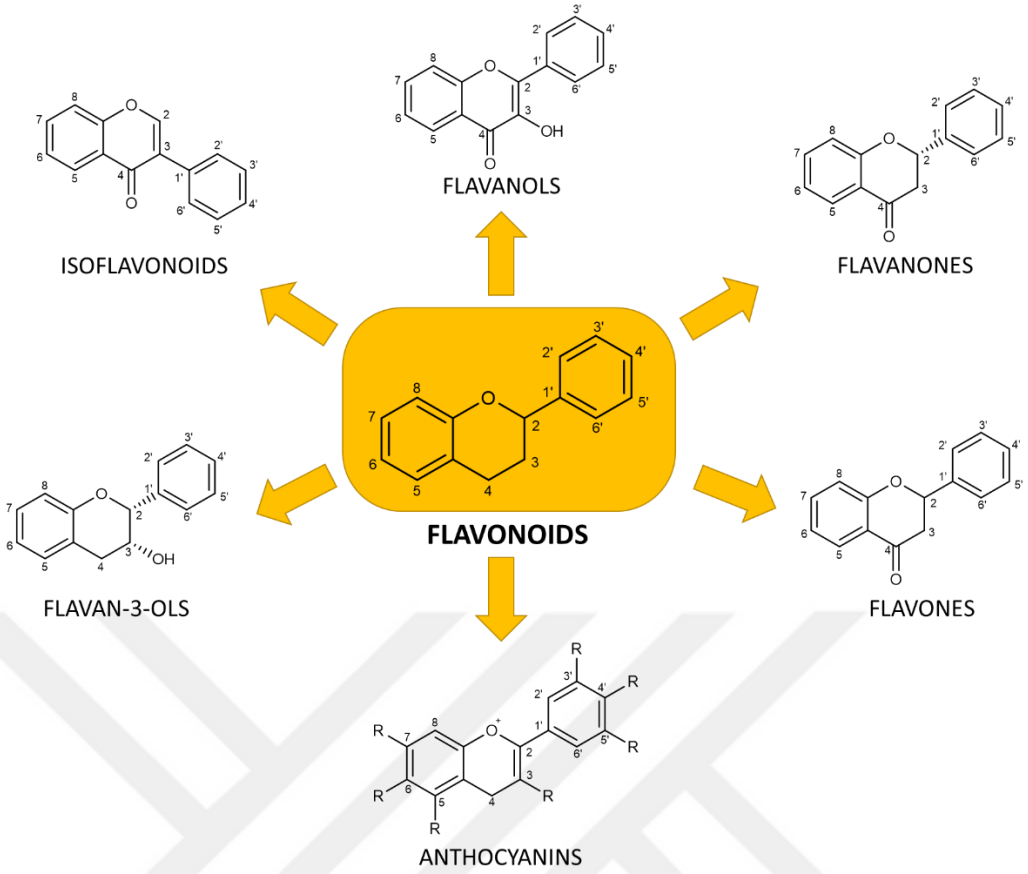


Figure 1.3 Classification of flavonoids

1.1.3 Curcumin

Curcumin is a yellow-orange phenolic compound isolated from the turmeric (*Curcuma longa*) plant, which is found in a spice widely used in Asian countries. Curcumin is classified as diarylheptanoid which is a comparatively small group of plant polyphenols [68]. It is used as a coloring and flavoring agent, listed as a food additive with the code of E 100 by the European Food Safety Authority since 1975 [69]. Curcumin contains a diketone group in its chemical structure, however, in the solvents at pH range of 3-9 it is available as keto-enol tautomer as shown in Figure 1.4 [70]. It is insoluble in aqueous media at acidic or neutral pH, soluble in alkaline pH, polar and nonpolar organic solvents [71]. Curcumin is too sensitive to environmental factors and degrades rapidly via oxidative degradation, photodegradation and solvolysis [72]. It can be degraded to several products via different degradation mechanisms. Vanillin, ferulic acid and feruloylmethane are the major products when curcumin is kept for 30 minutes in alkaline aqueous media

[73]. Autooxidation of curcumin takes part in a buffered solution and its main degradation product is bicyclopentadione [74]. When curcumin is dissolved in organic solvents, vanillin, vanillic acid, ferulic acid and ferulic aldehyde derives via photodegradation [75].

Although curcumin has a lot of properties as a phenolic compound, the chemical instability is the main drawback for its usage as a beneficial material. Its bioavailability is too limited and generally, its pharmacokinetic properties are referred as poor [76]. Especially when it has given orally, the metabolic pathways affect curcumin's pharmacokinetic properties with intestinal sulfation, glucuronidation and reduction [77]. In addition, low water solubility and poor intestinal permeability are crucial factors which limit curcumin's medical applications [78]. To overcome the problems of bioavailability, micro-nano encapsulation, emulsification, synergistic enhancers and other controlled release concepts are extensively studied in the literature [72, 76, 79]. Due to the disadvantages of the bioavailability of curcumin, there are several studies for its medical usage in topical formulations [80]. The topical usage also has to be optimized for obtaining an efficient delivery of curcumin to protect it from degradation and improve its solubility and permeability. Therefore, nano-formulations, emulsions, hydrogels, film and fiber formulations are studied in literature [80].

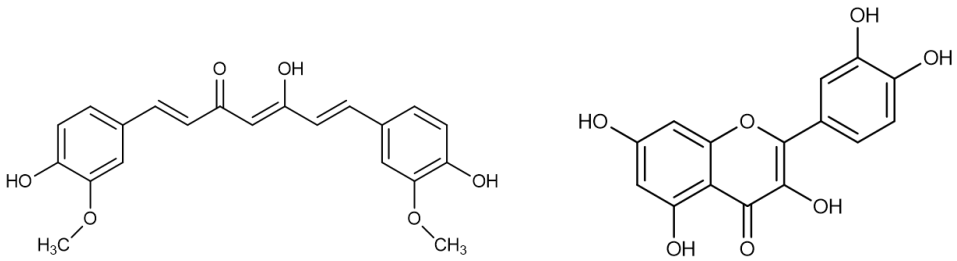


Figure 1.4 Chemical structures of curcumin and quercetin

1.1.4 Quercetin

Quercetin is a flavonoid, among the sub-group of flavanols. It is abundant in many plants' seeds, barks, leaves, flowers and fruits including berries, apples, grapes,

tomatoes, onions, tea, broccoli, brussels sprouts, cabbage, cauliflower [81]. It is practically not soluble in water, soluble in organic solvents. Quercetin is famous with its free radical scavenging capabilities, which makes it a great antioxidant molecule [82, 83]. Besides its antioxidant features, quercetin is an anti-inflammatory [84], anticarcinogenic [85], cardioprotective [86], gastroprotective [87] molecule which can seem to be used for medical purposes.

In contrast to quercetin's beneficial effects, some toxic effects have been observed due to its oxidation products. After antioxidative activities, quercetin turns into four different quinone forms, which can be displayed toxic effects via arylating protein thiols [88]. Only glutathione is able to bind to quinone type quercetin and prevent its toxic effects. However, their product is not stable and when glutathione concentration is low, toxic effects of quercetin quinones occur as membrane permeability or decreased function of enzymes [89, 90]. Furthermore, there are in vitro reports that indicate the genotoxic effect of quinone type quercetin [91, 92]. However, in vivo studies did not confirm the genotoxicity of quercetin which can be related to the metabolic protective activities [92].

Similar to curcumin, quercetin has poor bioavailability and absorption properties in humans. Glycosylated quercetin is the most abundant form in the fruits and vegetables and their bioavailability and absorption is much higher than quercetin's pure form [93, 94]. Quercetin glucoside is mainly absorbed in the upper segment of the small intestine, methylation, sulfonation occur in the small intestine and liver, and is finally excreted by the kidney [94]. For improved bioavailability and absorption, simultaneous ingestion of quercetin with ascorbic acid, folic acid and additional flavonoids is beneficial [95].

1.1.5 Antifungal Activity of Curcumin and Quercetin

There is an extensive literature about the antibacterial, antifungal and antiviral activity of polyphenolic compounds. Polyphenolic molecules' antimicrobial mode of action is mainly because of their antioxidant properties [67]. Antimicrobial activity of phenolic compounds is also observed to be dependent on their hydroxyl group moieties. Hydroxyl groups are able to disrupt membrane integrity of bacteria and

fungi besides generating reactive oxygen species and altering cell metabolism via affecting enzymatic pathways [96].

Fungal pathogens cause disruptive losses in the food and agriculture industry and they are responsible for a lot of diseases in especially immunocompromised humans [97]. Polyphenolics can be used to inhibit the disease-causing fungal pathogens [98]. Curcumin is able to prevent fungal growth via disintegrating fungal cell wall, inhibition of ergosterol synthesis and respiration of the fungi [99]. Quercetin is found to be effective in inhibiting fatty acid synthase pathway and mitochondrial activity, as well as disruption of fungal membrane and generating reactive oxygen species [98]. Some of the antifungal studies of curcumin and quercetin have been shown in Table 1.2.

Table 1.2 Antifungal studies of curcumin and quercetin

Microorganism	Description	Reference
CURCUMIN		
Candida sp.	Antifungal assays have indicated that, curcumin has MIC values between 250-2000 ppm against 6 different *Candida* species and their different strains. Its mode of actions is also investigated, and curcumin has affected Plasma membrane ATPase, ergosterol biosynthesis, and proteinase secretion.	[100]
Candida sp.	Antifungal activity of curcumin and methyl cinnamaldehyde was investigated against 38 different strains of *Candida* including fluconazole resistant ones. MIC doses of curcumin have been determined between 250-500 ppm. Efflux pump disruption is determined in the curcumin and methyl cinnamaldehyde treated candida.	[101]
Candida albicans	In this study, curcumin's anticandidal activity with photodynamic properties have been investigated. In vivo studies showed that, curcumin mediated photodynamic therapy against oral candidasis is able to decrease colony forming at 80 μM concentration in mice.	[102]
Candida sp.	Antifungal activity of curcumin against different *Candida* species have been investigated and its synergistic effect with ascorbic acid have been examined. Curcumin's MIC doses have been determined between 0.625-25 ppm and synergistic activity with 250 ppm of ascorbic acid have been decreased MIC doses to 0.3125-1.25 ppm.	[103]
Aspergillus flavus	Photodynamic treatmet with curcumin on maize kernels have been investigated as an alternative agricultural solution. Reduction of 3log CFU/ml have been observed.	[104]

Table 1.2 Antifungal studies of curcumin and quercetin (continued)

Penicillium expansum	Curcumin mediated photodynamic activity against *Penicillium expansum* have been examined in vitro and in planta on apples. Photodynamic treatment with curcumin has decreased sporulation by 99.7%.	[105]
Aspergillus fumigatus	Curcumin and Quercetin's antifungal activity against *Aspergillus fumigatus* and 3 different *Candida* species, their synergistic activity and encapsulated forms activity have been investigated. Curcumin's and Quercetin's MIC against *Aspergillus fumigatus* is determined as 64 ppm. Their MIC doses varies between 4-64 ppm against *Candida* species. Synergistic effect has been observed.	[106]
Different filamentous fungi	This study indicates curcumin's antifungal activity is significantly increased with photodynamic therapy against different type of phytopathogens and human pathogens. Curcumin with photosanitization against *Fusarium oxysporum, Candda albicans, Aspergillus niger, Aspergillus flavus, Penicillium griseofulvum, Penicillium chrysogenum* and *Zygosaccharomyces bailii* was inhibited sporulation as 100%, 100%, 99%, 88.9%, 78%, 99.7% and 99.2% respectively at different concentrations of curcumin.	[107]
QUERCETIN		
Aspergillus flavus	Quercetin is able to inhibit the morphogenesis of conidia of *Aspergillus flavus*, and interrupt germination, by modulating the gene expression from calcium signalling pathway, Hsp90 and 70. It also elucidated the role of conserved elements of signal transduction cascades under the phytochemical stress.	[108]
Aspergillus flavus	*Aspergillus flavus* was cultured in corn flour and MIC dose is observed as 226 ppm. It has been revealed that quercetin can inhibit biosynthesis of Aflatoxin B1 via damaging MAPK pathway which is a crucial pathway for aflatoxin biosynthesis.	[109]
Aspergillus fumigatus	MIC doses of quercetin have been determined 200 and 100 ppm against different strains of *Aspergillus fumigatus*. Gold nanoparticles with quercetin shows 380 and 150 ppm against different strains.	[110]
Botrytis cinerea	Quercetin shows half-maximal inhibitory concentration (IC50) at 120.8 ppm	[111]
Candida albicans	MIC value of quercetin has been determined as 10 ppm and increased intracellular magnesium accumulation has been observed. Mitochondrial dysfunction and DNA damage induced apoptosis via quercetin	[112]
Candida sp.	A review study about flavonoids which indicates quercetin's MIC doses between 7.8-128 against different *Candida* species.	[98]

Table 1.2 Antifungal studies of curcumin and quercetin (continued)

Cryptococcus neoformans	Quercetin MIC dose is bigger than 125 ppm. It shows synergistic effect with rutin and Amphotericin B.	[113]
Penicillium expansum	250 ppm is determined as the MIC value against *Penicillium expansum*. With the quercetin treatment, kiwifruit infected with *P. expansum* had higher chitinase, β-1,3-glucanase, phenylalanine ammonialyase, polyphenol oxidase and peroxidase activities, compared with those of kiwifruit only treated with quercetin or inoculated with *P. expansum*. which shows its toxic properties against fungal pathogens and inhibition of defense responses.	[114]
Trichophyton rubrum	MIC of the quercetin has been determined as 33.54 ppm against wild-type and 56.15 ppm against mutant strains of dermatopyhtic yeast *Trichophyton rubrum*. It has also been observed that fatty acid and ergosterol synthesis was inhibited.	[115]

1.1.6 Microencapsulation

Microencapsulation is a technology that allows entrapping active ingredients also known as core materials using a surrounding material namely as encapsulating or shell material. The main target is to create a physical barrier to protect the active ingredients from the external environment via loading them into the encapsulating material and allowing a possible controlled release. This technology has been primarily used by the pharmaceutical and food industry. Besides it is extensively used by agriculture, cosmetic and textile industries [116]. The primary reason for microencapsulation is found to be either for sustained or prolonged drug release. It can also be used for masking taste and odor of molecules, stabilization of the sensitive molecules like polyphenols, preventing evaporation of volatile molecules, reducing toxicity of medical or agricultural drugs and increasing biocompatibility of encapsulated material [117].

As microencapsulating material, biopolymers can be used especially in pharmaceutical and food industry regarding to increase biocompatibility and decrease toxicity [118]. Animal-origin proteins like collagen, gelatin, casein, fibroin, plant-based proteins such as zein and soy protein are used as shell materials for encapsulating various molecules [119-122]. Polysaccharides like alginate, agar,

carrageenan, dextran, cellulose, starch and chitosan are able to be used for encapsulating materials [123-128].

Microencapsulation production techniques can be classified as top-down and bottom-up methods depending on the physical or chemical applications during the production stage. Top-down methods include extrusion-gelation emulsification-gelation and high energy homogenization techniques, physical breaking down via milling, grinding and shredding [118]. Bottom-up techniques are identified as self-organization of molecules to build microparticle structures due to the electrostatic attraction, pH, temperature and chemical affinities [129]. This approach includes anti-solvent precipitation, coacervation, inclusion complexation, drying and gelation methods [118]. For gelation methods shear forces are applied to form liquid microparticles so it can be included in both top-down and bottom-up categories [130].

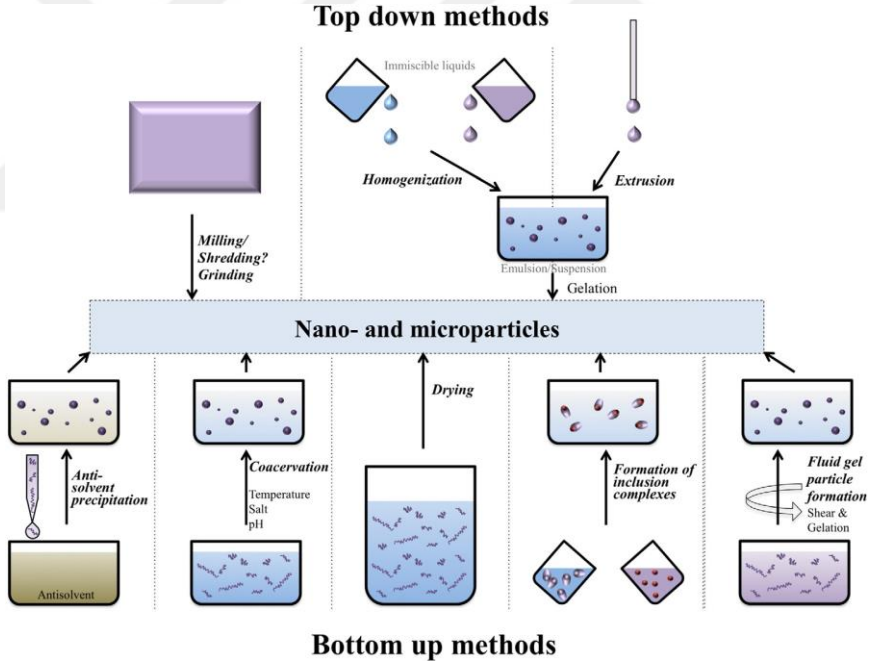


Figure 1.5 Different microencapsulation techniques for producing biopolymer-based nanosized and microsized particles [118]

For obtaining chitosan microparticles, emulsification, polyelectrolyte complexation and ionotropic gelation methods are applied to load active ingredients [131]. Ionotropic gelation is a simple method, based on the contact and complexation of

oppositely charged molecules and their molecular crosslinking [132]. Chitosan is a weak base with protonated amino groups in acidic media which makes chitosan a positively charged polysaccharide. Ionic gelation occurs in the presence of anionic molecules such as tripolyphosphate (TPP) [133, 134].

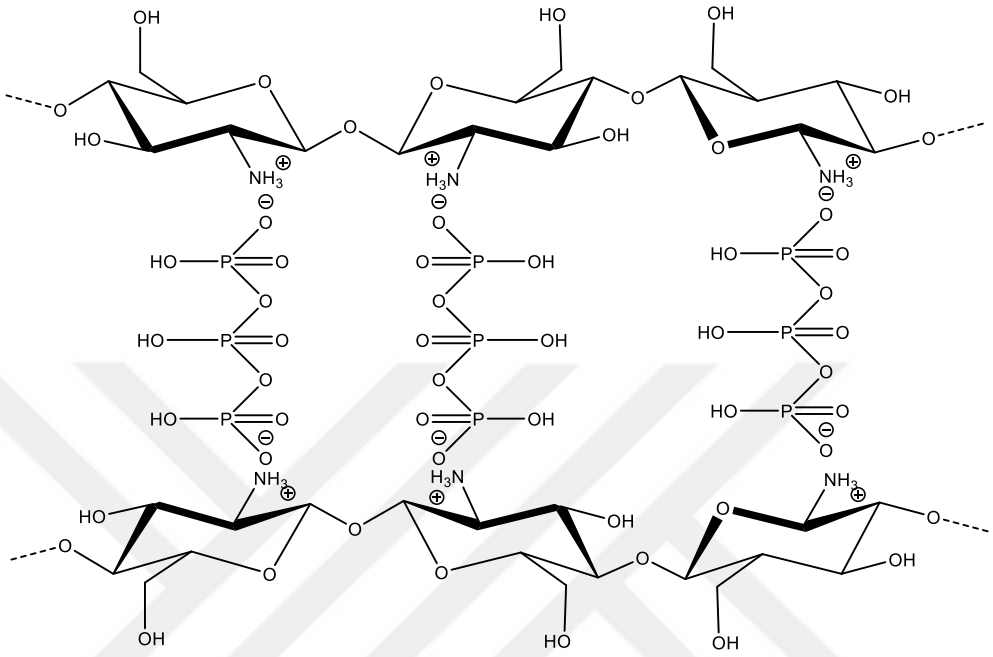


Figure 1.6 Ionic interaction between chitosan and tripolyphosphate in acidic media

Chitosan/TPP crosslinking mechanism has been used to encapsulate curcumin in many articles [135, 136]. In one study, curcumin is encapsulated into chitosan/TPP microcapsules and release kinetics have been observed between different formulations [137]. In another study, Ch/TPP-Cur nanoparticles were synthesized, and their transdermal delivery properties have been investigated. In that study, zero-order drug release kinetic has been observed at the pH value of 5.0 and HaCat cells viability enhanced with nanoparticles [138]. Ch/TPP-Cur nanoparticles also have been prepared as a stable emulsion formulation which can be functional as a delivery system [139]. Another study showed Ch/TPP-Cur microparticles can be effective in vitro for inhibition of *Pseudomonas aeruginosa* [140]. For quercetin encapsulation, Ch/TPP microencapsulation has been performed in literature. In one study, antitumor effects of Ch/TPP-Quer nanoparticles have been investigated on mice with colorectal cancer and results demonstrated that it may be a promising

alternative in site-specific treatments [58]. Another study demonstrated a Ch/TPP nanogel formulation to be one of the effective ways to transport quercetin and glibenclamide in transdermal delivery [141]. Effects of microencapsulation to the antioxidant activity of quercetin have been studied in literature and results demonstrated that the antioxidant properties does not change [142].

Modified chitosan is also used in microencapsulation studies in literature. Cinnamic acid modified chitosan via esterification is used to encapsulate *Mentha piperita* essential oil and final formulation antimicrobial activity against *Aspergillus flavus* is tremendously increased compared to the essential oil and cinnamic acid grafted chitosan alone [143]. In another study, *Gaultheria procumbens* essential oil has been encapsulated in chitosan-cinnamic acid microgels and antimicrobial and antiaflatoxigenic properties have been observed against *Aspergillus flavus* pathogen [144]. Another study has been shown the better antimicrobial activity of different essential oil microcapsules encapsulated with cinnamic acid grafted chitosan against dermatophytic fungus *Microsporum canis* [145].

1.2 Objective of the Thesis

This study aims to prove the antimicrobial and antifungal activity of modified cinnamyl chitosan and usage of the modified polysaccharide as an encapsulation material. The main reason of the encapsulation is increasing the antifungal activity of curcumin and quercetin and providing a novel alternative functional material as an antifungal agent.

1.3 Hypothesis

Chitosan is a widely used biopolymer for microencapsulation of different types of molecules. It can be modified via reductive amination with cinnamaldehyde depending on the literature and its antimicrobial activity increases. Quercetin and Curcumin are antioxidant polyphenolic molecules with antimicrobial and antifungal properties. Literature shows the microencapsulation concept is enabling to increase in vivo activity of antimicrobial molecules via increasing bioavailability. Furthermore, when microencapsulation is performed with modified antimicrobial biopolymers, a synergistic effect occurs and antimicrobial activity increases. This

study hypothetically asserts that the cinnamaldehyde modified chitosan (CinCh) can be used as an encapsulation material for delivery of Curcumin and Quercetin and this increases the antifungal activity of the polyphenols against wild-type *Aspergillus niger* and *Penicillium digitatum* species.

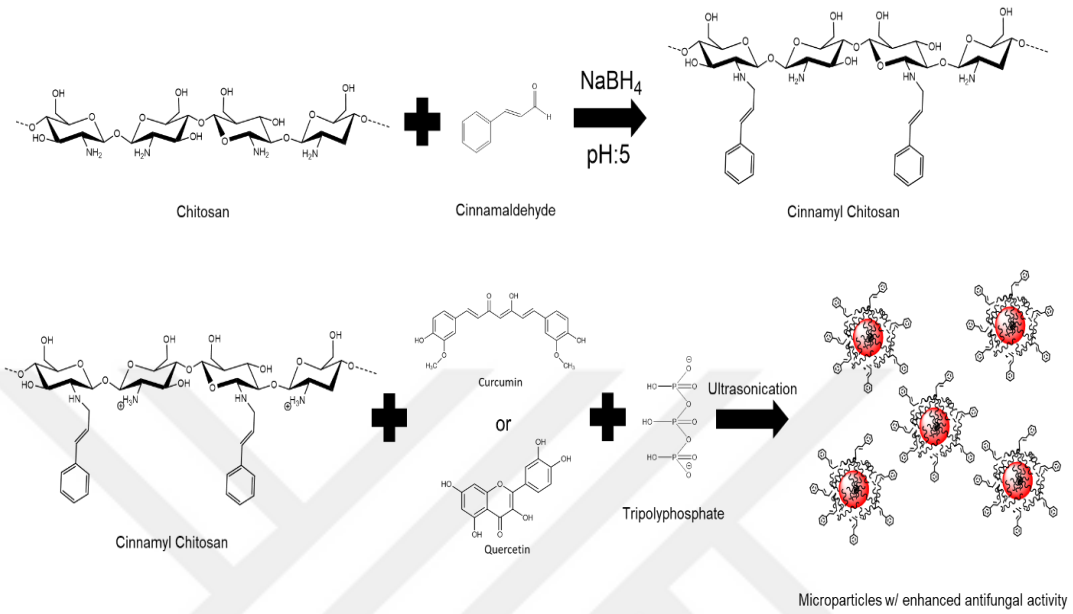


Figure 1.7 Hypothetical strategy for obtaining microparticles with enhanced antifungal activity

2

MATERIALS AND METHODS

2.1 Materials

Low molecular weight chitosan (Deacetylation degree: 76%, 122 centipoise, Batch Number: STBH7664), Sodium Borohydride ($NaBH_4$), TPP, Sodium hydroxide (NaOH), TNBS, Curcumin and Quercetin were purchased from Sigma-Aldrich. Cinnamaldehyde (Batch Number: 191013) was purchased from Salus Nutra Inc. Extra pure Methanol and Lactic Acid were product of Tekkim. Dimethyl sulfoxide (DMSO) and Sodium bicarbonate ($NaHCO_3$) was purchased from Merck. For antifungal activity assays, Dichloran Rose Bengal Chloramphenicol (DRBC) Agar purchased from Neogen. Saboraud Dextrose Agar (SDA) has been purchased from Merck. Peptone and Yeast Extract have been purchased from Biolife and Glucose purchased from Neofroxx. Milli-Q Direct 3 UV Water Purification System has been used to obtain purified and ultrapure water.

2.2 Synthesis of Cinnamyl Chitosan

Cinnamaldehyde modified chitosan was prepared via reductive amination mechanism based on a method prescribed in literature with modifications [33, 41]. For synthesis, 1% of low molecular weight chitosan (Mw:110 kDa) was dissolved in 1% lactic acid solution and 2 molar equivalents of cinnamaldehyde in methanol, was added into chitosan solution stirred for 12 hours under argon atmosphere (pH:5). 5 molar aldehyde equivalents of $NaBH_4$ in methanol was added and stirred for 24 hours. After the reaction, pH was adjusted to 12 and precipitated material was collected via centrifugation for 20 minutes at 9000 RPM. Precipitate washed with ethanol 3 times to remove unreacted cinnamaldehyde. After purification, cinnamyl chitosan was dispersed in dH_2O and lyophilized for 5 days.

2.3 Microencapsulation of Curcumin and Quercetin with Chitosan and Cin-Ch

Microencapsulation of curcumin and quercetin has been performed via ionotropic gelation method. Process has been optimized with chitosan at different concentrations of active ingredients and solvents. Briefly, chitosan was prepared at 1% concentration within 1% lactic acid solution. TPP solution was prepared at 0.5% concentration and pH of TPP solution was adjusted do 3 with 2 M HCl solution. Tween 80 was used as a surfactant for optimization of the encapsulation process occasionally which was added into chitosan solution before introducing active ingredients. Curcumin or quercetin were dissolved in 5 ml of EtOH or DMSO with concentrations of 4 mg/ml, 20 mg/ml and 50 mg/ml. After active ingredient solutions were prepared, they were added into 25 ml of chitosan solution and homogenized with ultrasonication device at 40% amplitude for 2.5 minutes. After sonication, 25 ml of TPP solution was added into the mixture via 50 ml syringe drop by drop. Another sonication with same amplitude and time was applied and samples were stirred at 600 rpm for 2 hours. All samples were centrifuged for determination of encapsulation efficiency and further analysis. The optimized procedure was applied with same parameters using Cin-Ch instead of chitosan.

2.4 Characterization of Modified Chitosan and Microcapsules

2.4.1 Spectrometric Analysis

UV spectrum of chitosan and cinnamyl chitosan was obtained within quartz cuvettes between 200-400 nm range using Epoch II Multiplate Reader (Biotek Instruments). Fourier Transform Infrared Spectroscopy (FTIR) analysis was performed with dry samples using attenuated total reflection (ATR) equipment.

2.4.2 Determination of Free Amine Groups with TNBS Assay

Trinitrobenzene sulfonic acid assay was performed to determine the free primary amine groups of chitosan and Cin-Ch based on the procedure reported previously [146, 147]. 1 mg of each sample were taken triplicated and dispersed in 250 µL of 1 M HCl solution. 250 µL of 0,25 M sodium bicarbonate solution and 250 µL of 0.1% TNBS (diluted with $NaHCO_3$ solution) were added into each Eppendorf and all

samples were incubated for 2 hours at 30°C. After incubation 250 µL of 2 M HCl was added and UV absorption at 344 nm was measured.

2.4.3 Contact Angle Analysis

Contact angle analysis performed with Attention Theta Flex (Biolin Scientific) device, for comparison of hydrophobicity between chitosan and Cin-Ch. All experiment has been performed with 3 repetitions. Contact angles have been recorded for 15 seconds on a parafilm surface. Chitosan has a greater contact angle on parafilm surface, compared to cinnamyl chitosan. The hydrophobicity of cinnamyl chitosan is much more than chitosan due to the conjugation of hydrophobic molecules onto polymer chains.

2.4.4 Determination of Encapsulation Efficiency and Loading Efficiency

Standard curves have been prepared with concentrations between 0-31,25 µg/ml for curcumin and 0-78,125 µg/ml for quercetin with three replicates in EtOH. For determination of non-encapsulated active ingredient content, supernatants of microcapsules were analyzed at 429 nm for curcumin, and 372 nm for quercetin with proper dilutions in EtOH and concentrations were determined based on the standard curves. Encapsulation efficiency was determined according to this formula:

$$EE\% = \frac{(Total\ Active\ Added - Free\ Active)}{Total\ Active\ Added} \times 100 \qquad (2.1)$$

For determining loading efficiency, dried microcapsules weight measured and loading efficiency was determined according to this formula:

$$LE\% = \frac{Active\ Ingredient\ in\ Capsules}{Total\ Weight\ of\ Dried\ Microcapsules} \times 100 \qquad (2.2)$$

2.4.5 DLS/Zeta Potential Analysis

Optimized microcapsules were analyzed with dynamic light scattering (DLS) for determination of the size and its distribution. All samples were filtered with 0,45 µm Whatman filter and analyzed with Zetasizer Nano ZS (Malvern Instruments Ltd., UK) via 173° backscatter measurement at a temperature of 25°C. The surface charge of the microparticles corresponds to zeta potential that represents its electrophoretic

mobility and is determined by the Zetasizer Nano ZS (Malvern Instruments Ltd., UK) at a temperature of 25°C.

2.4.6 Release Studies

Release studies have been performed with slight modifications of a procedure described in literature [144, 148]. Briefly, 10 mg of lyophilized microcapsules added into 50 ml of release media consisting of dH_2O and EtOH in 3:2 volumetric ratio. Suspension mixed with a magnetic stirrer at 500 rpm, 1 ml of samples were taken hourly within the first 8 hours and 24th hour. Taken samples were centrifuged for 10 minutes at 9000 rpm and supernatants were analyzed at the proper wavelengths with Epoch II Multiplate Reader (Biotek Instruments). Released amount of active ingredients were determined with calibration curve calculations and cumulative release percentage was calculated depending on this formula:

$$Cumulative\ Release\ \% = \frac{Released\ Active\ Ingredient}{Loaded\ Active\ Ingredient} \times 100 \qquad (2.3)$$

2.5 Antifungal Activity Assays

2.5.1 Preparation of Culture Media

DRBC Agar, Saboraud Dextrose Agar (SDA) and Yeast Peptone Dextrose Broth (YPD) have been used as fungi culture media. For DRBC Agar, 31.7 g of culture media powder is dispersed in 1 L of dH_2O and mixed for 10 minutes. For SDA, 65 g of SDA powder was dispersed in 1 L of dH_2O and mixed. For YPD, 20 g of peptone, 20 g of glucose and 10 g of yeast extract was added into 1 L of dH_2O and mixed until the ingredients are dissolved. All culture media have been autoclaved at 121^{o}C for 15 minutes via OT 40 Autoclave (Nüve) after prepared as mixtures.

2.5.2 Antifungal Activity Assays

Antifungal activity assays were performed using a procedure with modifications [149].

For solid culture, 100 µl of active ingredient dispersion and 100 µl solid culture media (DRBC or SDA) at 65^{o}C were mixed in 96-Wellplate wells and kept until solidifying. *Penicillium digitatum* and *Aspergillus niger* spores were dispersed in

ddH$_2$O with 1% Tween80 and their turbidity arranged at 0.5 McFarland with McFarland Densitometer (Biosan). After solidification of culture media, 10 µl of 0.5 McFarland *Penicillium digitatum* and *Aspergillus niger* spores were inoculated into wells and plates were incubated at 30^{o}C. Growth of fungi investigated after 1 week of incubation.

For liquid culture, 100 µl of active ingredient dispersion and 50 µl of YPD media were mixed within the 96-Wellplate wells. *Penicillium digitatum* and *Aspergillus niger* spores were dispersed in YPD and their turbidity was arranged at 2 McFarland with McFarland Densitometer (Biosan). 50 µl of 2 McFarland spore suspension was added into each well and plates were incubated at 30^{o}C. Growth of fungi investigated after 1 week of incubation.

For the determination of antifungal activity of chitosan and CinCh, DRBC agar has been used as culture media and dilutions were made with ddH$_2$O in triplicates between 5000-250 ppm in final concentrations. The plate layout has been shown in Figure 2.1.

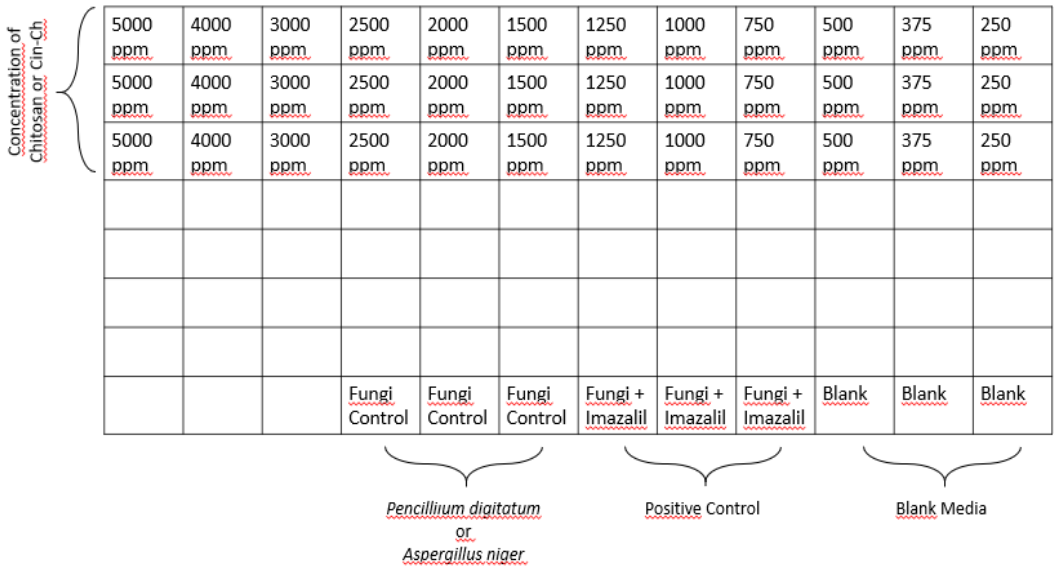


Figure 2.1 Plate layout of MIC assay of chitosan and cinnamyl chitosan

For determination of antifungal activity of curcumin and quercetin, YPD has been used as culture media. Curcumin and quercetin stock solutions were prepared in

DMSO and dilutions were made with ddH_2O in triplicates between 5000-250 ppm in final concentrations. The plate layout has been shown in Figure 2.2.

Concentration of Actives Penicillium	6666 ppm	5000 ppm	3333 ppm	2500 ppm	1666 ppm	1250 ppm	833 ppm	625 ppm	416.5 ppm	312.5 ppm	208.25 ppm	0 ppm
	6666 ppm	5000 ppm	3333 ppm	2500 ppm	1666 ppm	1250 ppm	833 ppm	625 ppm	416.5 ppm	312.5 ppm	208.25 ppm	0 ppm
	6666 ppm	5000 ppm	3333 ppm	2500 ppm	1666 ppm	1250 ppm	833 ppm	625 ppm	416.5 ppm	312.5 ppm	208.25 ppm	0 ppm
				P. digitatum	P. digitatum	P. digitatum	Blank	Blank	Blank	Imazalil +Pen.	Imazalil +Pen.	Imazalil +Pen.
				A. Niger	A. Niger	A. Niger				Imazalil +Asp.	Imazalil +Asp.	Imazalil +Asp.
Concentration of Actives Aspergillus	6666 ppm	5000 ppm	3333 ppm	2500 ppm	1666 ppm	1250 ppm	833 ppm	625 ppm	416.5 ppm	312.5 ppm	208.25 ppm	0 ppm
	6666 ppm	5000 ppm	3333 ppm	2500 ppm	1666 ppm	1250 ppm	833 ppm	625 ppm	416.5 ppm	312.5 ppm	208.25 ppm	0 ppm
	6666 ppm	5000 ppm	3333 ppm	2500 ppm	1666 ppm	1250 ppm	833 ppm	625 ppm	416.5 ppm	312.5 ppm	208.25 ppm	0 ppm

Figure 2.2 Plate layout of MIC assay of curcumin and quercetin

For determination of antifungal activity of microcapsules, SDA used as solid media and YPD used as liquid media. Dilutions were made with ddH2O in triplicates between 50%-0% in final concentrations. Plate layout has been shown in Figure 2.3.

Concentration of Microcapsules Penicillium	50%	37.5%	25%	18.75%	12.5%	9.375%	6.25%	4.687%	3.125%	2.343%	1.562%	0%
	50%	37.5%	25%	18.75%	12.5%	9.375%	6.25%	4.687%	3.125%	2.343%	1.562%	0%
	50%	37.5%	25%	18.75%	12.5%	9.375%	6.25%	4.687%	3.125%	2.343%	1.562%	0%
	Blank	Blank	Blank	Imazalil +Pen.	Imazalil +Pen.	Imazalil +Pen.	P. digitatum	P. digitatum	P. digitatum			
				Imazalil +Asp.	Imazalil +Asp.	Imazalil +Asp.	A. Niger	A. Niger	A. Niger			
Concentration of Microcapsules Aspergillus	50%	37.5%	25%	18.75%	12.5%	9.375%	6.25%	4.687%	3.125%	2.343%	1.562%	0%
	50%	37.5%	25%	18.75%	12.5%	9.375%	6.25%	4.687%	3.125%	2.343%	1.562%	0%
	50%	37.5%	25%	18.75%	12.5%	9.375%	6.25%	4.687%	3.125%	2.343%	1.562%	0%

Figure 2.3 Plate layout of MIC assay of microcapsules in SDA and YPD

3

RESULTS AND DISCUSSION

3.1 Characterization of Cin-Ch

3.1.1 Spectrometric Analysis

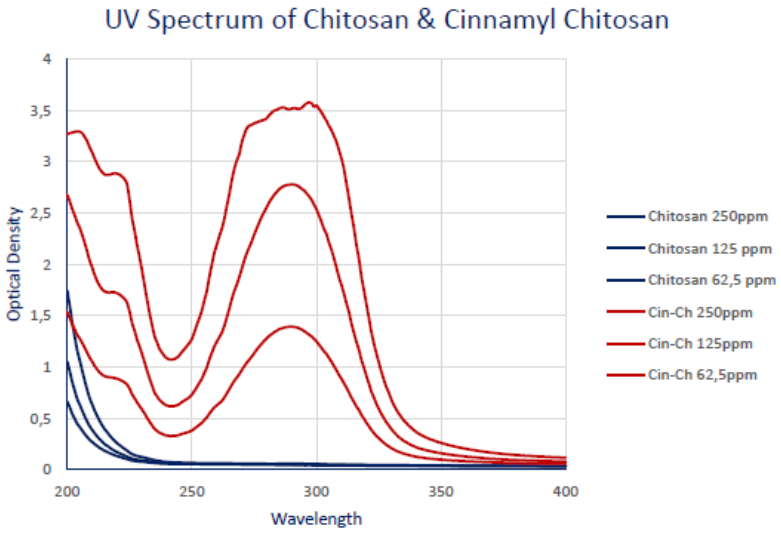


Figure 3.1 Comparison between UV spectrum of chitosan and cinnamyl chitosan

UV Spectrum comparison of chitosan and synthesized Cin-Ch showed significant difference between the samples. Normally chitosan has no optical density after the wavelength of 225 nm. As it has been observed, Cin-Ch has a significant peak at 290 nm, which shows the aromatic ring abundance on the polymer backbone.

FTIR spectrum of cinnamaldehyde (1), chitosan (2) and cinnamyl chitosan (3) can be observed at Figure 3.2. A strong band occurs at 1631 cm^{-1}, because of the alkene stretches of the aromatic rings. Also, there is a strong band observed at 748 cm^{-1} due to the aromatic ring C-H bond. FTIR spectra confirms the linkage between cinnamaldehyde and chitosan. The deformation of the NH_2 group band observed at 1585 cm^{-1}, which suggests formation of the amide bond between the amino group of chitosan with the cinnamaldehyde [143, 145].

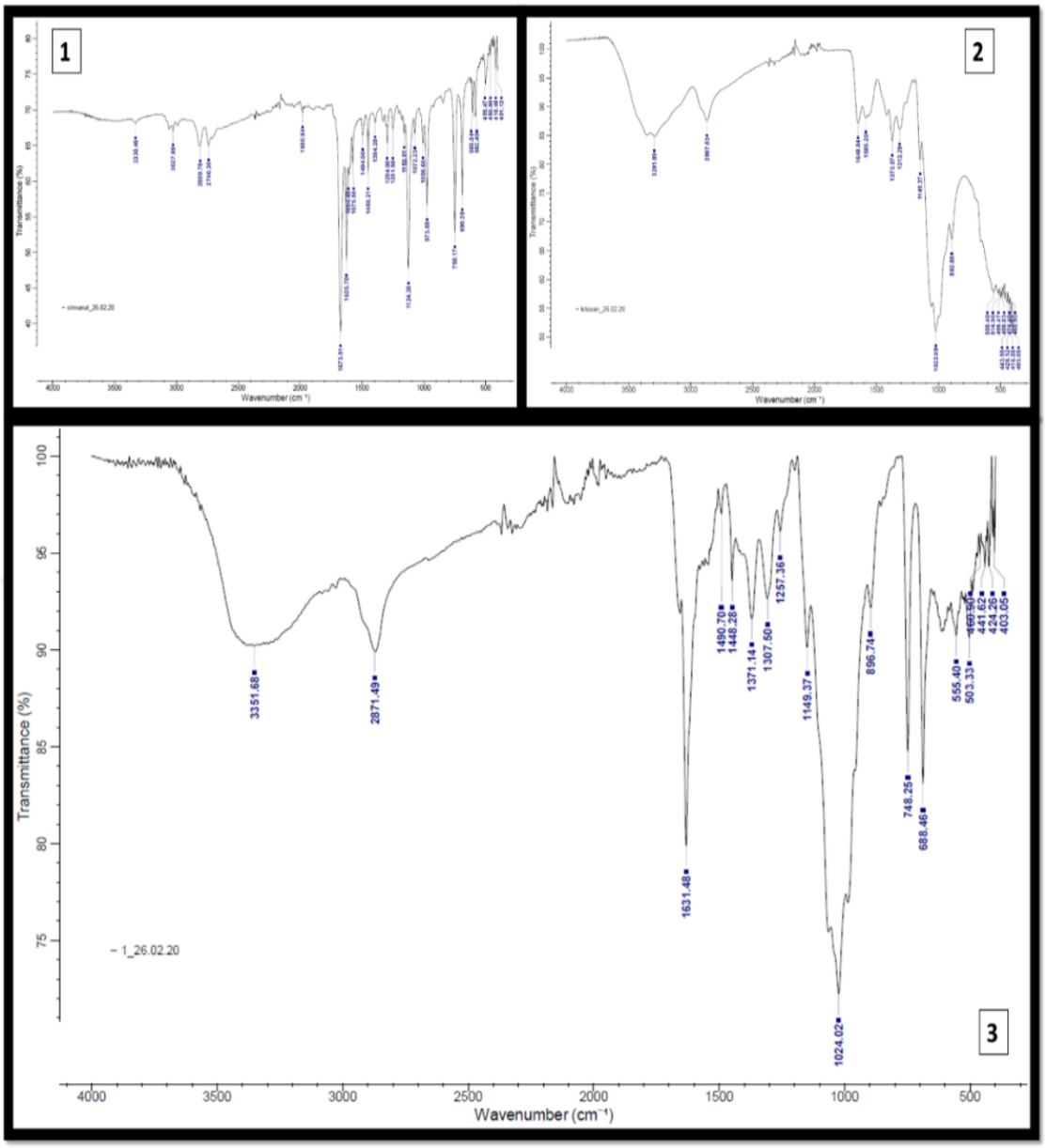


Figure 3.2 FTIR Spectra of cinnamaldehyde (1), chitosan (2) and cinnamyl chitosan (3)

3.1.2 Determination of Free Amine Groups with TNBS Assay

For determination of free primary amine groups, TNBS assay was performed. Optical Density values and statistical analysis are indicated at Table 3.1. Degree of substitution was calculated based on the formula:

$$DS\% = \frac{(\text{CHmean} - \text{CinChmean})}{CHmean} \times 100 \qquad (3.1)$$

Degree of substitution was calculated as 18.09%.

Table 3.1 TNBS assay results

Well ID	Well	OD344	Count	Mean	Std Dev	CV (%)
CH	F10	2,216	9	2,227	0,028	1,268
	G10	2,280				
	H10	2,264				
	F11	2,225				
	G11	2,218				
	H11	2,198				
	F12	2,234				
	G12	2,202				
	H12	2,205				
Cin-Ch	F7	1,812	9	1,824	0,024	1,326
	G7	1,825				
	H7	1,842				
	F8	1,790				
	G8	1,806				
	H8	1,807				
	F9	1,873				
	G9	1,829				
	H9	1,828				
Degree of Substitution (%)			**18.09609**			

3.1.3 Contact Angle Analysis

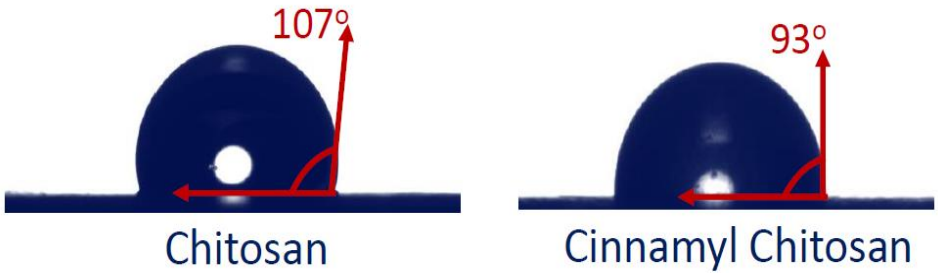


Figure 3.3 Contact angle of chitosan and cinnamyl chitosan on parafilm surface

Depending on the optical tensiometer analysis, the contact angle of chitosan and cinnamyl chitosan was measured as 107° and 93° respectively on the parafilm surface. Chitosan has a greater contact angle, compared to cinnamyl chitosan. The hydrophobicity of cinnamyl chitosan is much more than chitosan due to the conjugation of hydrophobic molecules onto polymer chains.

3.2 Characterization of Microcapsules

3.2.1 Optimization of Microencapsulation

For the optimization of microencapsulation procedure, 5 different experiments have been performed with same chitosan-TPP volumes and concentrations. In all experiments, 25 ml of 1% chitosan in 2% lactic acid solution and 0.5% TPP (pH:3) were used. Optimization studies were only involved in the surfactant (Tween80) concentration and active ingredient solutions. Encapsulation efficiency was determined via analyzing supernatant after the centrifugation of the microcapsules via using the prepared standard curves of curcumin and quercetin. Standard curves have been shown in Figure 3.4.

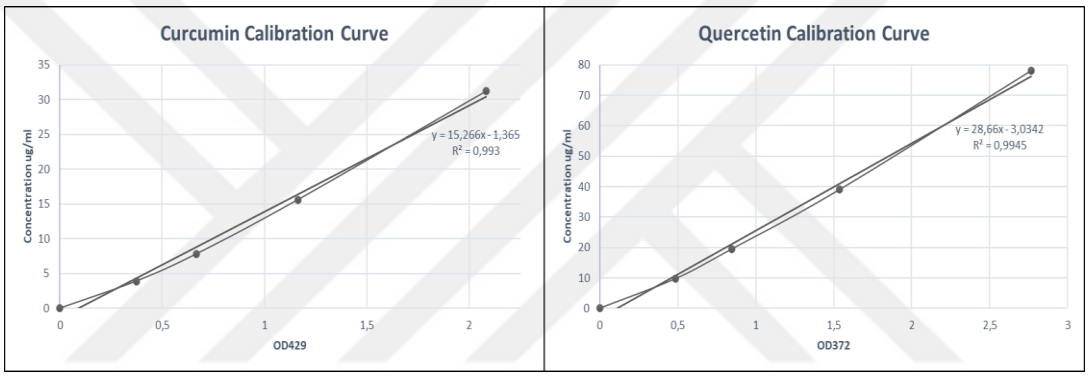


Figure 3.4 Calibration curves of curcumin and quercetin

Microencapsulation optimization trials have been shown in Table 3.2. Optimized microencapsulation method recipe is indicated below:

- 25 ml of 1% Chitosan or Cinnamyl Chitosan in 2% lactic acid solution.
- 25 ml of 0.5% TPP solution (pH:3)
- 250 mg of active ingredient dissolved in 5 ml of DMSO.

Compared to the other experiments, encapsulation efficiency was observed to be higher than 98%. In the first trials, EtOH was used as a solvent to dissolve less active ingredients. When ethanol used as a solvent, encapsulation efficiency was observed to be less than 57%. The solubility of quercetin in DMSO is much more higher than ethanol and its solubility in aqueous solutions is significantly affected [150]. Also, the aprotic nature of DMSO might be a key role in increasing the miscibility of curcumin and quercetin in chitosan and cinnamyl chitosan solution without any

surfactant. Tween 80 concentration was another parameter for the optimization, however, a significant effect of surfactants did not observe on the encapsulation efficiency. Loading efficiencies of Cin-Ch microcapsules were observed to be much more higher than chitosan microcapsules, which might be dependent on the increased hydrophobicity of cinnamyl chitosan.

Table 3.2 Microencapsulation trials and comparison

	Concentration of Tween80 (%)	Active Ingredient Added (mg)	Encapsulation Efficiency (%)	Loading Capacity (%)
ChCur v1 w/EtOH	0%	20	53.447776	-
ChCur v2 w/EtOH	1%	20	56.01604	-
ChCur v3 w/DMSO	1%	100	83.7131856	25.91739492
ChCur v4 w/DMSO	0%	100	97.949336	27.98552457
ChCur v5 w/DMSO	0%	250	99.9392248	43.07725207
ChQuer v1 w/EtOH	0%	20	30.58792	-
ChQuer v2 w/EtOH	1%	20	31.03652	-
ChQuer v3 w/DMSO	1%	100	90.368736	19.90500793
ChQuer v4 w/DMSO	0%	100	91.0272736	29.99425302
ChQuer v5 w/DMSO	0%	250	98.41907296	38.38497385
Cin-Ch Cur	0%	250	99.99386056	64.76286306
Cin-Ch Quer	0%	250	99.85115584	57.25410312

3.2.2 Release Studies

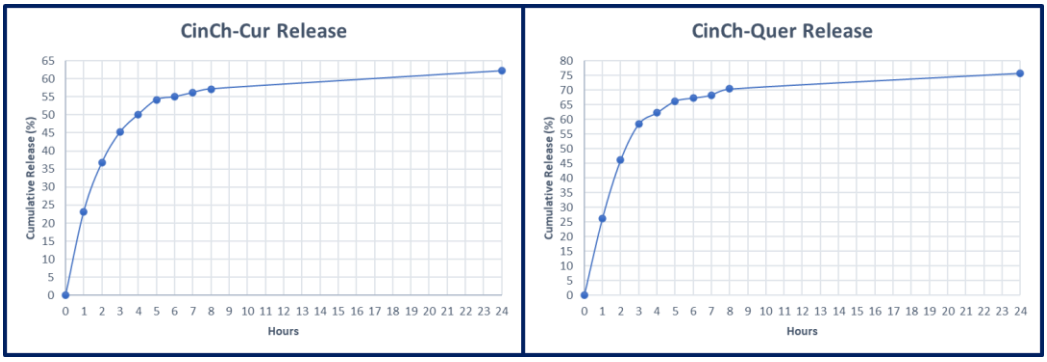


Figure 3.5 Cumulative release profile of Cin-Ch microcapsules within 24 hours

The cumulative release of Cin-Ch microcapsules has been shown in Figure 3.5. Release profiles were indicated as biphasic, which are burst release followed by a slower sustained release. For Cin-Ch-Cur microcapsules, burst release of curcumin was observed within the 5 hours. After 55% of curcumin released, sustained release occurs within the 24 hours. For Cin-Ch-Quer microcapsules, burst release of quercetin was observed within the first 3 hours. After 60% of quercetin released into media, slower release was observed.

Drug release from microparticles is mainly depending on several mechanisms including surface erosion, disintegration, diffusion and desorption [151]. The burst release of the microcapsules may be associated with the active ingredient molecules entrapped in the near of the surface of the polymer [148, 152, 153].

3.2.3 Particle Size and Zeta Potential Analysis

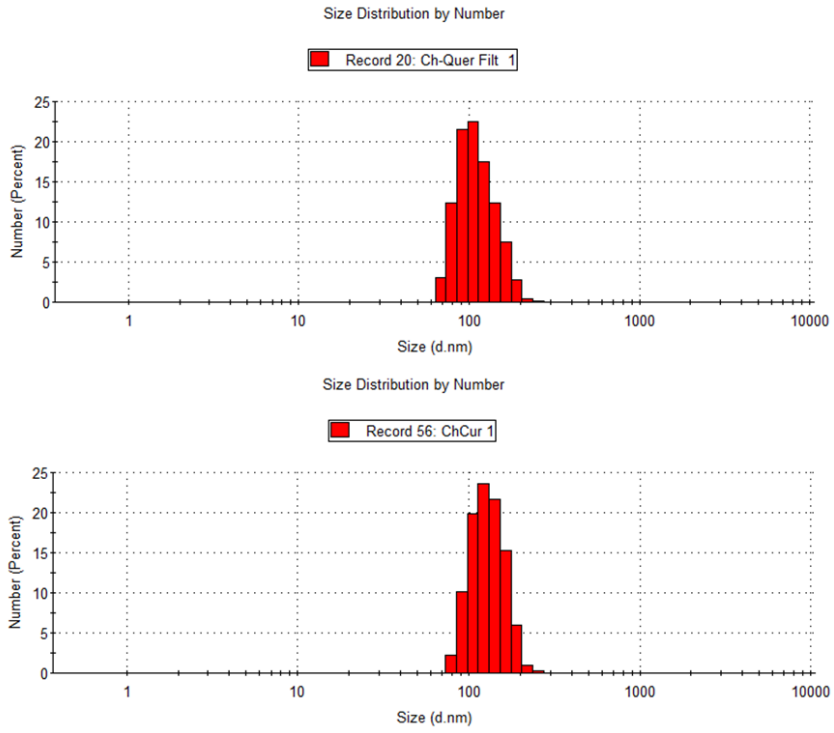


Figure 3.6 DLS measurements of Ch-Cur and Ch-Quer

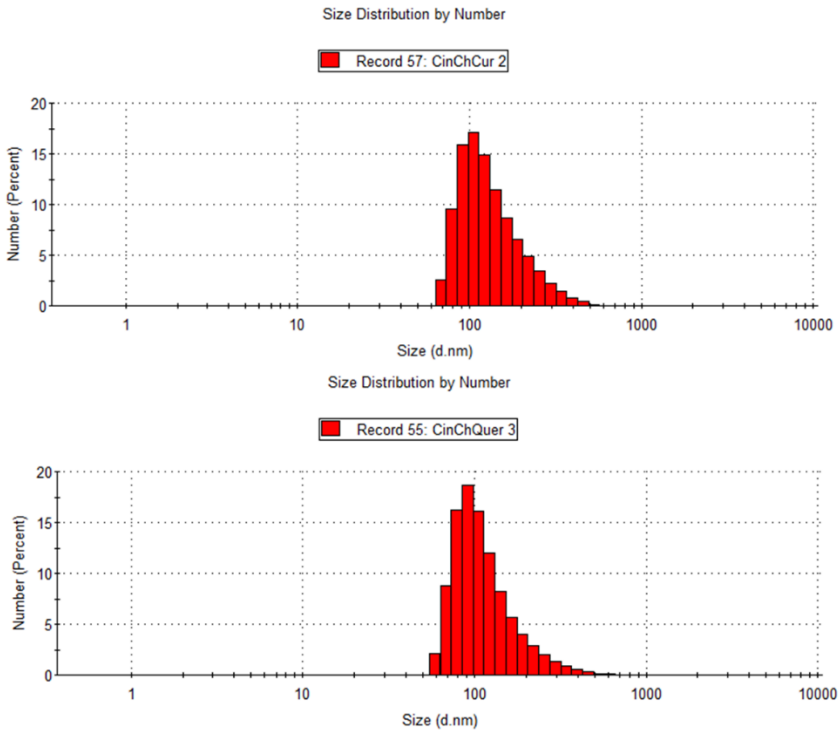


Figure 3.7 DLS measurements of CinCh-Cur and CinCh-Quer

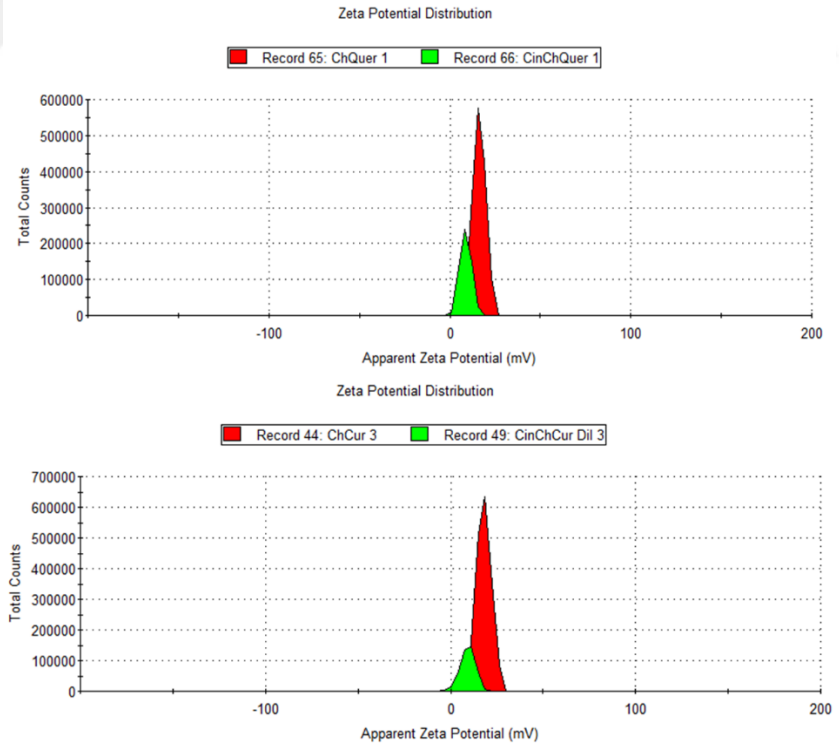


Figure 3.8 Zeta potential analysis of microparticles

DLS measurements have been performed at 25^{o}C. Ch-Cur microcapsules have 204 nm average diameter with 0.337 PDI. Ch-Quer microcapsules have average diameter of 182.1 nm with 0.276 PDI. CinCh-Cur microcapsules' average diameter was determined to be 200.2 nm with 0.240 PDI and CinCh-Quer microcapsules are 197.3 nm with 0.250 PDI. Zeta potentials of chitosan microparticles are determined to be 18.1 mV and 16.4 mV for ChCur and ChQuer, respectively. Cinnamyl chitosan microcapsules Zeta potentials were found to be 9.25 mV and 8.76 mV for CinCh-Cur and CinCh-Quer.

Chitosan's zeta potential is bigger than 30 mV and its ionic interaction may result as microparticles with decreased zeta potential. Amino groups are responsible from the cationic nature of chitosan and its positive zeta potential. These results indicate the decrease of zeta potential of chitosan via TPP interactions. Furthermore, cinnamyl chitosan microcapsules were found to be more decreased zeta potential compared to the chitosan microcapsules because of the cinnamyl groups linked to the amino groups of the chitosan.

3.2.4 FTIR Analysis of the Microcapsules

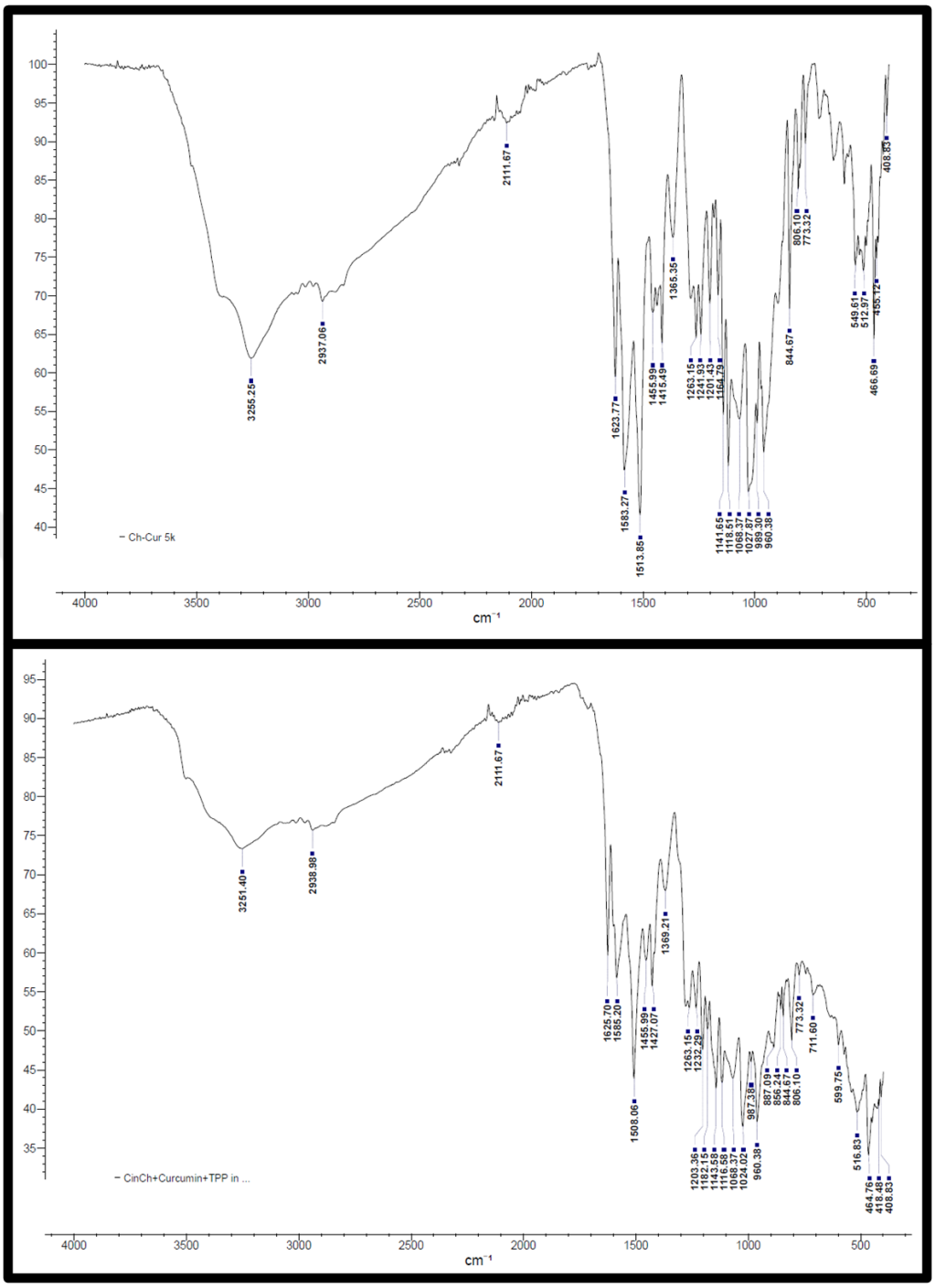


Figure 3.9 FTIR spectra of Ch-Cur (up) and CinCh-Cur (bottom) microcapsules

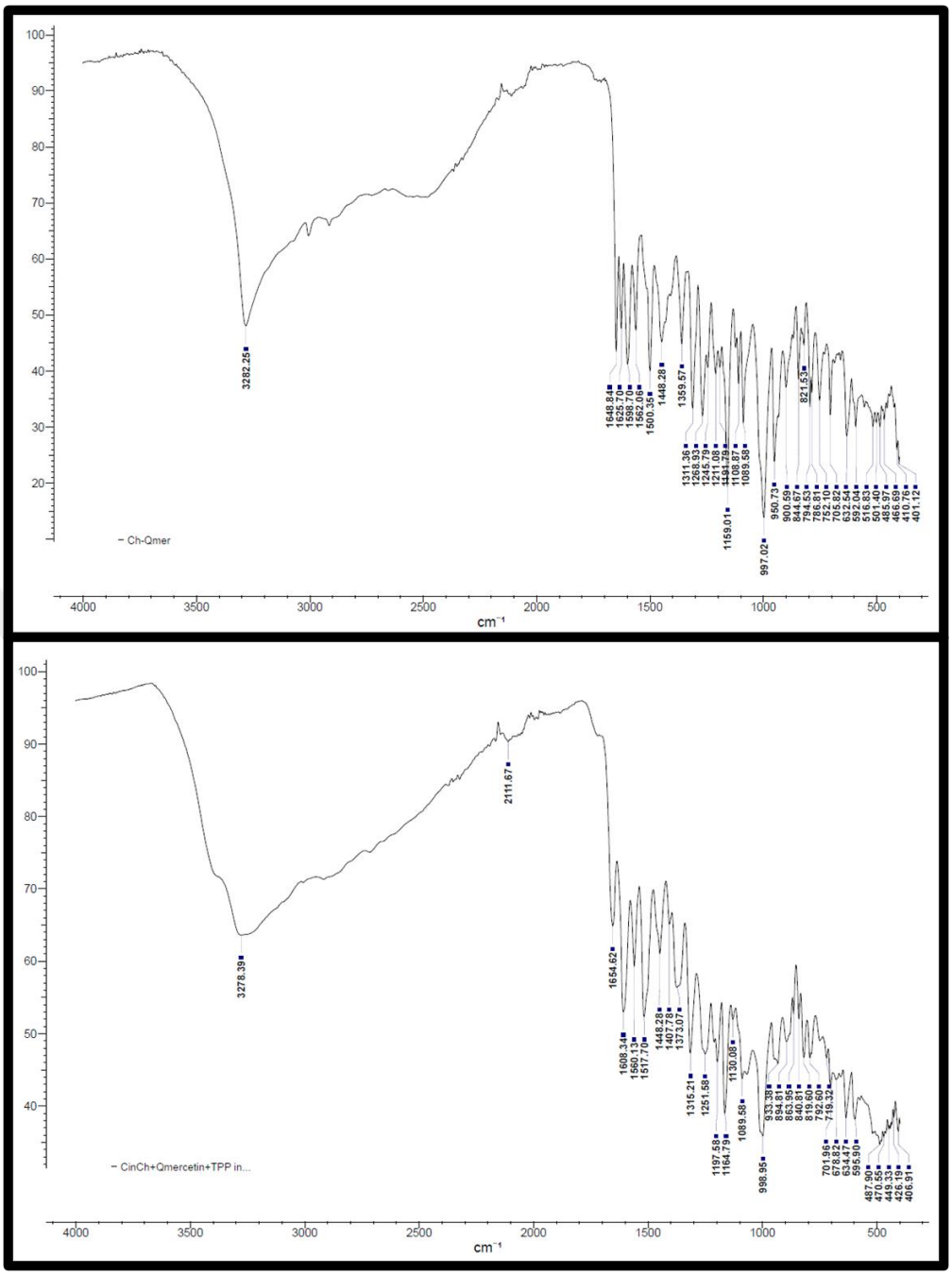


Figure 3.10 FTIR spectra of Ch-Quer (up) and CinCh-Quer (bottom) microcapsules

FTIR spectra of microcapsules was carried out to evaluate the difference of capsule materials and their possible interactions with the loaded molecules. The characteristic -NH_2 band of chitosan was observed to be broader in both of cinnamyl chitosan microcapsules compared to the chitosan microcapsules. Curcumin and quercetin have sharp absorption bands around 1650-1600 cm^{-1} due to the benzene

ring stretches [58, 154]. Both images approve loading of active ingredients by the presence of the main bands of the curcumin and quercetin on the spectrum.

3.3 Antifungal Activity Assays

3.3.1 Antifungal Activity of Cinnamyl Chitosan

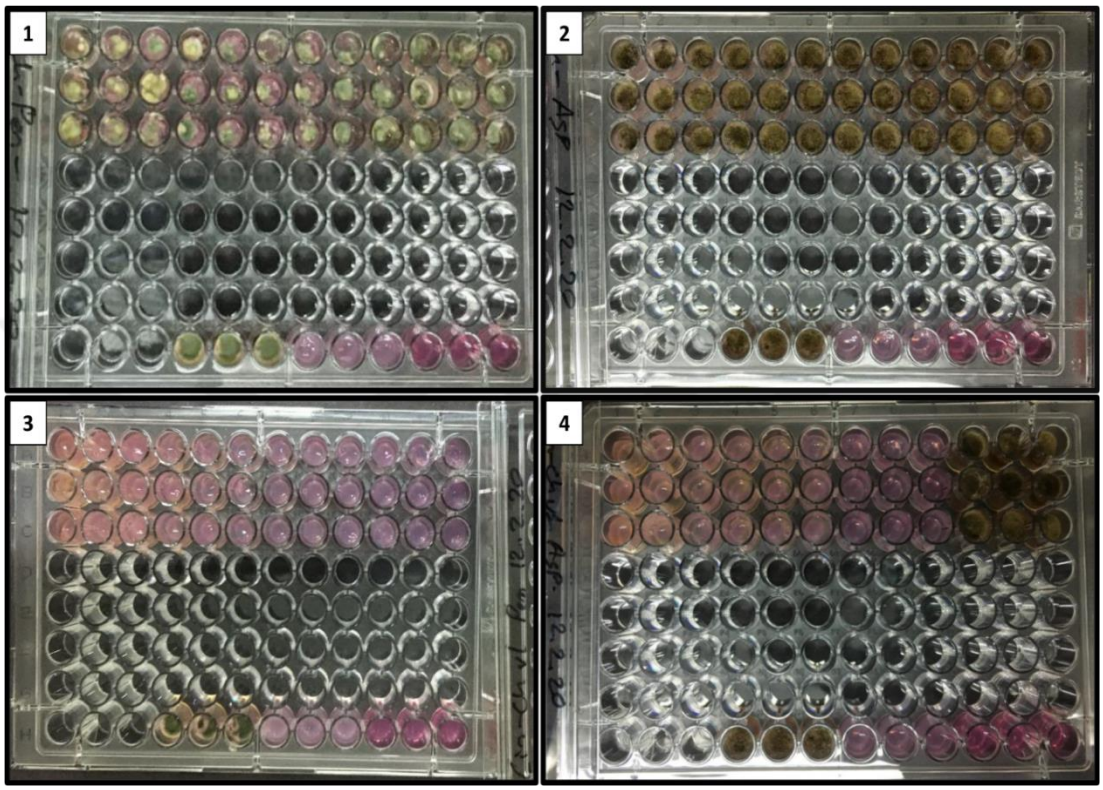


Figure 3.11 Antifungal activity assay of chitosan (1,2) and cinnamyl chitosan (3,4) against *P. digitatum* (1,3) and *A. niger* (2,4)

Chitosan inhibited the sporulation of *P. digitatum* (1) but MIC dose not observed. It also did not show any antifungal activity against *A. niger* (2). Cinnamyl chitosan has shown full inhibition against *P. digitatum* (3) until 250 ppm. Against *A. niger,* MIC dose of cinnamyl chitosan determined as 750 ppm (4).

3.3.2 Antifungal Activity of Active Ingredients and Microcapsules

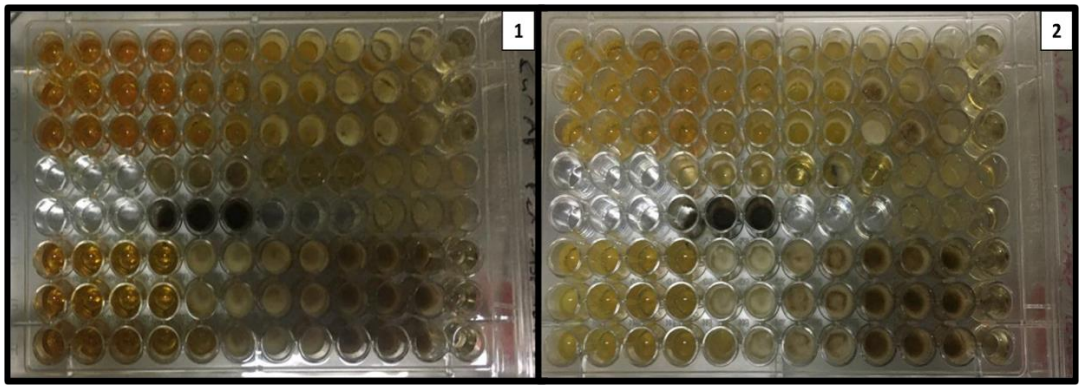


Figure 3.12 Antifungal activity assay of curcumin (1) and quercetin (2) against *P. digitatum* and *A. niger*

Antifungal activity assays of curcumin and quercetin have been performed in YPD media against *P.digitatum* and *A. niger.* MIC doses of curcumin and quercetin against *Aspergillus niger* have been determined as 2500 ppm. MIC doses of curcumin and quercetin against *Penicillium digitatum* have been determined as 1666 ppm.

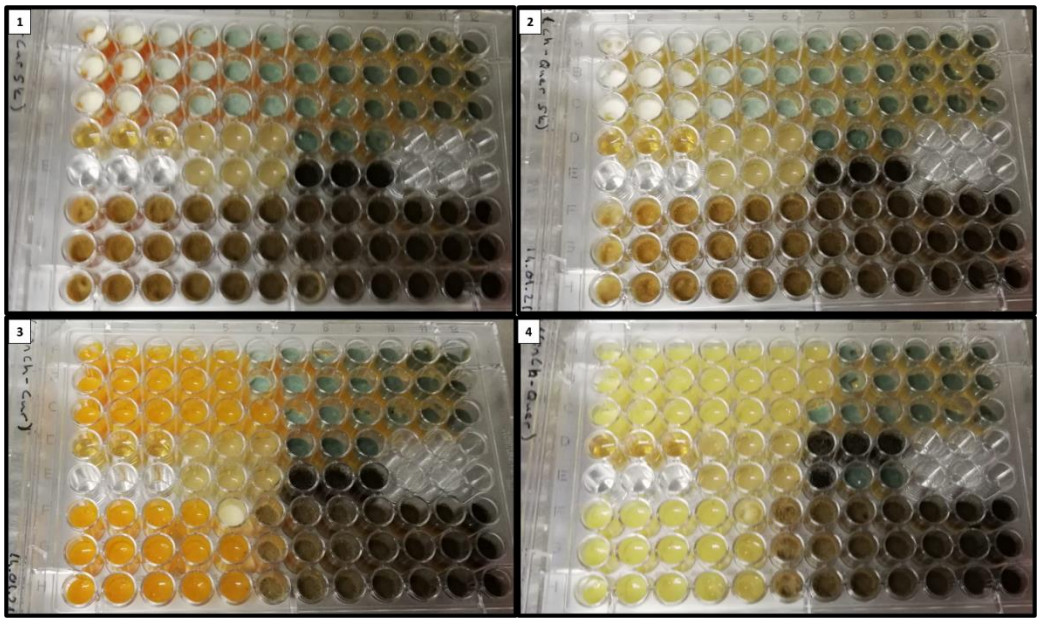


Figure 3.13 Antifungal activity assays of Ch-Cur (1), Ch-Quer (2), Cin-Ch-Cur (3) and Cin-Ch-Quer (4) against *P. digitatum* and *A. niger* in SDA media

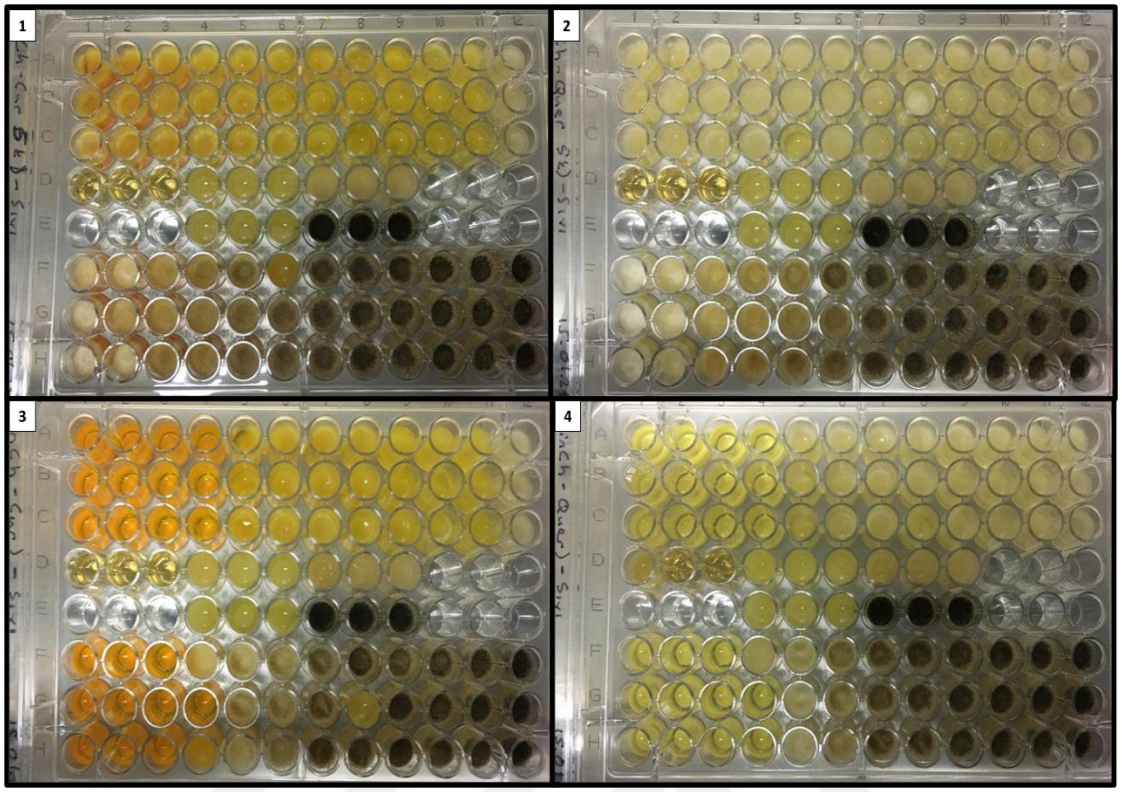


Figure 3.14 Antifungal activity assays of Ch-Cur (1), Ch-Quer (2), Cin-Ch-Cur (3) and Cin-Ch-Quer (4) against *P. digitatum* and *A. niger* in YPD media

In SDA media, the MIC doses of Cin-Ch-Cur and Cin-Ch-Quer microcapsules against *Penicillium digitatum* were determined as 12.5% and 9.375%, respectively. The MIC doses of Cin-Ch-Cur and Cin-Ch-Quer microcapsules against *Aspergillus niger* were determined as 18.75% for both. Sporulation retention observed for the Ch-Cur and Ch-Quer microcapsules, especially against *Penicillium digitatum*, however they were not able to inhibit the growth.

In YPD media, the MIC doses of Cin-Ch-Cur and Cin-Ch-Quer microcapsules against *Penicillium digitatum* were determined as 18.75%. the MIC doses of Cin-Ch-Cur and Cin-Ch-Quer microcapsules against *Aspergillus niger* were determined at 25% concentration. No inhibition occurred for the Ch-Cur and Ch-Quer microcapsules.

3.4 Conclusion

In this thesis, chitosan was modified with cinnamaldehyde via reductive amination mechanism and used as a microencapsulation material to encapsulate curcumin and quercetin. Degree of substitution of modified chitosan was found to be 18% and modification increased antifungal activity of chitosan. MIC doses of cinnamyl chitosan were found to be <250 ppm against *Penicillium digitatum* and 750 ppm against *Aspergillus niger*.

Microencapsulation of curcumin and quercetin was performed with chitosan and cinnamyl chitosan as wall material with high encapsulation efficiency. Release studies indicated the capsules have a biphasic release character; a burst release continue with a sustained release. DLS studies show that the microcapsules obtained with an average diameter between 180-220 nm with low polydispersity index. Also, loading of the active ingredients have been proved with FTIR analysis.

Antifungal assays were performed with active ingredient loaded chitosan and active ingredient loaded cinnamyl chitosan microcapsules within different culture media. Cinnamyl chitosan microcapsules were able to completely inhibit wild-type *Penicillium digitatum* and *Aspergillus niger* species. Chitosan microcapsules were observed to delay sporulation, but inhibition did not occur.

This thesis proves that, cinnamyl chitosan is a promising antifungal agent. It can be used as microencapsulating agent for controlled release of the active ingredients and increasing their antimicrobial activity. Antifungal mode of actions and possible synergistic effect with other antimicrobial molecules should be studied as a future perspective. Antimicrobial activity against different types of microorganisms should be evaluated.

REFERENCES

[1] J. Sebastian, T. Rouissi, and S. K. Brar, "Fungal chitosan: prospects and challenges," *Handbook of Chitin and Chitosan: Volume 1: Preparation and Properties,* p. 22, 2020.

[2] J. P. Martínez, M. P. Falomir, and D. Gozalbo, "Chitin: a structural biopolysaccharide with multiple applications," *eLS,* 2014.

[3] K. A. Rieger, N. M. Eagan, and J. D. Schiffman, "Encapsulation of cinnamaldehyde into nanostructured chitosan films," *Journal of Applied Polymer Science,* vol. 132, 2015.

[4] S. Ogawa, E. A. Decker, and D. J. McClements, "Production and characterization of o/w emulsions containing droplets stabilized by lecithin–chitosan– pectin mutilayered membranes," *Journal of Agricultural and Food Chemistry,* vol. 52, pp. 3595-3600, 2004.

[5] J. Hu, X. Wang, Z. Xiao, and W. Bi, "Effect of chitosan nanoparticles loaded with cinnamon essential oil on the quality of chilled pork," *LWT-Food Science and Technology,* vol. 63, pp. 519-526, 2015.

[6] F. Lopez-Moya, M. Suarez-Fernandez, and L. V. Lopez-Llorca, "Molecular mechanisms of chitosan interactions with fungi and plants," *International journal of molecular sciences,* vol. 20, p. 332, 2019.

[7] M. Rodríguez-Vázquez, B. Vega-Ruiz, R. Ramos-Zúñiga, D. A. Saldaña-Koppel, and L. F. Quiñones-Olvera, "Chitosan and Its Potential Use as a Scaffold for Tissue Engineering in Regenerative Medicine," *BioMed Research International,* vol. 2015, p. 821279, 2015/10/04 2015.

[8] A. R. Pandey, U. S. Singh, M. Momin, and C. Bhavsar, "Chitosan: Application in tissue engineering and skin grafting," *Journal of Polymer Research,* vol. 24, p. 125, 2017.

[9] Y. Zhu, Y. Liu, and Z. Pang, "Chitosan in drug delivery applications," in *Natural Polysaccharides in Drug Delivery and Biomedical Applications*, ed: Elsevier, 2019, pp. 101-119.

[10] X. Zhang, L. Li, and S. Mao, "Drug Delivery Applications of Chitosan and its Derivatives," in *Excipient Applications in Formulation Design and Drug Delivery*, A. S. Narang and S. H. S. Boddu, Eds., ed Cham: Springer International Publishing, 2015, pp. 637-678.

[11] M. Vakili, M. Rafatullah, B. Salamatinia, A. Z. Abdullah, M. H. Ibrahim, K. B. Tan*, et al.*, "Application of chitosan and its derivatives as adsorbents for dye removal from water and wastewater: A review," *Carbohydrate polymers,* vol. 113, pp. 115-130, 2014.

[12] N. A. S. Rozman, W. Y. Tong, C. R. Leong, W. N. Tan, M. A. Hasanolbasori, and S. Z. Abdullah, "Potential antimicrobial applications of chitosan nanoparticles (ChNP)," 2019.

[13] D. Raafat and H. G. Sahl, "Chitosan and its antimicrobial potential–a critical literature survey," *Microbial biotechnology,* vol. 2, pp. 186-201, 2009.

[14] E. I. Rabea, M. E.-T. Badawy, C. V. Stevens, G. Smagghe, and W. Steurbaut, "Chitosan as antimicrobial agent: applications and mode of action," *Biomacromolecules,* vol. 4, pp. 1457-1465, 2003.
[15] N. Liu, X.-G. Chen, H.-J. Park, C.-G. Liu, C.-S. Liu, X.-H. Meng*, et al.*, "Effect of MW and concentration of chitosan on antibacterial activity of Escherichia coli," *Carbohydrate polymers,* vol. 64, pp. 60-65, 2006.
[16] M. Kong, X. G. Chen, K. Xing, and H. J. Park, "Antimicrobial properties of chitosan and mode of action: a state of the art review," *International journal of food microbiology,* vol. 144, pp. 51-63, 2010.
[17] H. K. No, N. Y. Park, S. H. Lee, and S. P. Meyers, "Antibacterial activity of chitosans and chitosan oligomers with different molecular weights," *International journal of food microbiology,* vol. 74, pp. 65-72, 2002.
[18] T. Hongpattarakere and O. Riyaphan, "Effect of deacetylation conditions on antimicrobial activity of chitosans prepared from carapace of black tiger shrimp," *Songklanakarin Journal of Science & Technology,* vol. 30, 2008.
[19] G. Tsai, W.-H. Su, H.-C. Chen, and C.-L. Pan, "Antimicrobial activity of shrimp chitin and chitosan from different treatments," *Fisheries science,* vol. 68, pp. 170-177, 2002.
[20] X. Fei Liu, Y. Lin Guan, D. Zhi Yang, Z. Li, and K. De Yao, "Antibacterial action of chitosan and carboxymethylated chitosan," *Journal of applied polymer science,* vol. 79, pp. 1324-1335, 2001.
[21] A. Balicka-Ramisz, A. Wojtasz-Pajak, B. Pilarczyk, A. Ramisz, and L. Laurans, "Antibacterial and antifungal activity of chitosan," *ISAH,* vol. 2, pp. 406-408, 2005.
[22] S. Roller and N. Covill, "The antimicrobial properties of chitosan in mayonnaise and mayonnaise-based shrimp salads," *Journal of Food Protection,* vol. 63, pp. 202-209, 2000.
[23] K. Kurita, "Chitin and chitosan: functional biopolymers from marine crustaceans," *Marine Biotechnology,* vol. 8, pp. 203-226, 2006.
[24] Y. Omura, M. Shigemoto, T. Akiyama, H. Saimoto, Y. Shigemasa, I. Nakamura*, et al.*, "Antimicrobial activity of chitosan with different degrees of acetylation and molecular weights," *Biocontrol Science,* vol. 8, pp. 25-30, 2003.
[25] R. C. Goy, D. d. Britto, and O. B. Assis, "A review of the antimicrobial activity of chitosan," *Polímeros,* vol. 19, pp. 241-247, 2009.
[26] P. Sahariah and M. Masson, "Antimicrobial chitosan and chitosan derivatives: a review of the structure–activity relationship," *Biomacromolecules,* vol. 18, pp. 3846-3868, 2017.
[27] P. Venkatraman, "Chitosan: An Antimicrobial Polymer," *Journal of Undergraduate Materials Research,* vol. 5, 2015.
[28] V. Mourya and N. N. Inamdar, "Chitosan-modifications and applications: opportunities galore," *Reactive and Functional polymers,* vol. 68, pp. 1013-1051, 2008.
[29] E. I. Rabea, M. E. Badawy, W. Steurbaut, and C. V. Stevens, "In vitro assessment of N-(benzyl) chitosan derivatives against some plant pathogenic bacteria and fungi," *European Polymer Journal,* vol. 45, pp. 237-245, 2009.
[30] A. Verlee, S. Mincke, and C. V. Stevens, "Recent developments in antibacterial and antifungal chitosan and its derivatives," *Carbohydrate Polymers,* vol. 164, pp. 268-283, 2017.

[31] N. F. Kamaruzzaman, L. P. Tan, R. H. Hamdan, S. S. Choong, W. K. Wong, A. J. Gibson*, et al.*, "Antimicrobial polymers: The potential replacement of existing antibiotics?," *International journal of molecular sciences,* vol. 20, p. 2747, 2019.

[32] M. E. Badawy, "Structure and antimicrobial activity relationship of quaternary N-alkyl chitosan derivatives against some plant pathogens," *Journal of applied polymer science,* vol. 117, pp. 960-969, 2010.

[33] M. E. Badawy, E. I. Rabea, T. M. Rogge, C. V. Stevens, G. Smagghe, W. Steurbaut*, et al.*, "Synthesis and fungicidal activity of new N, O-acyl chitosan derivatives," *Biomacromolecules,* vol. 5, pp. 589-595, 2004.

[34] M. E. Badawy, E. I. Rabea, T. M. Rogge, C. V. Stevens, W. Steurbaut, M. Höfte*, et al.*, "Fungicidal and insecticidal activity of O-acyl chitosan derivatives," *Polymer bulletin,* vol. 54, pp. 279-289, 2005.

[35] E. I. RabEa and W. StEuRbaut, "Chemically modified chitosans as antimicrobial agents against some plant pathogenic bacteria and fungi," *Plant Protection Science,* vol. 46, pp. 149-158, 2010.

[36] X. Jin, J. Wang, and J. Bai, "Synthesis and antimicrobial activity of the Schiff base from chitosan and citral," *Carbohydrate research,* vol. 344, pp. 825-829, 2009.

[37] S. Zhaveh, A. Mohsenifar, M. Beiki, S. T. Khalili, A. Abdollahi, T. Rahmani-Cherati*, et al.*, "Encapsulation of Cuminum cyminum essential oils in chitosan-caffeic acid nanogel with enhanced antimicrobial activity against Aspergillus flavus," *Industrial Crops and Products,* vol. 69, pp. 251-256, 2015.

[38] C. V. Pardeshi and V. S. Belgamwar, "Controlled synthesis of N, N, N-trimethyl chitosan for modulated bioadhesion and nasal membrane permeability," *International journal of biological macromolecules,* vol. 82, pp. 933-944, 2016.

[39] W. Sajomsang, S. Tantayanon, V. Tangpasuthadol, M. Thatte, and W. H. Daly, "Synthesis and characterization of N-aryl chitosan derivatives," *International journal of biological macromolecules,* vol. 43, pp. 79-87, 2008.

[40] W. Sajomsang, "Synthetic methods and applications of chitosan containing pyridylmethyl moiety and its quaternized derivatives: A review," *Carbohydrate Polymers,* vol. 80, pp. 631-647, 2010.

[41] M. E. Badawy and E. I. Rabea, "Synthesis and structure–activity relationship of N-(cinnamyl) chitosan analogs as antimicrobial agents," *International journal of biological macromolecules,* vol. 57, pp. 185-192, 2013.

[42] Y. Kurita and A. Isogai, "N-Alkylations of chitosan promoted with sodium hydrogen carbonate under aqueous conditions," *International journal of biological macromolecules,* vol. 50, pp. 741-746, 2012.

[43] Q. Wang, J. Chen, K. Huang, X. Zhang, L. Xu, and Z.-g. Shi, "Preparation, characterization and application of N-methylene phosphonic acid chitosan grafted magnesia–zirconia stationary phase," *Analytica chimica acta,* vol. 854, pp. 191-201, 2015.

[44] X. Yang, C. Zhang, C. Qiao, X. Mu, T. Li, J. Xu*, et al.*, "A simple and convenient method to synthesize N-[(2-hydroxyl)-propyl-3-trimethylammonium] chitosan chloride in an ionic liquid," *Carbohydrate polymers,* vol. 130, pp. 325-332, 2015.

[45] A. O. Aytekin, S. Morimura, and K. Kida, "Synthesis of chitosan–caffeic acid derivatives and evaluation of their antioxidant activities," *Journal of bioscience and bioengineering,* vol. 111, pp. 212-216, 2011.
[46] T. J. Madera-Santana, C. H. Herrera-Méndez, and J. R. Rodríguez-Núñez, "An overview of the chemical modifications of chitosan and their advantages," *Green Materials,* vol. 6, pp. 131-142, 2018.
[47] I. Hamed, F. Özogul, and J. M. Regenstein, "Industrial applications of crustacean by-products (chitin, chitosan, and chitooligosaccharides): A review," *Trends in Food Science & Technology,* vol. 48, pp. 40-50, 2016/02/01/ 2016.
[48] A. Li, Q. Xue, Y. Ye, P. Gong, M. Deng, and B. Jiang, "Study on TEMPO-Mediated Oxidation of N-Succinyl Chitosan and the Water Retention Property," *Molecules,* vol. 25, p. 4698, 2020.
[49] K. Li, G. Guan, J. Zhu, H. Wu, and Q. Sun, "Antibacterial activity and mechanism of a laccase-catalyzed chitosan–gallic acid derivative against Escherichia coli and Staphylococcus aureus," *Food Control,* vol. 96, pp. 234-243, 2019.
[50] C. G. Boeriu and L. A. van den Broek, "Chemical and Enzymatic Modification of Chitosan to Produce New Functional Materials with Improved Properties," *Chitin and Chitosan: Properties and Applications,* pp. 245-258, 2019.
[51] A. M. Bakowska-Barczak and P. P. Kolodziejczyk, "Black currant polyphenols: Their storage stability and microencapsulation," *Industrial crops and products,* vol. 34, pp. 1301-1309, 2011.
[52] M. M. Cowan, "Plant products as antimicrobial agents," *Clinical microbiology reviews,* vol. 12, pp. 564-582, 1999.
[53] R. M. De Conti Lourenço, P. da Silva Melo, and A. B. A. de Almeida, "Flavonoids as Antifungal Agents," in *Antifungal Metabolites from Plants*, M. Razzaghi-Abyaneh and M. Rai, Eds., ed Berlin, Heidelberg: Springer Berlin Heidelberg, 2013, pp. 283-300.
[54] N. F. Santos-Sánchez, R. Salas-Coronado, B. Hernández-Carlos, and C. Villanueva-Cañongo, "Shikimic acid pathway in biosynthesis of phenolic compounds," in *Plant Physiological Aspects of Phenolic Compounds*, ed: IntechOpen, 2019.
[55] A. Mouradov and G. Spangenberg, "Flavonoids: a metabolic network mediating plants adaptation to their real estate," *Frontiers in plant science,* vol. 5, p. 620, 2014.
[56] R. S. Santhosh and B. Suriyanarayanan, "Plants: a source for new antimycobacterial drugs," *Planta medica,* vol. 80, pp. 9-21, 2014.
[57] L. Panzella, "Natural Phenolic Compounds for Health, Food and Cosmetic Applications," ed: Multidisciplinary Digital Publishing Institute, 2020.
[58] J. Rashedi, A. G. Haghjo, M. M. Abbasi, A. D. Tabrizi, S. Yaqoubi, D. Sanajou*, et al.*, "Anti-tumor Effect of Quercetin Loaded Chitosan Nanoparticles on Induced Colon Cancer in Wistar Rats," *Advanced pharmaceutical bulletin,* vol. 9, p. 409, 2019.
[59] P. Wang, C. Su, H. Feng, X. Chen, Y. Dong, Y. Rao*, et al.*, "Curcumin regulates insulin pathways and glucose metabolism in the brains of APPswe/PS1dE9 mice," *International Journal of Immunopathology and Pharmacology,* vol. 30, pp. 25-43, 2017.

[60] K. Bisht, K.-H. Wagner, and A. C. Bulmer, "Curcumin, resveratrol and flavonoids as anti-inflammatory, cyto-and DNA-protective dietary compounds," *Toxicology,* vol. 278, pp. 88-100, 2010.
[61] Z. Abdulwanis Mohamed, E. Mohamed Eliaser, E. Mazzon, P. Rollin, G. Cheng Lian Ee, and A. F. Abdull Razis, "Neuroprotective potential of secondary metabolites from melicope lunu-ankenda (rutaceae)," *Molecules,* vol. 24, p. 3109, 2019.
[62] H. Liu, C. Wang, Z. Qiao, and Y. Xu, "Protective effect of curcumin against myocardium injury in ischemia reperfusion rats," *Pharmaceutical biology,* vol. 55, pp. 1144-1148, 2017.
[63] Y. Cai, D. Lu, Y. Zou, C. Zhou, H. Liu, C. Tu*, et al.*, "Curcumin protects against intestinal origin endotoxemia in rat liver cirrhosis by targeting PCSK9," *Journal of food science,* vol. 82, pp. 772-780, 2017.
[64] M. Mulat, F. Khan, G. Muluneh, and A. Pandita, "Phytochemical profile and antimicrobial effects of different medicinal plant: current knowledge and future perspectives," *Current Traditional Medicine,* vol. 6, pp. 24-42, 2020.
[65] A. Pandey and S. Kumar, "Perspective on plant products as antimicrobial agents: A review," *Pharmacologia,* vol. 4, pp. 469-480, 2013.
[66] M. F. G. Rocha, J. A. Sales, M. G. da Rocha, L. M. Galdino, L. de Aguiar, W. d. A. Pereira-Neto*, et al.*, "Antifungal effects of the flavonoids kaempferol and quercetin: a possible alternative for the control of fungal biofilms," *Biofouling,* vol. 35, pp. 320-328, 2019.
[67] M. Daglia, "Polyphenols as antimicrobial agents," *Current opinion in biotechnology,* vol. 23, pp. 174-181, 2012.
[68] G. Brahmachari, *Chemistry and pharmacology of naturally occurring bioactive compounds*: CRC Press, 2013.
[69] E. P. o. F. Additives and N. S. a. t. Food, "Scientific Opinion on the re-evaluation of curcumin (E 100) as a food additive," *EFSA Journal,* vol. 8, p. 1679, 2010.
[70] F. Payton, P. Sandusky, and W. L. Alworth, "NMR study of the solution structure of curcumin," *Journal of natural products,* vol. 70, pp. 143-146, 2007.
[71] M. L. Lestari and G. Indrayanto, "Curcumin," in *Profiles of drug substances, excipients and related methodology*. vol. 39, ed: Elsevier, 2014, pp. 113-204.
[72] K. M. Nelson, J. L. Dahlin, J. Bisson, J. Graham, G. F. Pauli, and M. A. Walters, "The essential medicinal chemistry of curcumin: miniperspective," *Journal of medicinal chemistry,* vol. 60, pp. 1620-1637, 2017.
[73] Y.-J. Wang, M.-H. Pan, A.-L. Cheng, L.-I. Lin, Y.-S. Ho, C.-Y. Hsieh*, et al.*, "Stability of curcumin in buffer solutions and characterization of its degradation products," *Journal of pharmaceutical and biomedical analysis,* vol. 15, pp. 1867-1876, 1997.
[74] M. Griesser, V. Pistis, T. Suzuki, N. Tejera, D. A. Pratt, and C. Schneider, "Autoxidative and cyclooxygenase-2 catalyzed transformation of the dietary chemopreventive agent curcumin," *Journal of Biological Chemistry,* vol. 286, pp. 1114-1124, 2011.
[75] H. H. Tønnesen, J. Karlsen, and G. B. van Henegouwen, "Studies on curcumin and curcuminoids VIII. Photochemical stability of curcumin," *Zeitschrift für Lebensmittel-Untersuchung und Forschung,* vol. 183, pp. 116-122, 1986.

[76] A. Siviero, E. Gallo, V. Maggini, L. Gori, A. Mugelli, F. Firenzuoli*, et al.*, "Curcumin, a golden spice with a low bioavailability," *Journal of Herbal Medicine,* vol. 5, pp. 57-70, 2015.
[77] S. I. Hoehle, E. Pfeiffer, A. M. Sólyom, and M. Metzler, "Metabolism of curcuminoids in tissue slices and subcellular fractions from rat liver," *Journal of agricultural and food chemistry,* vol. 54, pp. 756-764, 2006.
[78] P. Ravindran, K. N. Babu, and K. Sivaraman, *Turmeric: the genus Curcuma*: CRC press, 2007.
[79] A. Rauf, M. Imran, I. E. Orhan, and S. Bawazeer, "Health perspectives of a bioactive compound curcumin: A review," *Trends in food science & technology,* vol. 74, pp. 33-45, 2018.
[80] C. Mohanty and S. K. Sahoo, "Curcumin and its topical formulations for wound healing applications," *Drug Discovery Today,* vol. 22, pp. 1582-1592, 2017.
[81] G. S. Kelly, "Quercetin," *Alternative medicine review,* vol. 16, pp. 172-195, 2011.
[82] A. W. Boots, G. R. Haenen, and A. Bast, "Health effects of quercetin: from antioxidant to nutraceutical," *European journal of pharmacology,* vol. 585, pp. 325-337, 2008.
[83] M. Zhang, S. G. Swarts, L. Yin, C. Liu, Y. Tian, Y. Cao*, et al.*, "Antioxidant properties of quercetin," in *Oxygen transport to tissue XXXII*, ed: Springer, 2011, pp. 283-289.
[84] M. Lesjak, I. Beara, N. Simin, D. Pintać, T. Majkić, K. Bekvalac*, et al.*, "Antioxidant and anti-inflammatory activities of quercetin and its derivatives," *Journal of Functional Foods,* vol. 40, pp. 68-75, 2018.
[85] F. Aghapour, A. A. Moghadamnia, A. Nicolini, S. N. M. Kani, L. Barari, P. Morakabati*, et al.*, "Quercetin conjugated with silica nanoparticles inhibits tumor growth in MCF-7 breast cancer cell lines," *Biochemical and Biophysical Research Communications,* vol. 500, pp. 860-865, 2018/06/12/ 2018.
[86] M. Pfeuffer, A. Auinger, U. Bley, I. Kraus-Stojanowic, C. Laue, P. Winkler*, et al.*, "Effect of quercetin on traits of the metabolic syndrome, endothelial function and inflammation in men with different APOE isoforms," *Nutrition, Metabolism and Cardiovascular Diseases,* vol. 23, pp. 403-409, 2013/05/01/ 2013.
[87] A. de la Lastra, M. J. Martin, and V. Motilva, "Antiulcer and gastroprotective effects of quercetin: a gross and histologic study," *Pharmacology,* vol. 48, pp. 56-62, 1994.
[88] D. Metodiewa, A. K. Jaiswal, N. Cenas, E. Dickancaité, and J. Segura-Aguilar, "Quercetin may act as a cytotoxic prooxidant after its metabolic activation to semiquinone and quinoidal product," *Free radical biology and medicine,* vol. 26, pp. 107-116, 1999.
[89] G.-C. Yen, P.-D. Duh, H.-L. Tsai, and S.-L. Huang, "Pro-oxidative properties of flavonoids in human lymphocytes," *Bioscience, biotechnology, and biochemistry,* vol. 67, pp. 1215-1222, 2003.
[90] A. W. Boots, J. M. Balk, A. Bast, and G. R. Haenen, "The reversibility of the glutathionyl-quercetin adduct spreads oxidized quercetin-induced toxicity," *Biochemical and Biophysical Research Communications,* vol. 338, pp. 923-929, 2005.

[91] I. D. Silva, J. Gaspar, G. G. Da Costa, A. Rodrigues, A. Laires, and J. Rueff, "Chemical features of flavonols affecting their genotoxicity. Potential implications in their use as therapeutical agents," *Chemico-biological interactions,* vol. 124, pp. 29-51, 2000.
[92] D. Utesch, K. Feige, J. Dasenbrock, T. Broschard, M. Harwood, B. Danielewska-Nikiel*, et al.*, "Evaluation of the potential in vivo genotoxicity of quercetin," *Mutation Research/Genetic Toxicology and Environmental Mutagenesis,* vol. 654, pp. 38-44, 2008.
[93] T. Walle, A. M. Browning, L. L. Steed, S. G. Reed, and U. K. Walle, "Flavonoid glucosides are hydrolyzed and thus activated in the oral cavity in humans," *The Journal of nutrition,* vol. 135, pp. 48-52, 2005.
[94] Y. Li, J. Yao, C. Han, J. Yang, M. T. Chaudhry, S. Wang*, et al.*, "Quercetin, inflammation and immunity," *Nutrients,* vol. 8, p. 167, 2016.
[95] M. Harwood, B. Danielewska-Nikiel, J. Borzelleca, G. Flamm, G. Williams, and T. Lines, "A critical review of the data related to the safety of quercetin and lack of evidence of in vivo toxicity, including lack of genotoxic/carcinogenic properties," *Food and chemical toxicology,* vol. 45, pp. 2179-2205, 2007.
[96] R. Gyawali and S. A. Ibrahim, "Natural products as antimicrobial agents," *Food control,* vol. 46, pp. 412-429, 2014.
[97] R. R. M. Paterson and N. Lima, "Filamentous fungal human pathogens from food emphasising Aspergillus, Fusarium and Mucor," *Microorganisms,* vol. 5, p. 44, 2017.
[98] Y.-S. Jin, "Recent advances in natural antifungal flavonoids and their derivatives," *Bioorganic & medicinal chemistry letters,* vol. 29, p. 126589, 2019.
[99] C. Chen, L. Long, F. Zhang, Q. Chen, C. Chen, X. Yu*, et al.*, "Antifungal activity, main active components and mechanism of Curcuma longa extract against Fusarium graminearum," *PloS one,* vol. 13, p. e0194284, 2018.
[100] K. Neelofar, S. Shreaz, B. Rimple, S. Muralidhar, M. Nikhat, and L. A. Khan, "Curcumin as a promising anticandidal of clinical interest," *Canadian Journal of Microbiology,* vol. 57, pp. 204-210, 2011.
[101] N. Khan, S. Shreaz, R. Bhatia, S. I. Ahmad, S. Muralidhar, N. Manzoor*, et al.*, "Anticandidal activity of curcumin and methyl cinnamaldehyde," *Fitoterapia,* vol. 83, pp. 434-440, 2012.
[102] L. N. Dovigo, J. C. Carmello, C. A. de Souza Costa, C. E. Vergani, I. L. Brunetti, V. S. Bagnato*, et al.*, "Curcumin-mediated photodynamic inactivation of Candida albicans in a murine model of oral candidiasis," *Sabouraudia,* vol. 51, pp. 243-251, 2013.
[103] O. A. K. Khalil, O. M. M. de Faria Oliveira, J. C. R. Vellosa, A. U. de Quadros, L. M. Dalposso, T. K. Karam*, et al.*, "Curcumin antifungal and antioxidant activities are increased in the presence of ascorbic acid," *Food Chemistry,* vol. 133, pp. 1001-1005, 2012.
[104] B. A. Temba, M. T. Fletcher, G. P. Fox, J. J. Harvey, and Y. Sultanbawa, "Inactivation of Aspergillus flavus spores by curcumin-mediated photosensitization," *Food control,* vol. 59, pp. 708-713, 2016.
[105] L. Song, F. Zhang, J. Yu, C. Wei, Q. Han, and X. Meng, "Antifungal effect and possible mechanism of curcumin mediated photodynamic technology

against Penicillium expansum," *Postharvest Biology and Technology,* vol. 167, p. 111234, 2020.
[106] Z. Sadeghi-Ghadi, A. Vaezi, F. Ahangarkani, M. Ilkit, P. Ebrahimnejad, and H. Badali, "Potent in vitro activity of curcumin and quercetin co-encapsulated in nanovesicles without hyaluronan against Aspergillus and Candida isolates," *Journal de Mycologie Médicale,* vol. 30, p. 101014, 2020.
[107] F. Al-Asmari, R. Mereddy, and Y. Sultanbawa, "A novel photosensitization treatment for the inactivation of fungal spores and cells mediated by curcumin," *Journal of Photochemistry and Photobiology B: Biology,* vol. 173, pp. 301-306, 2017.
[108] S. K. Shishodia, S. Tiwari, S. Hoda, P. Vijayaraghavan, and J. Shankar, "SEM and qRT-PCR revealed quercetin inhibits morphogenesis of Aspergillus flavus conidia via modulating calcineurin-Crz1 signalling pathway," *Mycology,* vol. 11, pp. 118-125, 2020.
[109] S. Tiwari and J. Shankar, "Integrated proteome and HPLC analysis revealed quercetin-mediated inhibition of aflatoxin B1 biosynthesis in Aspergillus flavus," *3 Biotech,* vol. 8, p. 47, 2018.
[110] F. G. Milanezi, L. M. Meireles, M. M. de Christo Scherer, J. P. de Oliveira, A. R. da Silva, M. L. de Araujo*, et al.*, "Antioxidant, antimicrobial and cytotoxic activities of gold nanoparticles capped with quercetin," *Saudi pharmaceutical journal,* vol. 27, pp. 968-974, 2019.
[111] L. Mendoza, K. Yañez, M. Vivanco, R. Melo, and M. Cotoras, "Characterization of extracts from winery by-products with antifungal activity against Botrytis cinerea," *Industrial crops and products,* vol. 43, pp. 360-364, 2013.
[112] M. S. Kwun and D. G. Lee, "Quercetin-induced yeast apoptosis through mitochondrial dysfunction under the accumulation of magnesium in Candida albicans," *Fungal Biology,* vol. 124, pp. 83-90, 2020.
[113] V. Oliveira, E. Carraro, M. Auler, and N. Khalil, "Quercetin and rutin as potential agents antifungal against Cryptococcus spp," *Brazilian Journal of Biology,* vol. 76, pp. 1029-1034, 2016.
[114] M. Zhang, L. Xu, L. Zhang, Y. Guo, X. Qi, and L. He, "Effects of quercetin on postharvest blue mold control in kiwifruit," *Scientia Horticulturae,* vol. 228, pp. 18-25, 2018.
[115] T. A. Bitencourt, T. T. Komoto, B. G. Massaroto, C. E. S. Miranda, R. O. Beleboni, M. Marins*, et al.*, "Trans-chalcone and quercetin down-regulate fatty acid synthase gene expression and reduce ergosterol content in the human pathogenic dermatophyte Trichophyton rubrum," *BMC complementary and alternative medicine,* vol. 13, pp. 1-6, 2013.
[116] F. Paulo and L. Santos, "Design of experiments for microencapsulation applications: A review," *Materials Science and Engineering: C,* vol. 77, pp. 1327-1340, 2017.
[117] S. S. Jyothi, A. Seethadevi, K. S. Prabha, P. Muthuprasanna, and P. Pavitra, "Microencapsulation: a review," *Int. J. Pharm. Biol. Sci,* vol. 3, pp. 509-531, 2012.
[118] I. J. Joye and D. J. McClements, "Biopolymer-based nanoparticles and microparticles: Fabrication, characterization, and application," *Current Opinion in Colloid & Interface Science,* vol. 19, pp. 417-427, 2014.

[119] A. MaHam, Z. Tang, H. Wu, J. Wang, and Y. Lin, "Protein-based nanomedicine platforms for drug delivery," *Small,* vol. 5, pp. 1706-1721, 2009.
[120] K. Numata and D. L. Kaplan, "Silk-based delivery systems of bioactive molecules," *Advanced Drug Delivery Reviews,* vol. 62, pp. 1497-1508, 2010/12/30/ 2010.
[121] S. Quispe-Condori, M. D. Saldaña, and F. Temelli, "Microencapsulation of flax oil with zein using spray and freeze drying," *LWT-Food Science and Technology,* vol. 44, pp. 1880-1887, 2011.
[122] M.-J. Sáiz-Abajo, C. González-Ferrero, A. Moreno-Ruiz, A. Romo-Hualde, and C. J. González-Navarro, "Thermal protection of β-carotene in re-assembled casein micelles during different processing technologies applied in food industry," *Food chemistry,* vol. 138, pp. 1581-1587, 2013.
[123] C. Barron and J.-Q. He, "Alginate-based microcapsules generated with the coaxial electrospray method for clinical application," *Journal of Biomaterials science, Polymer edition,* vol. 28, pp. 1245-1255, 2017.
[124] W. S. Cheow, T. Y. Kiew, and K. Hadinoto, "Amorphous nanodrugs prepared by complexation with polysaccharides: Carrageenan versus dextran sulfate," *Carbohydrate polymers,* vol. 117, pp. 549-558, 2015.
[125] A. Dafe, H. Etemadi, H. Zarredar, and G. R. Mahdavinia, "Development of novel carboxymethyl cellulose/k-carrageenan blends as an enteric delivery vehicle for probiotic bacteria," *International journal of biological macromolecules,* vol. 97, pp. 299-307, 2017.
[126] H. R. Sharif, H. D. Goff, H. Majeed, M. Shamoon, F. Liu, J. Nsor-Atindana*, et al.*, "Physicochemical properties of β-carotene and eugenol co-encapsulated flax seed oil powders using OSA starches as wall material," *Food hydrocolloids,* vol. 73, pp. 274-283, 2017.
[127] C. G. Hernández-Valencia, A. Román-Guerrero, Á. Aguilar-Santamaría, L. Cira, and K. Shirai, "Cross-linking chitosan into hydroxypropylmethylcellulose for the preparation of neem oil coating for postharvest storage of pitaya (Stenocereus pruinosus)," *Molecules,* vol. 24, p. 219, 2019.
[128] G. B. Martínez-Hernández, M. L. Amodio, and G. Colelli, "Carvacrol-loaded chitosan nanoparticles maintain quality of fresh-cut carrots," *Innovative Food Science & Emerging Technologies,* vol. 41, pp. 56-63, 2017.
[129] T. Panagiotou and R. J. Fisher, "Producing micron-and nano-size formulations for functional foods applications," *Functional Foods in Health and Disease,* vol. 3, pp. 274-289, 2013.
[130] I. Norton, D. Jarvis, and T. Foster, "A molecular model for the formation and properties of fluid gels," *International Journal of Biological Macromolecules,* vol. 26, pp. 255-261, 1999.
[131] L. Keawchaoon and R. Yoksan, "Preparation, characterization and in vitro release study of carvacrol-loaded chitosan nanoparticles," *Colloids and surfaces B: Biointerfaces,* vol. 84, pp. 163-171, 2011.
[132] K. Janes, P. Calvo, and M. Alonso, "Polysaccharide colloidal particles as delivery systems for macromolecules," *Advanced drug delivery reviews,* vol. 47, pp. 83-97, 2001.
[133] S. Sreekumar, F. M. Goycoolea, B. M. Moerschbacher, and G. R. Rivera-Rodriguez, "Parameters influencing the size of chitosan-TPP nano-and microparticles," *Scientific reports,* vol. 8, pp. 1-11, 2018.

[134] S. Kunjachan, S. Jose, and T. Lammers, "Understanding the mechanism of ionic gelation for synthesis of chitosan nanoparticles using qualitative techniques," *Asian Journal of Pharmaceutics (AJP): Free full text articles from Asian J Pharm,* vol. 4, 2014.
[135] J. Jacob, J. T. Haponiuk, S. Thomas, and S. Gopi, "Biopolymer based nanomaterials in drug delivery systems: A review," *Materials today chemistry,* vol. 9, pp. 43-55, 2018.
[136] Z. Ma, A. Garrido-Maestu, and K. C. Jeong, "Application, mode of action, and in vivo activity of chitosan and its micro-and nanoparticles as antimicrobial agents: A review," *Carbohydrate polymers,* vol. 176, pp. 257-265, 2017.
[137] A. L. Parize, H. K. Stulzer, M. C. M. Laranjeira, I. M. d. C. Brighente, and T. C. R. d. Souza, "Evaluation of chitosan microparticles containing curcumin and crosslinked with sodium tripolyphosphate produced by spray drying," *Química Nova,* vol. 35, pp. 1127-1132, 2012.
[138] R. S. Nair, A. Morris, N. Billa, and C.-O. Leong, "An evaluation of curcumin-encapsulated chitosan nanoparticles for transdermal delivery," *AAPS PharmSciTech,* vol. 20, p. 69, 2019.
[139] B. R. Shah, Y. Li, W. Jin, Y. An, L. He, Z. Li*, et al.*, "Preparation and optimization of Pickering emulsion stabilized by chitosan-tripolyphosphate nanoparticles for curcumin encapsulation," *Food Hydrocolloids,* vol. 52, pp. 369-377, 2016.
[140] A. V. Samrot, U. Burman, S. A. Philip, N. Shobana, and K. Chandrasekaran, "Synthesis of curcumin loaded polymeric nanoparticles from crab shell derived chitosan for drug delivery," *Informatics in Medicine Unlocked,* vol. 10, pp. 159-182, 2018.
[141] D. K. Chellappan, N. J. Yee, B. J. Kaur Ambar Jeet Singh, J. Panneerselvam, T. Madheswaran, J. Chellian*, et al.*, "Formulation and characterization of glibenclamide and quercetin-loaded chitosan nanogels targeting skin permeation," *Therapeutic delivery,* vol. 10, pp. 281-293, 2019.
[142] Y. Zhang, Y. Yang, K. Tang, X. Hu, and G. Zou, "Physicochemical characterization and antioxidant activity of quercetin-loaded chitosan nanoparticles," *Journal of Applied Polymer Science,* vol. 107, pp. 891-897, 2008.
[143] M. Beyki, S. Zhaveh, S. T. Khalili, T. Rahmani-Cherati, A. Abollahi, M. Bayat*, et al.*, "Encapsulation of Mentha piperita essential oils in chitosan–cinnamic acid nanogel with enhanced antimicrobial activity against Aspergillus flavus," *Industrial Crops and Products,* vol. 54, pp. 310-319, 2014.
[144] A. Kujur, S. Kiran, N. Dubey, and B. Prakash, "Microencapsulation of Gaultheria procumbens essential oil using chitosan-cinnamic acid microgel: Improvement of antimicrobial activity, stability and mode of action," *LWT,* vol. 86, pp. 132-138, 2017.
[145] S. Y. B. de Carvalho, R. R. Almeida, N. A. R. Pinto, C. de Mayrinck, S. S. Vieira, J. F. Haddad*, et al.*, "Encapsulation of essential oils using cinnamic acid grafted chitosan nanogel: Preparation, characterization and antifungal activity," *International Journal of Biological Macromolecules,* vol. 166, pp. 902-912, 2020.
[146] R. Fields, "[38] The rapid determination of amino groups with TNBS," in *Methods in enzymology*. vol. 25, ed: Elsevier, 1972, pp. 464-468.

[147] M. Malhotra, C. Tomaro-Duchesneau, S. Saha, I. Kahouli, and S. Prakash, "Development and characterization of chitosan-PEG-TAT nanoparticles for the intracellular delivery of siRNA," *International journal of nanomedicine,* vol. 8, p. 2041, 2013.
[148] S. F. Hosseini, M. Zandi, M. Rezaei, and F. Farahmandghavi, "Two-step method for encapsulation of oregano essential oil in chitosan nanoparticles: preparation, characterization and in vitro release study," *Carbohydrate polymers,* vol. 95, pp. 50-56, 2013.
[149] J. L. Xie, S. D. Singh-Babak, and L. E. Cowen, "Minimum inhibitory concentration (MIC) assay for antifungal drugs," *Bio-protocol,* vol. 2, p. e252, 2012.
[150] M. Kakran, R. Shegokar, N. G. Sahoo, L. Al Shaal, L. Li, and R. H. Müller, "Fabrication of quercetin nanocrystals: comparison of different methods," *European Journal of Pharmaceutics and Biopharmaceutics,* vol. 80, pp. 113-121, 2012.
[151] S. Hariharan, V. Bhardwaj, I. Bala, J. Sitterberg, U. Bakowsky, and M. R. Kumar, "Design of estradiol loaded PLGA nanoparticulate formulations: a potential oral delivery system for hormone therapy," *Pharmaceutical research,* vol. 23, pp. 184-195, 2006.
[152] A. Anitha, V. Deepagan, V. D. Rani, D. Menon, S. Nair, and R. Jayakumar, "Preparation, characterization, in vitro drug release and biological studies of curcumin loaded dextran sulphate–chitosan nanoparticles," *Carbohydrate Polymers,* vol. 84, pp. 1158-1164, 2011.
[153] C. Dima, L. Pătraşcu, A. Cantaragiu, P. Alexe, and Ş. Dima, "The kinetics of the swelling process and the release mechanisms of Coriandrum sativum L. essential oil from chitosan/alginate/inulin microcapsules," *Food chemistry,* vol. 195, pp. 39-48, 2016.
[154] M. M. Yallapu, M. Jaggi, and S. C. Chauhan, "β-Cyclodextrin-curcumin self-assembly enhances curcumin delivery in prostate cancer cells," *Colloids and Surfaces B: Biointerfaces,* vol. 79, pp. 113-125, 2010.

PUBLICATIONS FROM THE THESIS

Contact Information: askican.h@gmail.com

Conference Paper

1. A.Hacıoğlu and C.B. Üstündağ, "Synthesis of cinnamyl chitosan and investigation of its antimicrobial activity," *3rd International Eurasian Conference on Biological and Chemical Science, EurasianBioChem2020*, Ankara, Turkey, 2020, p.670.