DEVELOPMENT OF NOVEL BIO-BASED METHODS FOR INHIBITION OF PLANT PATHOGENS

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ABSTRACT

DEVELOPMENT OF NOVEL BIO-BASED METHODS FOR INHIBITION OF PLANT PATHOGENS

In this thesis, to develop a post-harvest bio-based preservation method for spoilage of fresh fruits and vegetables, antimicrobial packaging commonly applied for inhibition of human pathogenic bacteria was adapted for inhibition of plant pathogenic bacteria including *Pseudomonas syringae, Erwinia amylovora, Xanthomonas vesicatoria* and *Erwinia carotovora.* The zein based films were developed by incorporation of pure phenolic acids such as gallic acid (GA), cinnamic acid (CA), vanillic acid (VA); essential oils such as carvacrol (CAR), thymol (THY), eugenol (EUG) and citral (CIT) and phenolic extracts obtained from clove, artichoke, oregano and walnut shells into film forming solutions. The films containing phenolic acids between 1 and 4 mg/cm^2 were effective on all bacteria. Essential oils at concentrations between 2 and 4 mg/cm^2 and clove extract at concentrations between 4 and 8 mg/cm^2 were found effective against pathogens except *P. syringae.* The HPLC analysis showed that the clove extracts owe its inhibitory activity to high concentration of GA which is a potent antimicrobial. The incorporation of natural compounds and extracts into films caused significant changes in morphologies and mechanical properties of films. This thesis showed the good potential of antimicrobial films against plant pathogens for the first time in the literature.

ÖZET

BİTKİ PATOJENLERİNİN İNHİBİSYONU İÇİN BİYOTABANLI YÖNTEMLER GELİŞTİRİLMESİ

Bu tez çalışmasında taze meyve ve sebzelerin bozulmasını engelleyecek bir muhafaza yöntemi geliştirmek amacıyla normalde insan patojeni olan bakterilerin gıdalarda üremesini önlemek için kullanılan antimikrobiyel paketleme *Pseudomonas syringae, Erwinia amylovora, Xanthomonas vesicatoria* and *Erwinia carotovora* gibi bitki patojenlerini inhibe etmek üzere uyarlanmıştır. Bu amaçla yenilebilir zein filmler içerisine gallik asit (GA), sinnamik asit (CA), vanillik asit (VA) gibi fenolik asitler; karvakrol (CAR), timol (THY), eugenol (EUG) ve sitral (CIT) gibi esansiyel yağlar ve karanfil, enginar, kekik ve ceviz kabuğu fenolik ekstraktları ilave edilmiştir. Elde edilen sonuçlara göre zein filmler içerisine 1 ile 4 mg/cm^2 arasında fenolik asit ilavesi tüm bitki patojenleri üzerinde inhibisyon etkisi oluştururken, esansiyel yağlarda 2 ile 4 mg/cm^2 ve karanfil ekstraktında ise 4 ile 8 mg/cm^2 ekstrakt ilavesi *P. syringae* hariç diğer bakteriler üzerinde antimikrobiyel etki göstermiştir. Karanfil ekstraktında HPLC kullanılarak gerçekleştirilen testlerle bu ekstraktın etkili bir bitki patojeni olan gallik asitçe zengin olduğu tesbit edilmiştir. Kullanılan doğal maddeler ve ekstraktlar filmlerin mekaniki özellikleri ve morfolojisinde kayda değer değişikliklere neden olmaktadır. Bu tez çalışmasıyla literatürde ilk kez antimikrobiyel ambalajlamanın bitki patojenlerine karşı kullanılabileceği gösterilmiştir.

DEDICATED TO

DENİZ AND MERCAN

TABLE OF CONTENTS

LIST OF FIGURES xi

LIST OF TABLES xiv

CHAPTER 1. INTRODUCTION 1

CHAPTER 2. FRUITS AND VEGETABLES 4

2.1. Microbiological Spoilage of Fruits and Vegetables 4

2.2. Spoilage Microorganisms 5

2.3. Prevention and Control of Microbiological Spoilage 9

CHAPTER 3. FOOD PACKAGING 12

3.1 .Food Packaging Polymers 12

3.2 .Edible Films and Coatings 13

3.2.1. Polysaccharide-based Coatings 14

3.2.2. Lipid-based Coatings 15

3.2.3. Protein-based Coatings 16

CHAPTER 4. ANTIMICROBIAL PACKAGING 19

4.1 .Organic Acids and Salts 22

4.2 .Polypeptides 25

4.2.1. Lysozyme 25

4.2.2. Lactoperoxide 25

4.2.3. Lactoferrin 26

4.2.4. Nisin 26

4.2.5. Natamycin 27

4.3. Plant Extracts, Essential Oils, Spices 27

CHAPTER 5. NATURAL ANTIMICROBIAL COMPOUNDS 29

5.1. Phenolic Compounds 29

5.1.1. Vanillic Acid 31
5.1.2. Gallic Acid 31
5.1.3. Cinnamic Acid 32
5.1.4. Antimicrobial Studies with Phenolic Compounds Derived from Plants 33
5.1.5. Antimicrobial Films Incorporated with Phenolic Compounds Derived from Plants 37
5.2 .Essential Oils and Plant Extracts 40
5.2.1. Oregano and Its Active Compounds; Thymol and Carvacrol 40
5.2.2. Clove and Its Active Compound; Eugenol 43
5.2.3. Lemongrass and Its Active Compound; Citral 44
5.2.4. Antimicrobial Studies with Essential Oils and Plant Extracts 45
5.2.5. Antimicrobial Films Incorporated with Essential Oils and Plant Extracts 49

CHAPTER 6. MATERIALS AND METHODS 55
6.1 .Materials 55
6.1.1. Bacterial cultures 55
6.1.2. Chemicals 55
6.2 .Methods 56
6.2.1. Extraction of Phenols from Plant Material 56
6.2.2. Total Phenolic Content (TPC) 57
6.2.3. Determination of Minimum Inhibitory Concentration (MIC)..... 57
6.2.4. Film Making 57
6.2.5. Antimicrobial Activity of Films against Plant Pathogens 60
6.2.6. Release Tests in Distilled Water 61
6.2.7. Mechanical Properties of Films 62
6.2.8. Scanning Electron Microscopy (SEM) 62
6.2.9. HPLC Analysis 62
6.2.10. Statistical Analysis 63

CHAPTER 7. RESULTS AND DISCUSSION 64
7.1. Inhibition of Plant Pathogens by Edible Films Containing Phenolic acids 64

7.1.1. Minimum Inhibitory Concentrations (MIC) of Phenolic Acids 64
7.1.2. Antimicrobial Activity of Edible Films Containing Phenolic Acids 68
7.1.3. Antimicrobial Activity of Edible Films Containing Mixture of Different Phenolic Acids 76
7.1.4. Release Tests of Edible Films Containing Phenolic Acids 80
7.1.5. Mechanical Properties of Edible Films Containing Phenolic Acids 81
7.1.6. Scanning Electron Microscopy (SEM) of Edible Films Containing Phenolic Acids 84
7.2. Inhibition of Plant Pathogens by Edible Films Containing Essential Oils 89
7.2.1. Antimicrobial Activity of Edible Films Containing Essential Oils 89
7.2.2. Mechanical Properties of Edible Films Containing Essential Oils 99
7.2.3. Scanning Electron Microscopy (SEM) of Edible Films Containing Essential Oils 101
7.3. Inhibition of Plant Pathogens by Edible Films Containing Plant Extracts 106
7.3.1. Minimum Inhibitory Concentrations of Plant Extracts 106
7.3.2 Antimicrobial Activity of Edible Films Containing Clove Extract 108
7.3.3. Release Tests of Edible Films Containing Clove Extract 110
7.3.4. Mechanical Properties of Edible Films Contaning Clove Extract 111
7.3.5. Scanning Electron Microscopy (SEM) of Edible Films Containing Clove Extract 113
7.3.6. Total Phenol Contents of Plant Extracts 115
7.3.7. Phenol Composition of Clove Extract 116

CHAPTER 8. CONCLUSION 118

REFERENCES 120

APPENDICES
APPENDIX A. PHOTOMETRIC MEASUREMENTS OF VARIOSKAN FLASH PLATE READER AT 600 NM 132
APPENDIX B. STANDARD CURVES OF GA AND VA FOR RELEASE TEST..... 141
APPENDIX C. ANALYSIS OF VARIANCE TABLES FOR MECHANICAL PROPERTIES..... 143

LIST OF FIGURES

Figure		Page
Figure 2.1.	Soft rot (E. carotovora) in potato (Patateste Bakteriyel Yumuşak Çürüklük ve Karabacak Hastalığı ve Mücadelesi)	6
Figure 2.2.	Dieback (Pseudomonas syringae) of peach (Fruit trees-Peach Pseudomonas syringae pv. syringae	8
Figure 2.3.	Fire blight (Erwinia amylovora) of pear (HungryPlanet: Stories of Plant Diseases)	8
Figure 2.4.	Black spot (Xanthomonas vesicatoria) in tomato (Vegetables-tomato)	9
Figure 3.1.	Different categories of bio-based materials	14
Figure 4.1.	Antimicrobial packaging systems: package-food and package-headspace	20
Figure 5.1.	Chemical structure of Vanillic acid	31
Figure 5.2.	Chemical structure of Gallic acid	32
Figure 5.3.	Chemical structure of Cinnamic acid	33
Figure 5.4.	Chemical structure of Rosmarinic acid	41
Figure 5.5.	Chemical structure of Caffeic acid	41
Figure 5.6.	Chemical structures of Apigenin, Luteolin and Quercetin	41
Figure 5.7.	Chemical structures of thymol and carvacrol	42
Figure 5.8.	Chemical structures of Eugenol and Eugenol acetate	44
Figure 5.9.	Chemical structure of the citral	45
Figure 6.1.	Peeled films containing clove extract used in released test	61
Figure 7.1.	Antimicrobial activity of GA, VA, and CA containing zein films on E. carotovora	68
Figure 7.2.	Antimicrobial activity of GA, VA and CA containing zein films on E. amylovora	70
Figure 7.3.	Growth inhibition of E. amylovora by zein film containing CA	70
Figure 7.4.	Growth inhibition of E. amylovora by zein film containing GA	71
Figure 7.5.	Growth inhibition of E. amylovora by zein film containing VA	72

Figure 7.6. Antimicrobial activity of GA, VA, and CA containing zein films on P. syringae 73
Figure 7.7. Growth inhibition of P. syringae by zein film containing CA..... 73
Figure 7.8. Growth inhibition of P. syringae by zein film containing GA 73
Figure 7.9. Growth inhibition of P. syringae by zein film containing VA 74
Figure 7.10. Antimicrobial activity of GA, VA, and CA containing zein films on X. vesicatoria 75
Figure 7.11. Effect of GA, CA, and VA combinations incorporated zein film against plant pathogens..... 77
Figure 7.12. Growth inhibition of E. amylovora by zein film containing combinations of phenolic acids 78
Figure 7.13. Growth inhibition of E. carotovora by zein film containing combinations of phenolic acids 79
Figure 7.14. Growth inhibition of P. syringae by zein film containing combinations of phenolic acids 80
Figure 7.15. Release profiles of GA and VA from zein films incubated in distilled water at 4 ℃..... 81
Figure 7.16. SEM photographs of different film cross-sections. 85
Figure 7.17. SEM photographs of different film cross-sections. 86
Figure 7.18. SEM photographs of different film cross-sections. 87
Figure 7.19. SEM photographs of different film cross-sections. 88
Figure 7.20. Antimicrobial activities of essential oils containing zein films on E. amylovora..... 90
Figure 7.21. Growth inhibition of E. amylovora by zein film containing CAR 90
Figure 7.22. Growth inhibition of E. amylovora by zein film containing EUG 91
Figure 7.23. Growth inhibition of E. amylovora by zein film containing CIT 92
Figure 7.24. Antimicrobial activities of essential oils containing zein films on E. carotovora..... 93
Figure 7.25. Growth inhibition of E. carotovora by zein film containing THY 94
Figure 7.26. Growth inhibition of E. carotovora by zein film containing CAR 94
Figure 7.27. Growth inhibition of E. carotovora by zein film containing EUG 94
Figure 7.28. Growth inhibition of E. carotovora by zein film containing CIT 95
Figure 7.29. Antimicrobial activities of essential oils containing zein films on X. vesicatoria 96

Figure 7.30. Growth inhibition of X. vesicatoria by zein film containing THY 97
Figure 7.31. Growth inhibition of X. vesicatoria by zein film containing CIT 97
Figure 7.32. Growth inhibition of X. vesicatoria by zein film containing EUG 98
Figure 7.33. Antimicrobial activities of essential oils containing zein films on P.syringae 98
Figure 7.34. Growth inhibition of P. syringae by zein film containing EUG................ 99
Figure 7.35. SEM photographs of different film cross-sections. 102
Figure 7.36. SEM photographs of different film cross-sections. 103
Figure 7.37. SEM photographs of different film cross-sections. 104
Figure 7.38. SEM photographs of different film cross-sections................................. 105
Figure 7.39. Antimicrobial activities of clove extract containing zein film on plant pathogens.. 109
Figure 7.40. Growth inhibition of E. amylovora by zein film containing clove extract 109
Figure 7.41. Growth inhibition of X. vesicatoria by zein film containing clove extract 110
Figure 7.42. Release profiles of clove extract from zein films incubated in distilled water at 4 ℃... 111
Figure 7.43. SEM photographs of different film cross-sections. 114
Figure 7.44. TPCs of different plant extracts (mean ± SD) 115
Figure 7.45. Hplc profile of clove extract (retention time of gallic acid: 3.5 min).. 117

LIST OF TABLES

Table **Page**

Table 3.1. Examples of edible coating applications on fruits and vegetables 18
Table 4.1. Antimicrobial compounds used on edible films and coatings 21
Table 4.2. Organic acids incorporated into the films for inhibition of different microorganisms 24
Table 4.3. Polypeptides incorporated into the films for inhibition of different microorganisms 28
Table 5.1. Phenolic classes in Plants 30
Table 5.2. Plant extracts and phenolic constituents using for inhibition of various spoilage or pathogenic microorganisms 35
Table 5.3. Plant extracts and phenolic constituents used in films and coatings showing antimicrobial activity 39
Table 5.4. Essential oils and plant extracts using for inhibition of various spoilage or pathogenic microorganisms 48
Table 5.5. Essential oils and plant extracts used in films and coatings showing antimicrobial activity 53
Table 6.1. Chemicals used in the analysis 56
Table 6.2. Phenolic acids contents of zein films 59
Table 6.3. Essential oils contents of zein films 60
Table 7.1. The MIC of phenolic acids against plant pathogens 64
Table 7.2. Resistances of plant pathogens to different concentrations of phenolic compounds by means of lag periods and inhibitions in their growth 66
Table 7.3. Mechanical properties of zein films containing phenolic acids 83
Table 7.4. Mechanical properties of zein films containing essential oils 100
Table 7.5. Antimicrobial effects of plant extracts at different concentrations on the growth of plant pathogens 107
Table 7.6. Mechanical properties of zein films containing clove extract 112

CHAPTER 1

INTRODUCTION

The coating of fresh fruits and vegetables by natural edible films has recently gained a particular importance since this application can improve quality, safety and shelf-life of products by reducing microbial growth at their surface and by controlling transfer of respiration gases, volatile aromas and flavors and moisture (Cutter 2006, Lin 2007, Vermeiren, et al. 1999). The indicated functional benefits obtained by edible coatings could also be obtained by using flexible plastic films for individual packaging or modified atmosphere packaging of fresh fruits and vegetables. However, environmental problems and increasing recycling costs of plastics make edible films unique environmentally friend alternatives for packaging/coating applications of fresh fruits and vegetables. Thus, extensive studies are continuing to develop novel edible coating materials from biopolymers including polysaccharides, proteins and lipids (Ponce, et al. 2008, Lin 2007, Lopez-Rubio, et al. 2004).

Incorporation of antimicrobial agents into food packaging films and coatings, antimicrobial packaging, is a classical method to inhibit the growth of pathogenic and spoilage microorganisms and to increase safety and quality of processed foods. However, the current consumer demands to minimize use of chemical antimicrobials or to use natural antimicrobial substances in place of chemical antimicrobials caused a great challenge in controlling growth of bacteria, yeast and molds in packed foods (Çağrı, et al. 2004, Güçbilmez, et al. 2007, Appendini and Hotchkiss 2002). The major natural antimicrobial substances suitable for antimicrobial packaging include bacteriocins, antimicrobial enzymes, phenolic compounds and phenolic rich plant extracts and essential oils (Du, et al. 2011, Seydim and Sarıkuş 2006, Ponce, et al. 2008, Gutierrez, et al. 2009). The use of phenolic compounds and phenolic rich plant extracts and essential oils in edible films has become increasingly popular since these compounds have strong antimicrobial and antioxidant activity in food systems and they show bioactivities beneficial to human health (Du, et al. 2011, Coma 2008, Quattara, et al. 2000). Moreover, the bioactive phenolic compounds could easily be extracted from agroindustrial wastes of wine, oil and fruit and vegetable processing industries

(Schieber, et al. 2001). The bioactive properties of plant phenolic extracts have been reported by many different workers. The phenolic compounds are not only effective on inhibiting vegetative microbial cells but they also inhibit the microbial spores. For example, Sakanaka et al. (2000) showed the inhibition effects of tea polyphenols on *Bacillus* and *Clostridium* spores and suggested to employ tea polyphenols for preservation of canned drinks with high carbohydrate contents (Sakanaka, et al. 2000). Alberto et al. (2006) and Ganan, et al. (2009) showed antibacterial effects of apple peel and wine polyphenols on some important human pathogens while Baidez, et al. (2006) reported antifungal effects of olive polyphenols. The antimicrobial potential of essential oils obtained from clove, oregano, rosemary, thyme, lemongrass, sage and vanillin is also due to their phenolic content (Altıok, et al. 2010, Moon et al., 2011, Chun, et al. 2005, Kim, et al. 1995). For example, the oregano essential oil has inhibited bacteria due to the presence of phenolic compounds such as thymol and carvacrol while antimicrobial properties of clove oil originate from eugenol (Mytle, et al. 2006).

The fresh fruits and vegetables form an important part of diet for billions of people living in different parts of world. However, about 20 % of fruits and vegetables produced each year are wasted due to physiological ageing, undesired biochemical changes and microbial spoilage. The microbial spoilage is one of the major causes of fruit and vegetable wastes. Spoilage microorganisms can be introduced into the crop on the seed, during growth in the field, during harvesting or during storage. The distribution and economic losses due to plant pathogens are estimated at 10-20 % of the attainable yield for many crops (Western Association of Agricultural Experiment Station Directors). The bacteria including *Pseudomonas, Erwinia, Xanthomonas, Flovabacterium, Alcaligenes, Acinetobacter, Leuconostoc, Lactobacillus, Enterobacter, Micrococcus, Serratia and Streptococcus* species constitute natural microflora of plants and contribute to their spoilage (Barth, et al. 2009). However, there are no studies in the literature to use edible antimicrobial coatings against these bacterial plant pathogens.

In this work, for the first time in the literature antimicrobial packaging used frequently for inhibition of human pathogens in processed food was employed as a bio-based method to control plant pathogenic bacteria important for spoilage of fresh fruits and vegetables. The edible coatings were developed by incorporation of pure phenolic compounds such as gallic acid (GA), cinnamic acid (CA), vanillic acid (VA), essential oils such as carvacrol (CAR), thymol (THY), eugenol (EUG) and citral (CIT) and plant

extracts from clove, artichoke, oregano and walnut shells into zein film forming solutions. The antimicrobial performances of edible films were tested on selected plant pathogenic bacteria including *P. syringae, E. amylovora, X. vesicatoria* and *E. carotovora,* and release properties, morphologies and mechanical properties of developed films were characterized with details. This work opens a new perspective for reducing waste fruits and vegetables worldwide. The use of zein as potential antimicrobial coating material is also important since this biopolymer is the major co-product of the oil and rapidly growing bioethanol industries.

CHAPTER 2

FRUITS AND VEGETABLES

2.1. Microbiological Spoilage of Fruits and Vegetables

Consumption of fruit and vegetable products has increased in the world during the past few decades. A well-balanced diet, rich in fruit and vegetables, promotes good health. Because fresh fruit and vegetables are important source of substances with antioxidants and free-radical scavenging properties like anthocyanins and other phenolic compounds, they may reduce the risk of certain diseases. Carotenoids, tocopherols and vitamin C also play important role in the prevention of some human diseases. The important part of all fruits and vegetables produced is lost each year due to spoilage by microorganisms. The survival and growth of pathogenic microorganisms are the major food safety concerns in fresh fruits and vegetables.

Undesirable qualities, including off-aromas, off-flavors, or textural and appearance changes such as sliminess, moldiness, blemishes of fruit and vegetables typically result from the action of microorganisms such as bacteria and fungi (Ukuku, et al. 2005). Most microorganisms that are initially observed on whole fruit or vegetable surfaces are soil inhabitants, members of a very large and diverse community of microbes that collectively are responsible for maintaining a dynamic ecological balance within most agricultural systems. Spoilage microorganisms can be introduced to the crop on the seed itself, during crop growth in the field, during harvesting and postharvest handling, or during storage and distribution. Those same types of soil-borne spoilage microbes that occur on produce are the same spoilage microorganisms that are present on harvesting equipment, on handling equipment in the packinghouse, in the storage facility, and on food contact surfaces throughout the distribution chain (Barth, et al. 2009).

Many fruits and vegetables present nearly ideal conditions for the survival and growth of many types of microorganisms. The internal tissues are nutrient rich and many, especially vegetables, have a pH near neutrality. Their structure is comprised mainly of the polysaccharides cellulose, hemicellulose, and pectin. The principal

storage polymer is starch. Spoilage microorganisms exploit the host using extracellular lytic enzymes that degrade these polymers to release water and the plant's other intracellular constituents for use as nutrients for their growth (Barth, et al. 2009).

In general, plants defend themselves against pathogen attack. One of the defense mechanisms is structural characteristic that act as physical barriers and inhibit the pathogen from gaining entrance and spreading through the plant. Other forms of defense are biochemical reactions that take place in the cells and tissues of the plant and produce substances which either toxic to the pathogen or create conditions that inhibit growth of the pathogen in the plant. These substances include hypersensitivity responses and induction of phytoalexins and/or pathogenesis-related proteins (PRP). Induced resistance responses can act as a form of "immunization" of the plant against subsequent pathogen attack in the field and/or in storage (Agrios 1997).

However, many obligate phytopathogens having specialized enzymes that can degrade the cellulose, hemicellulose, pectin, and cutin of the epidermal and endodermal tissues can penetrate the plants' defenses. These organisms use a breach of the surface integrity to gain entry to the inner tissues of the leaf, fruit, tuber, etc. Poorly designed equipment and mishandling cause some wounds in product at a variety of stages during the growth, harvest, washing, packing, or shipping of the produce. Thus, products exposed to some mishandling treatments are especially vulnerable to microbiological spoilage (Ukuku, et al. 2005).

2.2. Spoilage Microorganisms

Postharvest spoilage can be caused by a wide variety of different organisms. About 100 species of bacteria cause diseases in plants. Bacteria may be rod shaped, spherical, spiral, or filamentous. Some bacteria can move through liquid media by means of flagella, whereas others have no flagella and cannot move themselves. Some can transform themselves into spores, and certain filamentous forms can produce spores, called conidia, at the end of the filament. Other bacteria, however, do not produce any spores. Bacterial diseases of plants occur in every place that is moist or warm, and they affect all kinds of plants. Plant pathogenic bacteria induce as many kinds of symptoms on the plants. They cause leaf spots and blights, soft rots of fruits, roots, and storage organs, wilts, overgrowths, scabs, and cankers. *Erwinia*,

Pseudomonas, Xanthomonas, Clavibacter, Streptomyces species cause important plant diseases (Agrios 1997).

The bacterium *Erwinia carotovora* subsp. *carotovora* is a highly effective spoilage microbe that causes soft rot across a broad host range of vegetables and some fruits. One of six known genera of soft-rot bacteria (including *Xanthomonas*, *Pseudomonas*, *Clostridium*, *Cytophaga,* and *Bacillus*), *E. carotovora* subsp. *carotovora* is one of several species of *Erwinia* that infect and destroy plant tissues both pre- and postharvest and is the species that causes the greatest damage to harvested vegetables (Figure 2.1). Soft rot is a form of decay characterized by a watery transparency in infected leafy plant parts and watery disintegration of nonleafy plant materials. "Soft-rot erwinia" tend to initiate infection and decay at wound sites and, once established, can quickly advance to total destruction of the product. Soft-rot erwinia express four pectin-degrading extracellular enzymes: pectin lyase, polygalacturonase, pectin methylesterase, and pectate lyase. Of these enzymes, pectate lyase is primarily responsible for extensive decay. *E. carotovora* has built-in redundancy for this apparently critical pathogenicity factor, expressing four distinct extracellular pectate lyase isozymes. Soft-rot erwinia are active only at temperatures of 20°C and above, which reinforces the need to maintain a continuous cold chain from immediately postharvest to retail to successfully manage this ubiquitous spoilage bacterium (Ravensdale, et al. 2007).

Figure 2.1. Soft rot (*E. carotovora*) in potato (Patateste Bakteriyel Yumuşak Çürüklük ve Karabacak Hastalığı ve Mücadelesi)

Another group of soft-rotting bacteria, the fluorescent pseudomonads (i.e., *Pseudomonas fluorescens* , *Pseudomonas syringae* and *Pseudomonas viridiflava*), can decay plant tissue at temperatures at or below 4°C. This is one explanation for the high prevalence of these bacteria on decayed vegetables at wholesale and retail markets. The soft-rotting fluorescent pseudomonads, when considered together with soft-rot erwinia, present a formidable challenge to commercial fresh product operations, and fresh vegetables in particular, from the farm to retail and wholesale outlets. *Pseudomonas tolaasii,* another fluorescent pseudomonad and fresh produce spoilage bacterium, has a much narrower range of host-specificity than *P. fluorescens* and *P. viridiflava*. *P. tolaasii* causes spoilage of the white mushroom, *Agaricus bisporus*. Similar to *P. fluorescens* and *P. viridiflava*, *P. tolaasii* produces siderophores that fluoresce under ultraviolet light. However, unlike the soft-rot pseudomonads, *P. tolaasii* does not cause soft rot on plants (i.e., it does not produce pectin depolymerases) but instead creates unsightly blemishes on the caps and stems of the *Agaricus* fruiting body as a result of localized infection and decay of those parts of the mushroom (Barth, et al. 2009).

Pseudomonas is straight to curved rod, 0.5-1 by 1.5-4 µm. They are motile by means of one or many polar flagella. Many species are common inhabitants of soil or of freshwater and marine environments. *Pseudomonas syringe,* another fluorescent pseudomonad produces yellow-green, diffusible, fluorescent pigments on a medium of low iron content. *Pseudomonas syringae* is a Gram-negative rod with two to three polar flagella. In culture at 23°C, it develops in 48 h creamy-white colonies 1-2 mm in diameter (on yeast-peptone-glucose medium). It causes bacterial dieback of peach (Figure 2.2). The characteristic symptom is an olive-green discoloration, rapidly browning, appearing round dormant buds on young shoots of peach (Agrios 1997).

Figure 2.2. Dieback (*Pseudomonas syringae*) of peach (Fruit trees-Peach *Pseudomonas syringae* pv. syringae

Erwinia amylovora, a member of the *Enterobacteriaceae*, is a Gram-negative rod-shaped bacterium and has peritrichous flagella. It has cells 1.1-1.6 × 0.6-0.9 µm in size. It requires nicotinic acid as a growth factor. It is identified from the symptoms it causes and by serological tests. It is the causal organism of fire blight which is a devastating disease of apple and pear trees. Fire blight causes damage to pear and apple orchard in many parts of the world (Figure 2.3). Certain apple and quince varieties are also very susceptible to the disease. Infected flowers become water soaked, then shrivel, turn brownish black, and fall or remain hanging in the tree (Roberts, et al. 1997).

Figure 2.3. Fire blight (*Erwinia amylovora*) of pear (HungryPlanet: Stories of Plant Diseases)

Xanthomonas cells are straight rods, 0.4-1.0 by 1.2-3 µm, and are motile by means of a polar flagellum. Growth on agar media is usually yellow, and most are slow

growing. All species are plant pathogens and are found only in association with plants or plant materials. *Xanthomonas vesicatoria* is an aerobic, mobile, Gram-negative rod, occurring singly or in pairs, 0.6×1.0-1.5 μm, with a single polar flagellum. On yeast dextrose chalk agar and nutrient dextrose agar, colonies are large, smooth-domed, mucoid-fluidal and yellow with entire edges. It causes bacterial spot, scab, and black spot in tomato and *Capsicum* (Figure 2.4). Fruits of tomato show superficial corky spots or scabs, with water-soaked margins, oval or irregular in shape, 2-10 mm in diameter (Mohano and Raveesha 2006).

Figure 2.4. Black spot (*Xanthomonas vesicatoria)* in tomato (Vegetables-tomato)

2.3. Prevention and Control of Microbiological Spoilage

Bacterial diseases of plants are usually very difficult to control. Infestation of fields or infection of crops with bacterial pathogens should be avoided by using only health seeds or transplants. Resistant varieties, supplemented with proper cultural practices and chemical applications, are the most effective means of controlling bacterial diseases. Soil infested with pathogenic bacteria can be sterilized with steam or electric heat and with chemicals such as formaldehyde. Bordeaux mixture, fixed coppers, and cupric hydroxide are most frequently used for the control of bacterial leaf spots and blights. However, bacterial strains resistant to copper fungicides are quite common. Copper-based bactericides have been used extensively to prevent many bacterial plant diseases, although copper resistance has been reported in many bacterial

pathogens, including plasmid-borne resistance and chromosomal resistance. Antibiotics have been used against certain bacterial diseases with mixed results. They can be applied as sprays or as dips for transplants. The most important antibacterial antibiotics in agriculture are formulations of streptomycin or of streptomycin and oxytetracycline. Unfortunately, bacterial races resistant to antibiotics develop soon after widespread application of antibiotics; besides, no antibiotics are permitted on edible plant produce (Agrios 1997). Streptomycin, an aminoglycoside antibiotic, has been utilized in agriculture since the 1950s to control phytopathogenic bacteria. Extensive use of this antibiotic for control of various bacterial diseases resulted in increased prevalence of streptomycin-resistant strains in bacterial populations and reduced efficacy of control of bacterial spot of tomato and pepper, fire blight of apple and pear, as well as many other bacterial plant pathogens. Systemic acquired resistance (SAR) plant inducers have shown activity against bacterial diseases of tomato and pepper, *Xanthomonas* leaf blight on onion, and fire blight on apple. Plant inducers have also been ineffective for disease control in some pathosystems such as for control of citrus canker (Jones, et al. 2007).

The use of synthetic chemicals to control postharvest deterioration has been restricted due to their carcinogenicity, teratogenicity, high and acute residual toxicity, long degradation period, environmental pollution and their effects on food and other side-effects on humans. As well, phytotoxic and off-odor effects of some prevalent bactericides have limited their use (Tripathi and Dubey 2004). Given the difficulties in controlling bacterial diseases using conventional strategies, considerable efforts have been made to use biologically based strategies. Recently, there has been resurgence in interest in use of bacteriophages for control of bacterial plant diseases. For examples, bacteriophages of *Xanthomonas campestris* pv. campestris and *X. oryzae* pv. oryzae which are the causative agents of black rot in crucifers and bacterial leaf blight in rice were isolated (Lee, et al. 2006). In another study, bacteriophages of *Erwinia amylovora*, which is the causal organism of fire blight, were isolated for the investigation of the potential of using phages as biological control agents (Gill, et al. 2003). Moreover, isolates of *E. carotovora* subsp. *carotovora* phages reduced soft-rot incidence in greenhouse-grown calla lilies by up to 70% (Ravensdale, et al. 2007).

In order to prevent microbial contamination, surface sanitation and sterilization of fruit and vegetables in postharvest period has been achieved by sodium or calcium hypochlorite and other salts. However, it has been shown that many microorganisms

exhibit resistance to chlorine treatments. The use of chlorine may also cause taste and odor defects in products, health hazards due to the potential toxicity, carcinogenicity and mutagenicity. Thus, alternative chemicals disinfection agents have been tested such as chlorine dioxide, ozone, organic acids, hydrogen peroxide, quaternary ammonium compounds, trisodium phosphate, sucrose esters, iodine compounds, alcohols, anionic and non-ionic surface-active agents, aldehydes, phosphoric and peroxiacetic acids, cisteine, methyl jasmonate and bioflavonoids. One of the important factors affecting the shelf-life of fruit and vegetables is temperature condition during transit, distribution and retail display. Products must be kept at 1–5°C throughout the distribution chain to ensure shelf-life. Because chemical use in plant foods has been restricted by reason of their harms to human, alternative techniques including hot water treatments, biological control, cultural adaptations and physical methods such as controlled atmosphere (CA), MAP and irradiation are needed. Recently, many non-conventional methods, such as ultraviolet-C (UV-C) light, ozone, pulsed electric fields, magnetic fields, high-intensity pulsed light, high hydrostatic pressure, and antimicrobials of natural origin or new edible coatings are now being investigated (Artes and Allende 2005).

CHAPTER 3

FOOD PACKAGING

Food packaging has an important role in the food supply chain because most commercialized foods, including fruits and vegetables, are exhibited inside packages. A food package protects food from environmental conditions, such as light, oxygen, moisture, microorganisms, mechanical damage and dust. A long time packaging prevents the changes in food products during production, distribution, storage, retail and thus improves the safety and quality of foods for consumers (Ahvenainen 2003).

3.1. Food Packaging Polymers

The most well-known packaging materials are polyethylene or co-polymer based materials, which have been in use by the food industry for over 50 years. Packaging polymers are typically low cost materials suitable for high volume production (Cutter 2006). They have more limited stress and lower temperature resistance than engineering polymers. Packaging polymers mostly used in food industry are polyethylene (PE), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), polyethylene terephthalate (PET), polyvinylidene chloride (PVDC), ethylene vinyl alcohol copolymer (EVOH), ionomer, ethylene vinyl acetate (EVA) copolymer, nylons, and polycarbonate (PC).

PE is the most frequently used polymer in food packaging because of its low cost, easy processing, and good mechanical properties. PE is basically classified in three names, high density polyethylene (HDPE), low density polyethylene (LDPE), and linear low density polyethylene (LLDPE). PP is a linear, crystalline polymer that has the lowest density among all plastics. Compared to PE, PP has higher tensile strength, stiffness, and hardness. PS is an amorphous polymer that has excellent clarity. Because of its excellent clarity, it is used in paperboard boxes to display products. PVC is a clear, amorphous polymer that is used for films and containers. Plasticized PVC films are limp, tacky, and stretchable, and it is commonly used for packaging fresh meat. The amorphous form of PET is mostly used as injection blow-molded bottles for carbonated

soft drinks, water, edible oil, and juices. PVDC is a copolymer of vinylidene chloride (85-90%) and vinyl chloride. The most important advantages of PVDC are its excellent oxygen and moisture barriers. EVOH is a crystalline copolymer of ethylene (27-48%) and vinyl alcohol. EVOH is the most expensive of all food packaging materials and has exceptional high oxygen barrier. An ionomer is a polymer that consists of a small but significant portion of ionic units. Its sealing performance, formability, clarity, oil/grease resistance, and high hot draw strength make it excellent for food packaging applications. EVA are long chains of ethylene hydrocarbons with acetate groups that are leading polymers for hot-melt manufacturing due to their high versatility in hot-melt formulations. Nylons are used for food packaging films because of their good gas barrier, puncture resistance, and heat resistance properties. PC is an amorphous thermoplastic which can be injaection-molded, blow-molded, and thermoformed (Lee, et al. 2008).

Food packaging polymers are safe, inexpensive, versatile, and flexible. However, these polymers are based on nonrenewable materials and thus the use of these materials is limited. Recently, there is a large amount of research underway to improve the performance of packaging materials obtain from renewable resources. Replacement of nonrenewable with renewable resources can solve the solid waste problem and the litter problem (Robertson 2006)

3.2. Edible Films and Coatings

New and novel foodgrade packaging materials have been developed in order to overcome recycling problem of polymers and meet consumer demands for safer and better quality foods. These packaging materials are bio-based polymers, bioplastic or biopolymer packaging products made from agricultural or marine sources (Figure 3.1). Examples of these polymers include polysaccharides such as starch, alginate, cellulose ethers, chitosan, carrageenan, or pectin; lipids; proteins such as casein, whey protein, gelatin/collagen, fibrinogen, soy protein, wheat gluten, corn zein, and egg albumen (Cutter 2006, Ponce, et al. 2008).

An edible film is defined as a thin layer of edible material applied on a food as a coating or placed on or between food components (Robertson 2006). It can improve shelf life and food quality by serving as selective barriers to moisture transfer, oxygen

uptake, lipid oxidation, and losses of volatile aromas, antioxidants, vitamins, and colorants and flavors (Rojas-Graü, et al. 2007). Edible coatings and films prepared from polysaccarides, proteins and lipids have a variety of advantages such as biodegradability, edibility, biocompatibility, appearance and barrier properties (Perez, et al. 2006, Ponce, et al. 2008). Wax coatings on fresh fruits and vegetables, collagen films for sausage casings, hydroxymethyl cellulose films of soluble pouches for dried food ingredients, zein coatings on candies, sugar coatings on drug pills, and gelatin films for soft capsules are the important examples of use of edible coatings and films (Lee, et al. 2008). Table 3.1 shows the examples of different edible coating incorporating bio-based additivies applied to some fruit and vegetables.

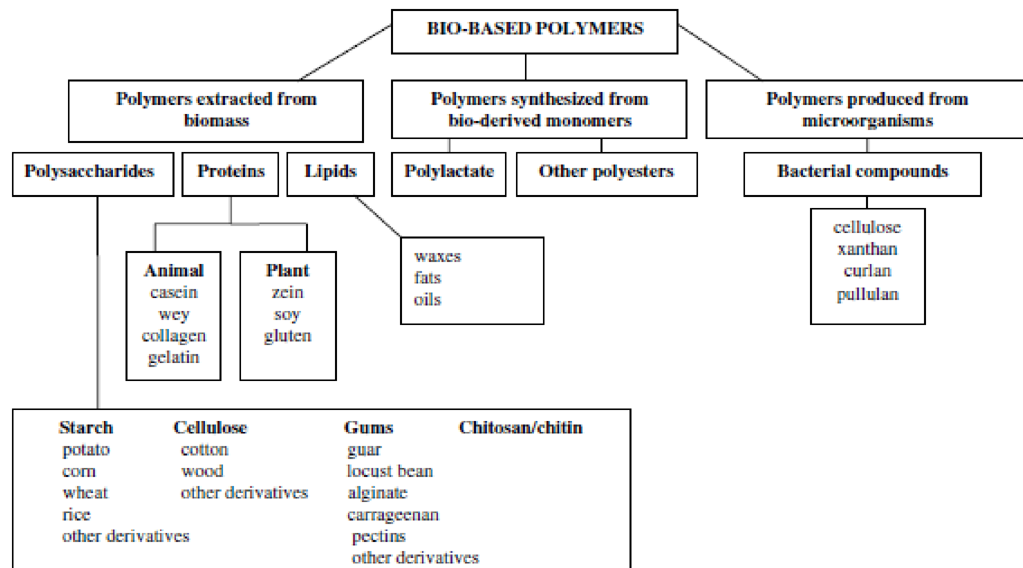

Figure 3.1. Different categories of bio-based materials
(Source: Cutter 2006)

3.2.1. Polysaccharide-based Coatings

Polysaccarides used as edible films include alginate, pectin, carrageenan, starch, starch hydrolysates and cellulose derivatives. They have limited moisture-barrier properties because of the hydrophilic nature of polysaccarides. Amylose is the linear fraction of starch and forms coherent, strong, free-standing films whereas amylopectin film is brittle and noncontinuous. Alginates are extracted from brown seaweeds of the *Phaephyceae* family and are the salts of alginic acid, a linear copolymer of D-

mannuronic and L-guluronic acid monomers. Films produced by evaporation of water from a thin layer of alginate solution are impervious to oils and greases but have high water vapor permeability. In the study of Rojas Graü et al. (2007), alginate (2% w/v) and gellan (0.5% w/v)-based edible coatings were developed and the effect of glycerol and antibrowning agents on water vapor resistance was studied. The ability of the coatings to carry antibrowning agents was investigated following color changes of coated fresh-cut Fuji apples. As a result of that study, the addition of sunflower oil in gellan was more effective than in alginate to increase the water vapor resistance of coated apples. Alginate and gellan-based coatings proved to be good carriers for antibrowning agents. Carrageenan is a complex mixture of at least five distinct polymers based on galactose and extracted from red seaweeds. Carrageenan-based coatings have been applied to fresh fruits and vegetables such as fresh apples for reducing moisture loss, oxidation, or disintegration of the apples (Lin 2007). Like carrageenan, agar is a galactose polymer and derived from a variety of red seaweeds of the *Rhodophyceae* class. Dextrans are microbial gums formed from sucrose fermentation and composed solely of α-D-glucopyranosyl units with various types of glycosidic linkages. They have been applied to preserve the flavor, color and freshness of food during refrigeration or frozen storage. Cellulose ethers are polymer substances obtained by partial substitution of three hydroxyl groups at positions 2, 3 and 6 on the glucosyl units of cellulose. They have good film-forming properties but are too expensive for large-scale commercial usage (Robertson 2006). The most common commercially produced cellulose derivatives are carboxymethyl cellulose (CMC), methyl cellulose (MC), hydroxypropyl cellulose (HPC), and hydroxypropylmethyl cellulose (HPMC). These materials are nonionic and compatible with surfactants, other water-soluble polysaccharides, and salt, and can be dissolved in aqueous or aqueous-ethanol solutions, producing films that are water-soluble and resistant to fats and oils (Lin 2007).

3.2.2. Lipid-based Coatings

Lipid compounds are not polymers and thus they do not generally form coherent stand alone films. They can provide gloss and a moisture barrier because of their relatively low polarity. Waxes such as carnauba wax, beeswax, and paraffin wax, and

mineral and vegetable oils have been used as protective coatings for fresh fruits and vegetables in order to block moisture transport, reduce surface abrasion during fruit handling, and control soft scald formation (browning of the skin) in fruits such as apples by improving mechanical integrity and controlling internal gas composition of the fruits. Saucedo-Pompa et al. (2009) studied the effect of addition of ellagic acid into candelilla wax matrix on shelf life and quality of whole avocado. They concluded that application of edible films based on candelilla wax and ellagic acid significantly minimized the changes in appearance, solid content, pH, aw, luminosity and weight loss, maintaining the quality of avocado fruits and prolonging their shelf life. Wax-, fat- and oil-based coatings can be difficult to apply due to their thickness and greasy surface, and may also confer a waxy or rancid taste (Robertson 2006). Triglycerides or neutral lipids can form a continuous stable layer on the food surface based on their high polarity relative to waxes. Most fatty acids derived from vegetable oils are considered GRAS (generally recognized as safety) substances and have been suggested as substitutes for the petroleum-based mineral oils used in the preparation of edible coatings. However, these coatings may suffer from flavor instability, while partially hydrogenated vegetable oil that is resistant to rancidity sometimes gives better results (Lin 2007).

3.2.3. Protein-based Coatings

Edible coatings made of animal proteins (such as milk protein) and plant proteins (such as zein, soy protein, and wheat gluten) exhibit excellent oxygen, carbon dioxide, and lipid-barrier properties, particularly at low relative humidity. Protein-based films and coatings are brittle and susceptible to cracking due to the strong cohesive energy density of the polymers. Because of their inherent hydrophilicity, and the significant amounts of hydrophilic plasticizers such as glycerin and sorbitol incorporated into films to give them flexibility, protein films have limited resistance to water vapor. Zein formulations for application as edible films and coatings on pears were developed with different concentrations of oleic acid as plasticizer (Scramin, et al. 2011). The ability of different proteins to form films and coatings is highly dependent on their molecular characteristics: molecular weight, conformations, electrical properties (charge vs pH), flexibilities, and thermal stabilities. In general, film

formation involves heat treatment to denature the protein, followed by solvent evaporation (casting). Protein-based films could have impressive gas barrier properties and mechanical properties compared with those prepared from polysaccharides and fat-based films, since proteins have a unique structure which confers a wider range of functional properties, especially a high intermolecular binding potential. Milk proteins are some of the most common source of proteins used to obtain films and coatings. Casein and whey proteins, the main milk protein fractions (80% and 20%, respectively), have acquired particular interest since they can provide a high nutritional added value and good taste in addition to their barrier and filmogenic properties (Campos, et al. 2011).

Table 3.1. Examples of edible coating applications on fruits and vegetables (Source: Lin 2007)

Commodity	Coating material	Primary functions	References
Apple	Caseinate; whey protein	O_2 barrier; carrier (antioxidant)	Le Tien and others (2001)
Apple (fresh-cut)	HPMC	O_2/H_2O barrier	Cisneros-Zevallos and Krochta (2003)
	Alginate; gelatin, CMC	$O_2/CO_2/H_2O$ barrier	Moldão-Martins and others (2003)
	Polysaccharide/lipid bilayer	O_2/CO_2 barrier, gloss	Wong and others (1994a, 1994b)
	Zein	$O_2/CO_2/H_2O$ barrier, gloss	Bai and others (2003a)
	Wax; shellac	O_2/CO_2 barrier	Bai and others (2003b)
	Carrageenan; WPC	O_2/H_2O barrier	Lee and others (2003)
	WPI-BW emulsion	O_2 barrier	Perez-Gago and others (2003b)
	WPI; WPC, HPMC; wax		Perez-Gago and others (2005)
Avocado	Methylcellulose	$O_2/CO_2/H_2O$ barrier	Maftoonazad and Ramaswamy (2005)
Carrot (peeled)	Xanthan gum	H_2O barrier; Ca^{2+}, Vit. E carrier	Mei and others (2002)
	Calcium caseinate; WPI; pectin; CMC	H_2O barrier	Lafortune and others (2005)
	Alginate	H_2O barrier; microbial barrier	Amanatidou and others (2000)
Celery	Caseinate	$O_2/CO_2/H_2O$ barrier; carrier (antimicrobial)	Avena-Bustillos and others (1997)
Cherry	Semperfresh™	O_2/H_2O barrier	Yaman and Bayoindirli (2002)
	Caseinate; milk protein	$O_2/CO_2/H_2O$ barrier	Certel and others (2004)
Corn	Zein	Microbial barrier	Carlin and others (2001)
Green bell pepper	Lipid-based	$O_2/CO_2/H_2O$ barrier	Conforti and Ball (2002)
			Conforti and Zinck (2002)
Kiwifruit	Pullulan (bacterial polysaccharide from starch)	$O_2/CO_2/H_2O$ barrier	Diab and others (2001)
Lettuce	Alginate-based	O_2/CO_2 barrier	Tay and Perera (2004)
Litchi fruit (peeled)	Chitosan	O_2/H_2O barrier	Dong and others (2004)
	Chitosan	O_2 barrier	Jiang and others (2005)
Mango fruit	Wax; shellac; zein; cellulose derivative	$O_2/CO_2/H_2O$ barrier	Hoa and others (2002)
Mushroom	Alginate	O_2/H_2O barrier	Hershko and Nussinovitch (1998)
Citrus	Chitosan	$O_2/CO_2/H_2O$ barrier	Fornes and others (2005)
Peach	Wax; CMC	H_2O barrier	Toğrul and Arslan (2004)
Pear (cut wedges)	Methylcellulose-based	$O_2/CO_2/H_2O$ barrier, carrier (antioxidant)	Guadalupe and others (2003)
	Methylcellulose	$O_2/CO_2/H_2O$ barrier	Olivas and others (2003)
Plum	HPMC/lipid composite	$O_2/CO_2/H_2O$ barrier	Perez-Gago and others (2003a)
Potato	Caseinate; whey protein	O_2 barrier; carrier (antioxidant)	Le Tien and others (2001)
Quince	Semperfresh™	$O_2/CO_2/H_2O$ barrier	Yurdugül (2005)
Raspberry	Chitosan	H_2O barrier; Ca^{2+}, Vit. E carrier	Han and others (2004b)
Strawberry	Cactus mucilage	O_2 barrier	Del-Valle and others (2005)
	Caseinate-whey protein	microbial barrier	Vachon and others (2003)
	Chitosan	H_2O barrier; Ca^{2+}, Vit. E carrier	Han and others (2004a, 2004b)
	Chitosan; HPMC	H_2O barrier; carrier (antimicrobial)	Park and others (2005)
	Pullulan (bacterial polysaccharide from starch)	$O_2/CO_2/H_2O$ barrier	Diab and others (2001)
	Starch-based	H_2O barrier, carrier (antimicrobial)	Garcia and others (1998)
	Wheat gluten-based	O_2/H_2O barrier	Tanada-Palmu and Grosso (2005)
Water chestnut (fresh-cut)	Chitosan	O_2 barrier	Pen and Jiang (2003)
Zucchini	Semperfresh™	$O_2/CO_2/H_2O$ barrier	Kaynas and Ozelkok (1999)

CHAPTER 4

ANTIMICROBIAL PACKAGING

Although there are various types of active packaging systems that depend on different principles, antimicrobial food packaging has increased significantly during the past 10 years as an alternative method to control undesirable microorganisms on foods by means of the incorporation of antimicrobial substances in or coated onto the packaging materials (Appendini and Hotchkiss 2002). Antimicrobial packaging is the packaging system that is able to kill or inhibit spoilage and pathogenic microorganisms that are contaminating foods. The primary goals of an antimicrobial food packaging system are (i) safety assurance, (ii) quality maintenance, and (iii) shelf-life extension, which is the reversed order of the primary goals of conventional packaging systems (Ahvenainen 2003). Antimicrobial food packaging systems can be divided into two parts; package / food systems and package / headspace / food systems (Figure 4.1). In package / food system, packaging materials contact with the food surfaces or low viscosity or liquid food without headspace (Han, et al. 2000). Main migration phenomena in this system is the diffusion between the packaging material and food and partitioning at the interface. In package / headspace / food systems, antimicrobial agents have to be volatile because of the migration through the headspace. Unlike nonvolatile substances, volatile substances can migrate through the headspace and air gaps between the package and the food. In order to prevent microbial contamination of food surface, direct surface application of antimicrobial substances has been made. However, this kind of application causes neutralization of the active substances or diffusion from the surface into the food mass. The use of antimicrobial films is preferred because of slow migration or action of the substances onto the surface of the product. The major potential food applications for antimicrobial films include meat, fish, poultry, bread, cheese, fruits, vegetables, and beverages (Lopez-Rubio, et al. 2004, Vermeiren, et al. 1999). Nowadays, antimicrobial food packaging is based on one of the following concepts (Appendini and Hotchkiss 2002):

* Addition of sachet containing antimicrobial substances into package from which the volatile bioactive substance is released during further storage.

* Direct incorporation of volatile and non-volatile antimicrobial agents into the packaging films.

* Coating of the packaging with a matrix that acts as a carrier for the antimicrobial agent.

* Immobilization of antimicrobials to polymers by ion or covalent linkages.

* Use of polymers that are inherently antimicrobial.

Antimicrobial compounds include some inorganic (carbonates, bicarbonates, etc.) or organic acids and their salts (propionates, sorbates, benzoates, etc.), parabens, chitosan, enzymes, bacteriocins, polypeptides, and essential oils or other natural extracts are shown in Table 4.1.

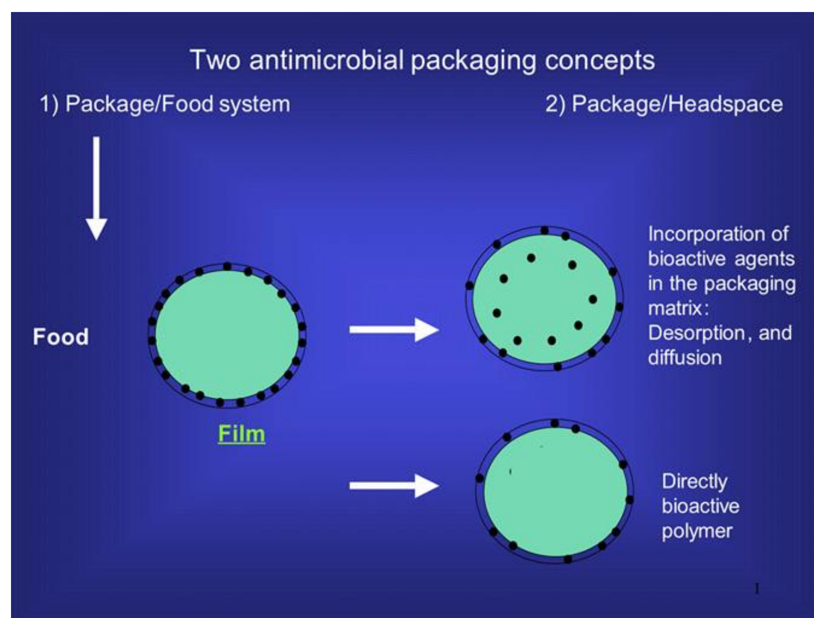

Figure 4.1. Antimicrobial packaging systems: package-food and package-headspace (Source: Coma 2008)

Table 4.1. Antimicrobial compounds used on edible films and coatings (Source: Valencia-Chamorro, et al. 2011)

Food preservatives			
Chemical compounds	E-Code[a]	Natural compounds	E-Code/ RegNum[b]
Organic acids		*Polypeptides*	
Acetic	E-260	Lysozyme	E-1105
Benzoic	E-210	Peroxidase	—
Citric	E-330	Lactoperoxidase	—
Lactic	E-270	Lactoferrin	—
Malic	E-296	Nisin	E-234
Propionic	E-280	Natamycin	E-235
Sorbic	E-200		
Tartaric	E-334		
Organic acid salts		*Plant extracts, essential oils, spices*	
Sodium acetate	E-262(I)	Cinnamon	182.10
Sodium diacetate	E-262(II)	Capsicum	182.10
Sodium benzoate	E-211	Lemongrass	182.20
Sodium citrate	E-331(I)	Oregano	182.10
Sodium formate	E-237	Rosemary	182.20
Calcium formate	E-238	Garlic	184.1317
Sodium L-lactate	E-325	Vanilla	182.10
Sodium propionate	E-281	Carvacrol	172.515
Calcium propionate	E-282	Citral	182.60
Potassium sorbate	E-202	Cinnamaldehyde	182.60
Sodium L-tartrate	E-335(I)	Vanillin	182.60
		Grape seed extracts	—
Parabens			
Methyl paraben	E-218		
Ethyl paraben	E-214		
Propyl paraben	E-216		
Sodium salt of methyl paraben	E-219		
Sodium salt of ethyl paraben	E-215		
Sodium salt of propyl paraben	E-217		
Mineral salts			
Sodium bicarbonate	E-500(I)		
Ammonium bicarbonate	E-237		
Sodium carbonate	E-500(II)		
Others			
EDTA-$CaNa_2$[c]	E-385		

[a]E-Code = code number for food additives approved by the European Union.
[b]RegNum = Regulation number in Title 21 of the U.S. Code of Federal Regulations where the chemical appears.
[c]EDTA-$CaNa_2$ = disodium calcium ethylenediaminetetraacetate.

4.1. Organic Acids and Salts

Organic acids are the most common synthetic antimicrobial agents and include acetic, benzoic, citric, fumaric, lactic, malic, propionic, sorbic, succinic, and tartaric acid, among others. Organic acids, natural constituents of many foods, have been used for a long time as additives in food preservation. Organic acids are either naturally present in fruits and vegetables or synthesized by microorganisms as a result of fermentation. The antimicrobial activity of organic acid is attributed to pH reduction, depression of internal pH of microbial cell by ionization of undissociated acid molecules, and disruption of substrate transport by altering cell membrane permeability or reduction of proton motive force (Eswaranandam, et al. 2004).

Benzoic acid and sodium benzoate; Benzoic acid is also called phenylformic acid or benzene-carboxylic acid. Sodium benzoate is the organic acid salt more widely used as antimicrobial food additives. It is soluble in most film solutions and remains active after film preparation. In the United States, benzoic acid and sodium benzoate are generally regarded as safe preservatives at levels up to 0.1%. The antimicrobial activity of benzoic acid and sodium benzoate is related to pH, and the most effective are the undissociated forms. Therefore, the use of these preservatives has been limited to those products that are acid in nature (Cagrı, et al. 2004).

Sorbic acid and potassium sorbate; Sorbic acid is a straight-chain unsaturated fatty acid. The carboxyl group of sorbic acid is highly reactive with calcium, sodium or potassium, and results in the formation of various salts and esters. Potassium sorbate, the most soluble form of sorbate is well known for its potent antifungal activity. Sorbic acid salts mostly used in carbohydrate and protein based edible films such as methylcellulose, whey protein isolate, and chitosan because they remain chemically active in the film matrix. The carboxyl group of sorbates forms hydrogen bonds with carbohydrate or protein chains in films (Cagrı, et al. 2004, Valencia-Chamorro, et al. 2011).

Propionic acid; Propionic acid is a naturally-occurring monocarboxylic acid. Salts of the acid have a slight cheeslike flavor. The antimicrobial activity of propionate salts is pH dependent, being also more effective at low pH because of the higher activity of the undissociated form. Propionic acid is primarily inhibitory to molds; however,

some yeasts and bacteria have also been satisfactorily controlled. Amounts of propionate used in foods are generally less than 0.4% (Valencia-Chamorro, et al. 2011).

Parabens; Parabens are produced from esterification of the carboxyl group of benzoic acid. Most parabens are active at pH 3.0 to 8.0 since they remain undissociated at pH values up to 8.5. Parabens having a longer alkyl chain show more antimicrobial activity than those having a shorter alkyl chain, which are inhibitorier to gram-positive than to gram-negative bacteria. However, they are generally more active against molds and yeasts than against bacteria (Cagrı, et al. 2004).

Different organic acids, included in different films, have been tested to suppress spoilage or pathogenic microorganisms in foods (Table 4.2).

Table 4.2. Organic acids incorporated into the films for inhibition of different microorganisms

Organic acids	Packaging materials	Microorganisms	References
Citric, lactic, malic acid	Soy protein	*L. monocytogenes, E. coli* O157:H7, *S. gaminara*	Eswaranandam et al., 2004
Acetic, propionic acid	Chitosan	Lactic acid bacteria, enterobacteriaceae, *L. sakei,S. liquefaciens*	Ouattara et al., 2000
Acetic, lactic, propionic, benzoic acid	Whey protein	*L. bulgaricus, S. thermophilus, E. coli, Salmonella* sp.	Manab et al., 2011
Benzoic, sorbic acid	Poly (ethylene-co-methacrylic acid)	*Aspergillus niger, Penicillium sp* .	Weng et al., 1999
p-aminobenzoic, sorbic acid	Whey protein	*L. monocytogenes, E. coli* O157:H7, *Salmonella* Typhimurium DT104	Cagrı et al., 2001
Sorbic acid	Low-density polyethylene	*E. coli, Staphylococcus spp* ., fungi, yeasts	Silveira et al., 2007

4.2. Polypeptides

Edible antimicrobial films produced by incorporating polypeptides, such as lysozyme, peroxidase, lactoperoxidase, lactoferrin, nisin, and natomycin, have been investigated recently (Table 4.3).

4.2.1. Lysozyme

Lysozyme is one of the most frequently used antimicrobial enzymes incorporated into packaging materials. It is an enzyme comprising 129 amino acids crosslinked by four disulfide bonds (Cagri, et al. 2004). This enzyme exhibits antimicrobial activity mainly against Gram-positive bacteria by splitting the bonds between *N*-acetylmuramic acid and *N*-acetylglucosamine of the peptidoglycan in their cell walls. Because of the protection of the cell wall by the outer membrane surrounding the peptidoglycan layer, Gram-negative bacteria are generally less sensitive than Gram-positive bacteria to lysozyme (Gucbilmez, et al. 2007, Valencia-Chamorro, et al. 2011). Plasticizers used in edible films help to stabilize lysozyme against heat through hydrophobic interactions that reduce the complete transfer of hydrophobic groups from an aqueous to a nonpolar environment. For this reason, lysozyme is mostly used for heat-processed films such as those prepared from corn zein (Cagri, et al. 2004).

4.2.2. Lactoperoxide

The lactoperoxidase system is a hemoprotein present in milk and in mammalians tears and saliva. It shows a broad antimicrobial spectrum because of its bactericidial and bacteriostatic effect on different bacteria, fungi, parasites, and viruses. The lactoperoxidase system consists of three components: lactoperoxidase, thiocyanate, and hydrogen peroxide. Lactoperoxidase catalyzes the oxidation of thiocyanate ion (SCN–) by the use of H_2O_2, generating oxidizing products such as hypothiocyanite (OSCN–) and hypothiocyanous acid (HOSCN), which inhibit microorganisms by the oxidation of sulphydryl (SH) groups of microbial enzymes and other proteins. The structural damage of microbial cytoplasmic membranes by the oxidation of SH-groups causes the eventual

death of the microbial cells (Campos, et al. 2011, Min, et al. 2006, Valencia-Chamorro, et al. 2011).

4.2.3. Lactoferrin

Lactoferrin, an iron-binding glycoprotein, is present in bovine milk and can bind two iron atoms per molecule. It inhibits microorganisms by binding iron and making this essential component unavailable to microorganisms. Some bacteria may be resistant to lactoferrin because of the presence of siderophores that cause the low-iron requirements. Lactoferricin B is the active region of lactoferrin. It was isolated from the N-terminal region of the molecule by means of the acid-pepsin hydroysis and comprises 25 amino acid residues. Inhibitory effect of lactoferricin may be related to its cationic nature that causes the alternation of membrane permeability (Min, et al. 2006, Cagrı, et al. 2004, Valencia-Chamorro, et al. 2011).

4.2.4. Nisin

Nisin is a natural, toxicologically safe, antibacterial food preservative. It is regarded as natural because it is a polypeptide produced by certain strains of the food-grade lactic acid bacterium *Lactococcus lactis* subsp. *lactis*, during fermentation. It shows antimicrobial activity against a wide range of Gram positive bacteria such as *L. monocytogenes* and *S. aureus*, and is particularly effective against spores. It shows little or no activity against Gram negative bacteria, yeasts, and moulds because of their less permeable cell walls. However, some treatments such as exposure to chelating agents, sub-lethal heat, osmotic shock and freezing of Gram negative bacteria weak the bacteria cell wall and make them susceptible to nisin. Nisin forms a complex with Lipid II, a precursor molecule in the formation of bacterial cell walls. The nisin–lipid II complex then inserts itself into the cytoplasmic membrane forming pores and allows the efflux of essential cellular components resulting in inhibition or death of the bacteria (Delves-Broughton 2005).

4.2.5. Natamycin

Natamycin, produced by *Streptomyces natalensis,* is a tetraene polyene macrolide. Natamycin has no effect on bacteria, but it is a natural antifungal agent against nearly all molds and yeasts. Natamycin has been used for many years in a large number of countries throughout the world as an authorized preservation treatment for cheeses and certain meat products such as dried sausages (Pintado, et al. 2010, Oliveira, et al. 2007).

4.3. Plant Extracts, Essential Oils, Spices

Plants, herbs, and spices, their derived essential oils and substances obtained from different extracts, contain a significant amount of compounds that are known to inhibit the metabolic activity of bacteria, yeasts, and molds. It has been known that essential oils of angelica, anise, carrot, cardamom, cinnamon, cloves, coriander, dill weed, fennel, garlic, nutmeg, oregano, parsley, rosemary, sage, or thymol have inhibitory effects against various spoilage or pathogenic bacteria, molds, and yeasts (Valencia-Chamorro, et al. 2011). Direct incorporation of essential oils to food such as meat products will result in the immediate reduction of the bacterial population but may alter the sensory characteristics of added food. Plants and spices are rich in phenolic compounds, such as flavonoids and phenolic acids. These compounds possess important biological effects, including antioxidant and antimicrobial properties. The availability of hydroxyl groups and conjugated double bonds in the reactive groups of phenolics determine the efficacy of natural extracts on pathogen inhibition. These natural compounds show lesser activity in food systems because of their strong interactions with food components (Coma 2008). Numerous researches showing the addition of phenolic compounds, plant extracts and essential oils and into edible antimicrobial film will be discussed in the next chapter.

Table 4.3. Polypeptides incorporated into the films for inhibition of different microorganisms

Polypeptides	Packaging materials	Microorganisms	References
Lysozyme	Zein	*E. coli* and *B. subtilis*	Güçbilmez et al., 2007
Lysozyme	PVOH	*Micrococcus lysodeikticus*	Buonocore et al., 2005
Lactoferrin, nisin	Polyvinylidene chloride copolymer	*L. monocytogenes*	Limjaroen et al., 2003
Lactoferrin, lysozyme, the lactoperoxidase system	Whey protein	*Salmonella enterica* *E.coli* O157:H7	Min et al., 2005
Nisin	Zein	*Lactobacillus plantarum*	Padgett et al., 2000
Natamycin	Cellulose polymeric	*Penicillium roquefortii*	Oliveira et al., 2007
Natamycin	Wheat gluten, methyl cellulose	*Aspergillus niger*, *Penicillium roquefortii*	Türe et al., 2008
Malic acid, nisin, natamycin	Whey protein	*P. aeruginosa*, *Y. lipolytica*, *L. monocytogenes*, *P. commune*, *P. chrysogenum*	Pintado et al., 2010

CHAPTER 5

NATURAL ANTIMICROBIAL COMPOUNDS

5.1. Phenolic Compounds

A large number of plants are used to overcome different diseases and possess antimicrobial activity. Aromatic plants such as herbs and spices are especially rich in their phenolic content, and have been widely used to extend the shelf life of foods and in traditional medicine as treatment for many diseases (Chun, et al. 2005). Polyphenols of plant origin have been reported to have a variety of biological effects, including antioxidant, anticarcinogenic, antiinflammatory and antimicrobial activities (Serra, et al. 2008). Phenolic compounds are complex class of chemicals including a hydroxyl group on a benzene ring. The plant phenols are aromatic secondary metabolites that contain a fundamental range of substances having an aromatic ring bearing one or more hydroxyl compounds. Plant phenols are defined based on metabolic origin and these substances derived from the shikimate pathway and phenylpropanoid metabolism (Ryan and Robards 1998). Although the presence of phenolic compounds is expansive along nature, respectable variation occurs between plant species. Phenolic compounds can be separated into different component classes listed in Table 5.1. The term phenolic acids represents the seven carbon benzoic acids ($C_6 - C_1$) and nine carbon cinnamic acids ($C_6 - C_3$). Hydroxycinnamic acid compounds occur most frequently as simple esters with hydroxy carboxylic acids or glucose. Hydroxybenzoic acid compounds are present mainly in the form of glucosides. *p*- hydroxybenzoic acid, protocatechuic acid, vanillic acid, gallic acid and syringic acid are the major benzoic acids. Phenolic acids may be conjugated with organic acids, sugars, amino compounds, lipids, terpenoids, or other phenolics. Many phenolic compounds are attached to sugar molecules and are called glucosides or glycosides, depending on the type of sugar. Vanillin is a single-ring phenolic compound derived from the breakdown of lignin. The coumarins contain an oxygen heterocyclic of six atoms fused with a benzene ring. Because they also possess the ($C_6 - C_3$) configuration, they can be considered along with the cinnamic acids. Coumarins are lactones of O - hydroxycinnamic acid. Some phenolic compounds occur

as polymers (often combined with glucose). Tannins are phenolic polymers that combine with the protein of animal skins (collagen) forming leather. Flavonoids are 3-ring phenolic compounds consisting of a double ring attached by a single bond to a third ring. These components enclose the flavones, flavonols, flavanones, dihydroflavonols, anthocyanins, chalcones, and iso-flavonoids.

Table 5.1. Phenolic classes in Plants

Phenolic classes	**Chemical Structure**
Simple phenols, benzoquinones	C_6
Phenolic acids	$C_6 - C_1$
Acetophenones, phenylacetic acids	$C_6 - C_2$
Hydroxycinnamic, phenylpropenes, coumarins, isocoumarins, chromones	$C_6 - C_3$
Naphthoquinones	$C_6 - C_4$
Xanthones	$C_6 - C_1 - C_6$
Stilbenes, anthraquinones	$C_6 - C_2 - C_6$
Flavonoids, isoflavonoids	$C_6 - C_3 - C_6$
Lignans, neolignans	$(C_6 - C_3)_2$
Biflavonoids	$(C_6 - C_3 - C_6)_2$
Lignins	$(C_6 - C_3)_n$
Condensed tannins	$(C_6 - C_3 - C_6)_n$

5.1.1. Vanillic Acid

Text Vanillic acid (4-hydroxy-3-methoxybenzoic acid) is a dihydroxybenzoic acid derivative and is an oxidized form of vanillin (Figure 5.1). It is also an intermediate in the production of vanillin from ferulic acid. Antimicrobial and antioxidant activities of vanillic acid were investigated (Merkl, et al. 2010). As a result, ethyl, propyl, and buthyl esters of vanillic acid highly effectively inhibit the growth of *B. cereus* and *S. cerevisiae.* Antimicrobial activity of vanillic acid was evaluated against Gram-positive and Gram-negative bacteria clinical isolates and it was concluded that vanillic acid showed higher antimicrobial activity these studied bacteria (Alves, et al. 2013). On the other hand, inhibitory effect of vanillic acid was tested against Gram-positive (*Streptococcus pyogenes* and *Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae*) and resulted in no growth inhibition of the four bacterial strains (Tafesh, et al. 2011).

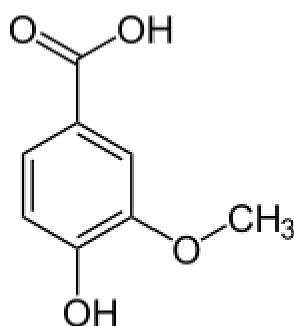

Figure 5.1. Chemical structure of Vanillic acid

5.1.2. Gallic Acid

Salicyclic acid and gentisic acid have an OH group ortho to the carboxylic acid function and gallic acid occurs as quinic acid esters in plants. Gallic acid exists in plant material in the form of free acids, esters, catechin derivatives and hydrolysable tannins (Figure 5.2). This phenolic acid is one of the most biologically- active phenolic compounds of plant origin. The antioxidant and antimicrobial activity of gallic acid and its derivatives has been reported in several studies (Karamac, et al. 2005). Antimicrobial effect of gallic acid on human pathogens (*S. aureus*, *C. accolans*), a plant pathogen (*E. carotovora*) and human pathogenic yeast (*Candida albicans*) was demonstrated by

Fogliani et al. (2005). The antifungal activity of gallic acid, isolated from *Oenothera biennis* roots, was also repoted by Shukla et al. (1999). In the study of Penna et al. (2001), different extracts of Argentine plants were obtained and quercetin, kaempferol, quercitrin, gallic acid, methylgallate and protocatechuic acid were identified as phenolic compounds. Methylgallate demonstrated activity against a number of Gram positive and Gram negative bacteria and fungi.

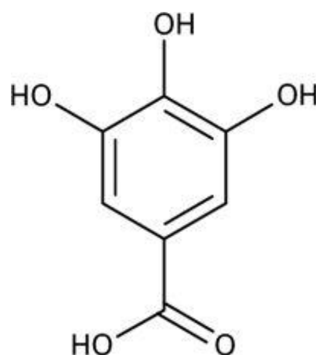

Figure 5.2. Chemical structure of Gallic acid

5.1.3. Cinnamic Acid

P- coumaric, caffeic, ferulic, and sinapic acids are also the most important cinnamic acids. Cinnamic acid can be found in the two isomeric forms cis- and trans-cinnamic acid because of the fact that they possess a double bond (Figure 5.3). It is a naturally occurring organic acid, found in some fruits and spices. It has antimicrobial activity against certain spoilage microorganisms and pathogenic bacteria. However, the acid is poorly soluble in water. Inclusion complexation of cinnamic acid with α-cyclodextrin greatly enhanced the solubility of the acid. The complexed cinnamic acid significantly contributed to the death of both *S. enterica* and *E. coli* O157:H7 (Truong 2007). A series of esters, substituted derivatives and amides of cinnamic acid were synthesized and evaluated for their in vitro antimicrobial activity against Gram positive *S. aureus, B. subtilis*, Gram negative *E. coli* and also against fungi *C. albicans* and *A. niger* (Narasimhan, et al. 2004). Antilisterial activity of cinnamic acid was also reported by Wen et al. 2003.

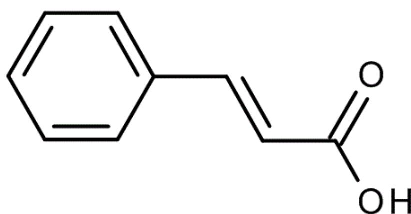

Figure 5.3. Chemical structure of Cinnamic acid

5.1.4. Antimicrobial Studies with Phenolic Compounds Derived from Plants

Different phenolic compounds obtained from plant material have been evaluated for their antimicrobial properties against several microorganisms (Table 5.2). Antimicrobial activity of artichoke leaf extracts was shown against several bacteria species, yeasts and molds by Zhu et al. (2004). Eight phenolic compounds were isolated from the n-butanol soluble fraction of artichoke leaf extracts: four caffeoylquinic acid derivatives, chlorogenic acid, cynarin, 3,5-di-O-caffeoylquinic acid, and 4,5-di-O-caffeoylquinic acid, and the four flavonoids, luteolin-7-rutinoside, cynaroside, apigenin-7-rutinoside, and apigenin-7-O-*â*-D-glucopyranoside, respectively. These compounds were analyzed for their antimicrobial activities on the several microorganisms and all eight compounds showed activity against most of the tested organisms. Antimicrobial capacity of walnut leaf was screened using different microorganisms, namely Gram negative and Gram positive bacteria and fungi by Pereira et al. (2007). The HPLC-DAD analysis of walnut leaves aqueous extracts revealed the presence of several hydroxycinnamic acid and flavonoid derivatives. The walnut leaves aqueous extracts were screened for their antimicrobial properties against *B. cereus, B. subtilis, S. aureus, E. coli, P. aeruginosa, K. pneumoniae, C. albicans* and *C. neoformans*. The results showed that the extracts inhibited only Gram + bacteria, although the tested Gram – bacteria and fungi species were resistant to walnut leave extracts. Negi and Jayaprakasha (2003) reported antibacterial activity of pomegranate extracts against Gram negative and Gram positive bacteria. The antibacterial activity of acetone, MeOH, and water extracts of pomegranate peels were tested against different bacteria. All Gram negative and Gram positive bacteria showed similar sensitivity against pomegranate extracts. Earlier studies have been shown the presence of gallic and ellagic acids in

pomegranate peel extracts. Due to the presence of these compounds, pomegranate peel extracts showed antibacterial activity against tested microorganisms. Apostolidis et al. (2008) found that natural antimicrobials from oregano and cranberry in combination with sodium lactate were able to control the growth of *L. monocytogenes* in broth and cooked ground beef. They concluded that the oregano-cranberry (50:50) combination with 2% sodium lactate among all the combination tested had the best inhibitory effect in broth and meat studies. It is thought that synergistic activity of the main phenolic compounds (ellagic acid and rosmarinic acid from cranberry and oregano, respectively) on the membrane could allow other smaller phenolic compounds and lactate to enter the cytosol through the membrane and act on specific enzymes involved in key energy pathways. Chun et al. (2005) compared the ethanol and water soluble phenolics of clonal oregano and heterogenous oregano from commercial sources. Antimicrobial activities of these extracts against gastric ulcer-associated bacterium *H. pylori* were analyzed. As a result of HPLC analysis, five major phenolic compounds were found and these were rosmarinic acid, caffeic acid, coumaric acid, protocatechuic acid and quercetin. It was found that the ethanol extracts of clonal and commercial oregano had higher activity against *H. pylori.* Klancnik et al. (2010) also investigated rosemary extract formulations with carnosic acid or rosmarinic acid, grape seed, olive leaves, green tea and sage extracts and extract mixtures on selected gram-positive (*S. aureus*, *B. cereus*, and *L. monocytogenes*) and gram- negative bacteria (*E. coli* O157:H7, *S. infantis*, *C. jejuni, C. coli*) with the disk diffusion, agar dilution, broth microdilution and macrodilution methods. Plant extracts with carnosic acid were more effective than plant extracts with rosmarinic acid. Grape seed, olive leaves, green tea extracts were much less efficient and sage more effective against all studied bacteria.

Table 5.2. Plant extracts and phenolic constituents using for inhibition of various spoilage or pathogenic microorganisms

Plant extracts and phenolic constituents	Microorganisms	References
Phenolic acids alkyl esters	*E. coli, B. cereus, L. monocytogenes, F. culmorum,* and *S. cerevisiae*	Merkl et al. (2010)
Benzoic and cinnamic acid derivatives; flavonoids; tannins	*S. aureus, S. epidermidis, E. faecalis, L. monocytogenes, E. coli, P. mirabilis* and *M. morganii*	Alves et al. (2013)
Ascorbic acid, tyrosol, protocatechuic acid, vanillic acid, caffeic acid, gallic acid, ferulic acid, hydroxytyrosol, 3,4-dihydroxyphenylacetic acid and p-coumaric acid	*S. pyogenes, S. aureus, E. coli* and *K. pneumoniae*	Tafesh et al. (2011)
Ellagic acid-4-*O*-β-D-xylopyranoside, mallorepanin, mallotinic acid along with corilagin, chebulagic acid, ellagic acid and gallic acid	*S. aureus, C. accolans, E. carotovora, C. albicans*	Fogliani et al. (2005)
Gallic acid	*F. fusiformi, F. semitectum* and *A. alternata*	Shukla et al. (1999)
Quercetin, kaempferol, quercitrin, gallic acid, methylgallate and protocatechuic acid	Gram positive and Gram negative bacteria and fungi	Penna et al. (2001)
Cinnamic acid	*E. coli* O157:H7, *S. enterica*	Truong et al. (2007)
Esters, amides and substituted derivatives of cinnamic acid	*E. coli* and *C. albicans*	Narasimhan et al. (2004)
Chlorogenic acid, hydroxycinnamic acids,caffeic acid, p-coumaric acid and ferulic acid	*L. monocytogenes*	Wen et al. (2003)

Table 5.2. (continued). Plant extracts and phenolic constituents using for inhibition of various spoilage or pathogenic microorganisms

Plant extracts and phenolic constituents	**Microorganisms**	**References**
Chlorogenic acid, cynarin, 3,5- di-O-caffeoylquinic acid, and 4,5-di-O-caffeoylquinic acid, and the four flavonoids, luteolin-7- rutinoside, cynaroside, apigenin-7-rutinoside, and apigenin-7-O-β-D-glucopyranoside from artichoke leaf extract	Gram-positive and Gram-negative bacteria, yeasts, and molds	Zhu et al. (2004)
3- and 5-caffeoylquinic acids, 3- and 4-pcoumaroylquinic acids, p-coumaric acid, quercetin 3-galactoside, quercetin 3-pentoside derivative, quercetin 3-arabinoside, quercetin 3-xyloside and quercetin 3-rhamnoside from walnut leaves	*B. cereus, B. subtilis, S. aureus, P. aeruginosa, E. coli, K. pneumoniae and C. albicans, C. Neoformans*	Pereira et al. (2007)
Phenolic compounds from pomegranate peel extract	*B. cereus, B. coagulens, B. subtilis, S. aureus, E. coli, and P. Aeruginosa*	Negi et al. (2003)
Water soluble phenolic extracts of oregano and cranberry	*L. monocytogenes*	Apostolidis et al. (2008)
Phenolic compounds from clonal oregano	*H. pylori*	Chun et al. (2005)
Rosemary extract with carnosic and rosmarinic acids	*S. aureus, B. cereus,L. monocytogenes, E. coli* O157:H7*, S. infantis, C. Jejuni, C. coli*	Klančnik et al. (2010)

5.1.5. Antimicrobial Films Incorporated with Phenolic Compounds Derived from Plants

The addition of phenolic compounds derived from plant extracts into film as a way of increasing the shelf life of food products has become increasingly popular (Table 5.3). Du et al. (2011) reported that apple edible film with apple skin polyphenols at concentration of 1.5 % was effective against *L. monocytogenes.* However, apple skin polyphenols did not show any antimicrobial activity at concentration of 10 % on *E. coli* O157:H7 and *S. enterica*. Preliminarily studies by Suppakul et al. (2003) showed the antimicrobial activities of linear low-density polyethylene (LLDPE) films containing linalool or methyl chavicol against the target microorganisms *S. aureus* (gram positive bacteria), *E. coli* (gram negative bacteria) and *S. cerevisiae* (yeast). The grape seed extract, nisin and EDTA incorporated soy protein edible film was found to have effective antimicrobial activities against *L. monocytogenes*, *E. coli* and *S. typhimurium* (Sivarooban, et al. 2008).

L. monocytogenes was more sensitive to various combinations of grape seed extract, nisin, and EDTA than the other two pathogens tested. The zein films containing different phenolic acids (gallic acid, p-hydroxy benzoic acid, ferulic acid), flavonoids (catechin, flavone, quercetin) were developed and tested against *L. monocytogenes* and *C. jejuni* by Arcan and Yemenicioğlu (2011).

They concluded that gallic acid containing zein film showed antimicrobial activity on these bacteria. Moreover, the zein and zein-wax composite films containing gallic acid between 2.5 and 10 mg/cm^2 were developed and antimicrobial effects of these films were tested on *C. jejuni* (Alkan, et al. 2011).

Incorporation of gallic acid at studied concentrations into films achieved the inhibition of isolated *C. jejuni*. Erdohan and Turhan (2011) reported that phenolics from olive leaf extract incorporated methyl-cellulose film have great potential in antimicrobial food packaging to reduce post-process growth of *S. aureus* and decrease staphylococcal food poisoning events. In the study of Rakchoy et al. (2009), the inhibitory effects of vanillin solutions for coating paperboard intended for packaging bakery products were investigated. For this purpose, three coating solutions; vanillin/dimethyl sulfoxide (DMSO) (10, 5, 2.5 and 1.25% (w/w)), vanillin/ethyl

alcohol (10, 5, 2.5 and 1.25% (w/w)) and vanillin/chitosan (10, 5, 2.5, 1.25, 0.625 and 0.3125 % (w/w)) were prepared. The inhibitory effects of all coating solutions were investigated against *E. coli*, *B. cereus* and *S. aureus* and significant inhibitory effects for all bacteria were observed.

Table 5.3. Plant extracts and phenolic constituents used in films and coatings showing antimicrobial activity

Plant extracts and phenolic constituents	**Edible film types**	**Microorganisms**	**References**
Apple skin polyphenol	Apple edible film	*L. monocytogenes, E. col and S. enterica*	Du et al. (2011)
Linalool or methyl chavicol	Low-density polyethylene film	*S. aureus, E. coli* and *S. cerevisiae*	Suppakul et al. (2002)
Phenols from grape seed extract	Soy protein edible film	*L. monocytogenes, E. coli* O157:H7 and *S. typhimurium*	Sivarooban et al. (2008)
Phenols from olive leaf extract	Methyl-cellulose film	*S. aureus*	Erdohan et al. (2011)
Vanillin	Vanillin/dimethyl sulfoxide (DMSO), vanillin/ethyl alcohol, and vanillin/chitosan	*E. coli, B. cereus* and *S. aureus*	Rakchoy et al. (2009)
Gallic acid	Zein-wax composite film	*C. jejuni*	Alkan et al. (2011)
Gallic acid, p-hydroxy benzoic acid, ferulic acids, flavonoids, catechin, flavone or quercetin	Zein film	*L. monocytogenes* and *C.*	Arcan et al. (2010)

5.2. Essential Oils and Plant Extracts

Essential oils are obtained from different parts of plant material by distillation methods, usually steam or hydrodistillation and responsible for the odour, aroma, and flavour of spices and herbs. They consist of terpenoids, specifically monoterpenes and sesquiterpenes, and a variety of low molecular weight aliphatic hydrocarbons (linear, ramified, saturated and unsaturated), acids, alcohols, aldehydes, acyclic esters or lactones and exceptionally nitrogen- and sulphur-containing compounds, coumarins and homologues of phenylpropanoids (Dorman and Deans 2000). Chemical composition of essential oil depends on the part of the plant (seed or leaves), the harvest time (before, during, or after flowering), the harvesting season and the geographical sources (Campos, et al. 2011). The advantage of essential oils is their bioactivity in the vapour phase, a characteristic that makes them attractive as possible antimicrobials for stored product protection. It is well known that essential oils from plants such as angelica, anise, carrot, cardamom, cinnamon, cloves, garlic, sage, oregano, rosemary, thyme, lemongrass are inhibitory to various spoilage or pathogenic bacteria, molds and yeasts (Cagrı, et al. 2004).

5.2.1. Oregano and Its Active Compounds; Thymol and Carvacrol

As an aromatic plant, oregano (*Origanum vulgare L.*) is an important plant widely used in medicine in the Mediterranean countries. The leaves, dried herbs as well as the volatile oil of oregano have been used as antimicrobial, anticoccidial, antifungal, antispazmolytic and antioxidant (Ertas, et al. 2005). The positive effects of oregano on human health are related to their essential oil and soluble phenolic fractions. Exarchou et al. (2002) found that the major component of ethanol extracts of oregano plant was rosmarinic acid and the presence of caffeic acid in trace amounts was confirmed (Figure 5.4 and 5.5). Rosmarinic acid is a caffeoyl ester and is shown to be an important antioxidant and anti-inflammatory compound. In addition to these compounds, flavonoids such as apigenin, quercetin and luteolin was detected on the HPLC chromatograms of oregano extract (Figure 5.6).

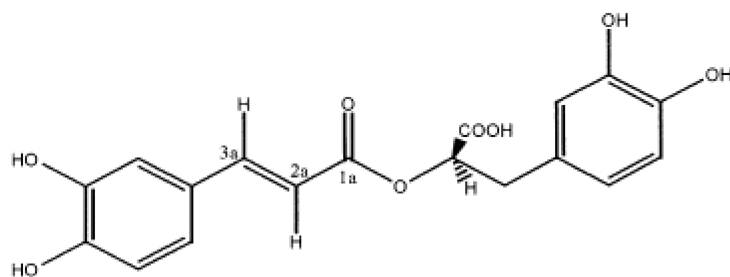

Figure 5.4. Chemical structure of Rosmarinic acid

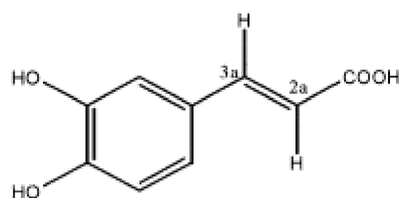

Figure 5.5. Chemical structure of Caffeic acid

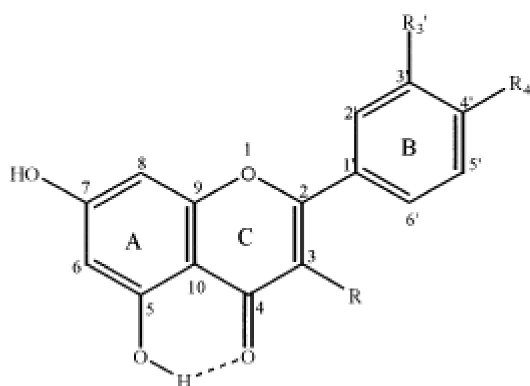

Figure 5.6. Chemical structures of Apigenin, Luteolin and Quercetin

The major component of essential oils of oregano is carvacrol, which was found to be as high as 60–75%. The other major component found in oregano oil is thymol (Figure 5.7). These two phenolic compounds, thymol and carvacrol, are generally reported in ratios of 1:10–1:20. The reported antimicrobial properties of carvacrol and thymol are 1.5 and 20.0 times that of phenol respectively. Seaberg et al. (2003) reported that thymol, carvacrol, and the clonal oregano line were all effective in inhibiting the

growth of *L. monocytogenes* in both broth and meat systems. For inhibition of *L. monocytogenes* in broth, 150-200 ppm of pure carvacrol or thymol was needed, while at least 1200 ppm (corresponding to 27.8 μg phenolics/ml) of the elite clonal oregano extract was needed. Inhibition of the growth of the pathogen in meat systems was achieved by using 800 ppm of the oregano extract. At 80 ppm, carvacrol alone did not have as significant effect as either oregano extract. The inhibitory effect of phenols could be explained by interactions with the cell membrane of micro-organisms and is often correlated with the hydrophobicity of the compounds. Hydrophobicity, the hydroxyl group and the presence of a system of delocalized electrons are important for the antimicrobial activities of carvacrol and thymol. Due to their appropriate hydrophobicity, carvacrol and thymol can be accumulated in the cell membrane and induce permeability alteration in the microorganisms' membranes with a consequent leakage of protons, phosphates and potassium. Their hydrogen-bonding ability and their proton-release ability may induce conformational modification of the membrane resulting in the cell death (Arfa, et al. 2006).

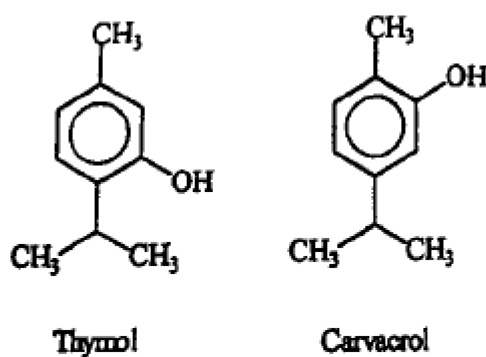

Figure 5.7. Chemical structures of thymol and carvacrol

5.2.2. Clove and Its Active Compound; Eugenol

Clove is an important plant that possesses antimicrobial properties (*Eugenia caryophyllata*). Clove oil obtained from the dried flower buds, stems and leaves of the clove tree by distillation is used as an anaesthetic for toothaches, headaches and joint pain. It is also used as a food flavouring agent, aromatherapy oil, mouth sterilizer or painkiller (Moon, et al. 2011). Clove essential oils contains 18 different components.

Eugenol (4-allyl-2-methoxyphenol), the active substance, comprises 90-95% of the essential oil extracted from cloves, and is classified as a food additive by the Food and Drug Administration (Figure 5.8). Other important essential oil constituents of clove oil include acetyl eugenol, beta-caryophyllene & vanillin crategolic acid tannins, gallotannic acid, methyl salicylate the flavonoids eugenin, kaempferol, rhamnetin, & eugenitin triterpenoids like oleanolic acid, stigmasterol & campesterol & several sesquiterpenes. Antiinflammatory, cytotoxic and anaesthetic properties of clove essential oil are related to its eugenol content (Gülçin, et al. 2010).

Eugenol ($C_{10}H_{12}O_2$), is an allyl chain-substituted guaiacol, i.e. 2-methoxy-4-(2-propenyl) phenol. Eugenol is a member of the allylbenzene class of chemical compounds. It is a clear to pale yellow oily liquid extracted from certain essential oils especially from clove oil, nutmeg and cinnamon and bay leaf. It is slightly soluble in water and soluble in organic solvents. It has a pleasant, spicy, clove-like odor. Eugenol has been used at least since the nineteenth century and is still used in perfumeries, as flavourings, in analgesics, biocides, antiseptics, and in local anaesthetic due to its anti-inflammatory, and antibacterial effects.

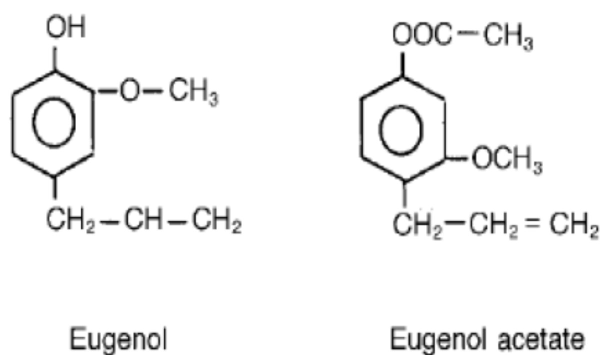

Figure 5.8. Chemical structures of Eugenol and Eugenol acetate

5.2.3. Lemongrass and Its Active Compound; Citral

Another important essential oil that has antimicrobial properties is lemongrass oil. Lemongrass is inhibitory to various spoilage or pathogenic bacteria, molds and yeasts. Lemongrass oil was investigated for activity against *S. aureus, B. cereus* , *B. subtilis, E. coli, K. pneumoniae* and *P. aeruginosa* by Naik et al. (2010). As a result of

that study, lemongrass oil was found to be effective against gram positive as compared to gram negative bacteria. *P. aeruginosa* was found to be highly resistant. Antimicrobial activity of lemongrass oil against microbes of environmental, clinical and food origin was evaluated in the study of Singh, et al. (2011). They concluded that all microbes are not equally susceptible to lemongrass oil as *Bacillus* spp. and streptococci among Gram positive and aeromonads and *E. tarda* among Gram negative bacteria are comparatively more susceptible to lemongrass oil than most of the other potentially pathogenic bacteria.

Lemongrass oil is characterized for monoterpenes compounds, and citral is the major component, present at levels of, approximately, 65-85%. Citral (3,7-dimethyl-2,6-octadienal) is the name given to a natural mixture of two isomeric acyclic monoterpene aldehydes: geranial (*trans*-citral, citral A) and neral (*cis*-citral, citral B) (Figure 5.9). In addition to citral, the lemongrass oil consists of small quantities of geraniol, geranylacetate and monoterpene olefins, such as myrcene (Tzortzakis and Economakis 2007, Silva, et al. 2008). It is insoluble in water but soluble in ethanol, diethyl ether, and mineral oil. It is used in perfumes and flavorings and in the manufacture of other chemicals.

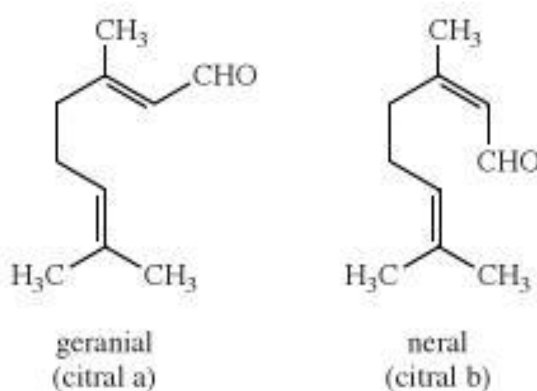

Figure 5.9. Chemical structure of the citral

5.2.4. Antimicrobial Studies with Essential Oils and Plant Extracts

Table 5.4 summarizes antimicrobial studies using essential oils and plant extracts against many microorganisms. The inhibitory effect of essential oils of Turkish oregano, bay laurel, Spanish lavender, and fennel on *E. coli* O157:H7, *L.*

monocytogenes, S. typhimurium, and *S. au*reus was documented (Dadalıoğlu and Evrendilek 2004). Seventeen constituents in the essential oil of Turkish oregano were determined, with the major components as 68.23% carvacrol, 11.84% *p*-cymene, 8.14% *γ*-terpinene, and 3.44% *β*-caryophyllene. Turkish oregano essential oils showed a higher antibacterial activity against the tested bacteria (except for *E. coli* O157:H7) due to the higher content of carvacrol. In another study, garlic oil, oregano extract and chitosan were evaluated to inhibit *S. enterica* under different thermal conditions (Marques, et al. 2008). This study demonstrated that the antimicrobial properties of garlic, oregano and chitosan against *S. enterica* were more effective at lower temperatures. Oregano had the strongest antibacterial properties, followed by chitosan and garlic.

Antimicrobial effect of clove oil and eugenol has been studied (Hsieh, et al. 2001). Various combinations of corni fructus, cinnamon and Chinese chive were used to evaluate their antimicrobial activities on common foodborne microorganisms, including bacteria, yeasts and moulds. When extracts of corni fructus, cinnamon and Chinese chive were combined at the ratio of 8:1:1 (v/v/v), the combined extract was found to have effective antimicrobial activity. The volatile oils of black pepper, clove, geranium, nutmeg, oregano and thyme were evaluated for antibacterial activity against 25 different genera of bacteria including animal and plant pathogens, food poisoning and spoilage bacteria (Dorman and Deans 2000). It was found that the component with phenolic structures, such as eugenol, was highly active against the test microorganisms. In addition, 21 terpenoids and the phenylpropanoid eugenol found in these volatile oils were also analyzed for antimicrobial activity. All the bacterial strains showed sensitivity to the volatile oils, although some microorganisms were uninhibited by volatile oils. *Erwinia carotovora* was inhibited by oils from black pepper, clove, nutmeg, oregano and thyme. Individual oil components with the widest spectrum of antibacterial activity were found to be thymol, carvacrol, α terpineol, terpinen-4-ol, and eugenol.

Arora and Kaur (1999) compared the sensitivity of some human pathogenic bacteria and yeasts to various spice extracts. Only garlic and clove extracts among different spices showed antimicrobial activity. Garlic extract had significant bactericidal activity against all tested organisms while clove only inhibited the growth of *Sh. flexneri*. All yeast cultures tested were sensitive to garlic and clove. Strong antimicrobial activity of garlic extract against yeasts and bacteria has been attributed to the oxidation of thiol groups present in essential proteins, causing enzyme inactivation

and inhibition of microbial growth. Eugenol and allicin has been found to be the major antimicrobial components present in clove and garlic extract, respectively. Another study demonstrated the inhibitory effect of clove oil on seven strains of *L. monocytogenes* on chicken frankfurters (Mytle, et al. 2006). All strains of pathogen on chicken frankfurters were inhibited with 1% and 2% clove oil under storage conditions at 5°C and 15°C.

Antimicrobial capacities of cinnamaldehyde and eugenol were investigated against 10 pathogenic and spoilage bacteria and three strains of yeast by Sanla-Ead et al. (2012). As a result of that study, minimum inhibitory concentration of eugenol in a range of 12.5−50 μl/ml inhibited the growth of all test microorganisms. Oyedemi et al. (2008) studied the mechanism of antimicrobial activity of essential oils components; α-terpineol, γ -terpinene and eugenol to evaluate their effect on the bacterial membrane against four strains of bacteria: *L. monocytogenes, S. pyogenes, P. vulgaris* and *E. coli*. The results showed that eugenol displayed a stronger effect on the protein leakage of cell membranes of *P. vulgaris* followed by *S. pyogenes* and *L. monocytogenes.* The efficacy of eugenol and cinnamaldehyde as anti *H.pylori* agents was evaluated by Ali et al. (2005). Results showed that eugenol and cinnamaldehyde at 2 μg/ml produced significant decrease of viability of the organism *H. pylori*. In another study, antibacterial activities of nisin, thymol and eugenol were assayed against four strains of common spoilage bacteria (Tippayatum and Chonhenchob 2007). In that study, it was shown that thymol and eugenol could inhibit all bacterial strains tested. These inhibitory effects of eugenol and thymol are related to their hydrophobicity. This property enables them to penetrate the lipopolysaccharide of the gram-negative bacterial cell membrane, and disturbed the cell structures. Therefore, leakage of ions and other cell contents occurred.

Studies on the antimicrobial, especially antibacterial and antifungal, activity of lemongrass oil and citral were reported. The antifungal activity of lemongrass and citral against *Candida* species was studied in order to estimate the possibility of using lemongrass oil as an antifungal agent for skin diseases (Silva, et al. 2008). Antifungal and antibacterial studies were carried out on citral and citral-epoxide by Saddiq and Khayyat (2010). Studies on the antifungal especially *Penicillium italicum* and *Rhizopus stolonifer* showed that citral and citral-epoxide have good antibacterial action. A more detailed study on antimicrobial compounds was done evaluating 52 plant oils and extracts (Hammer, et al. 1999). 52 plant oils and extracts were investigated for activity

against *A. baumanii*, *A. veronii* biogroup *sobria*, *C. albicans, E. faecalis, E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. enterica* subsp. *enterica* serotype *typhimurium, S. marcescens* and *S. aureus.* It was documented that lemongrass, oregano and bay inhibited all organisms at concentrations of 2.0% (v/v).

Table 5.4. Essential oils and plant extracts using for inhibition of various spoilage or pathogenic microorganisms

Essential oils	**Microorganisms**	**References**
Cinnamaldehyde and eugenol	pathogenic and spoilage bacteria	Sanla-Ead et al. (2012)
Garlic, ginger, clove, black pepper and green	Human pathogenic bacteria and yeasts	Arora et al. (1999)
Clove oil	*L. monocytogenes*	Mytle et al. (2006)
α- terpineol, γ -terpinene and eugenol	*L. monocytogenes, S. pyogenes, P. vulgaris* and *E. coli*	Oyedemi et al. (2009)
52 plant oils and extracts	Gram-positive, Gram-negative bacteria, a yeast	Hammer et al. (1999)
Black pepper, clove, geranium, nutmeg, orega	Gram-positive and Gram-negative bacteria	Dorman et al. (2000)
Eugenol and cinnamaldehyde	*H.pylori*	Ali et al. (2005)
Clove oil and eugenol	*B.subtilis, E. coli, Flavobacterium sp., L. monocytogenes, P. aeruginosa, S. typhimurium, S. aureus, V. parahaemolyticus, A. flavus and A. niger*	Hsieh et al. (2001)
Nisin, thymol and eugenol	pathogenic and spoilage bacteria	Tippayatum et al.(2007)
Lemongrass oil	pathogenic bacteria	Naik et al. (2010)
Lemongrass oil	molds, yeasts and bacteria	Singh et al. (2011)
Lemongrass oil and citral	*Candida* spp.	Silva et al. (2008)
Citral	*Penicillium italicum, Rhizopus stolonifer and S. aureus*	Saddiq et al. (2010)
Garlic oil, oregano extract, chitosan	*Salmonella enterica*	Marques et al. (2006)
Oregano, bay laurel, lavender, fennel oil	*E. coli O157:H7, L. monocytogenes,S. typhimurium, S. aureus*	Dadalıoğlu et al. (2004)
Oregano extract	*L. monocytogenes*	Seaberg et al. (2003)

5.2.5. Antimicrobial Films Incorporated with Essential Oils and Plant Extracts

Essential oils can be added to edible films to modify flavor, aroma and odor and to introduce antimicrobial properties. Antimicrobial studies including edible films wit essential oils and plant extracts are shown in Table 5.5. Antimicrobial properties of whey protein isolate films containing different ratios of oregano, rosemary and garlic essential oils were tested against several microorganisms (Seydim and Sarıkuş 2006). The incorporation of oregano and garlic oil into whey protein-based films was found to be effective against *S. aureus*, *S. enteritidis*, *L. monocytogenes*, *E. coli* and *Lactobacillus plantarum*. However, the use of rosemary essential oil did not exhibit any antimicrobial activity. An antimicrobial packaging material based on the combination of the most active compounds of essential oils (hydrocinnamaldehyde, oregano essential oil, cinnamaldehyde, thymol, and carvacrol) together with some aromas commonly used in the food industry was developed by Gutierrez et al. (2009). In another study, the antimicrobial activity in the vapor-phase of laboratory-made flexible films of polypropylene (PP) and polyethylene/ethylene vinyl alcohol copolymer (PE/EVOH) incorporating essential oil of cinnamon (*Cinnamomum zeylanicum*), oregano (*Origanum vulgare*), clove (*Syzygium aromaticum*), or cinnamon fortified with cinnamaldehyde was evaluated against a wide range of microorganisms (Lopez, et al. 2007). Films containing cinnamon or oregano essential oil (4% w/w) completely inhibited the growth of the fungi while higher concentrations were necessary to inhibit the Gram-positive and Gram-negative bacteria.

In addition, Altıok et al. (2010) tested the growth inhibition effects of the films containing different amount of thyme oil on the gram negative microorganisms of *E. coli, K. pneumoniae, P. aeruginosa* and a gram positive microorganism of *S. aureus*. The minimum thyme oil concentration in chitosan films showing the antimicrobial activity on all microorganisms used in the study was found as 1.2% (v/v). An antimicrobial multilayered polyethylene film was fabricated by incorporating grapefruit seed extract and tested in its antimicrobial activity against several selected microorganisms, and then applied to the packaging of ground beef (Ha, et al. 2001). Films with grapefruit seed extract effectively inhibited the microbial growth on

packaged beef at 3°C. Studies of Jutaporn et al. (2011) showed the antimicrobial effect of hydroxylpropylmethyl cellulose (HPMC) films containing phayom wood (*Shorea tolura*) extract and kiam wood (Cotylelobium lanceolatum craih) extract against *Escherichia coli* O175:H7, *Staphylococcus aureus* and *Listeria monocytogenes.* The antimicrobial effectiveness of oregano, cinnamon, and lemongrass oils in apple puree film-forming solution against *E. coli* was evaluated (Rojas-Grau, et al. 2006). It was concluded that the antimicrobial activity of oregano oil in apple purees edible films and film-forming solutions against *E. coli* O157:H7 was significantly greater than the activities of cinnamon oil and lemongrass oil. In the study of Armendariz et al. (2010), the antimicrobial activity of the zein films with carvacrol and eugenol against food pathogens was compared. They concluded that the interactions of essential oils and the protein matrix had a crucial effect on the antimicrobial activity of active films.

Antimicrobial efficacy of hydroxylpropyl methylcellulose edible film incorporated with encapsulated clove oil was assessed against *E. coli* O175:H7, *S. aureus* and *L. monocytogenes* (Nonsee, et al. 2011). The results showed that the minimum bactericidal concentration of clove oil inhibited tested microorganisms were 0.5, 1.0, and 1.5%. Ponce et al. (2008) studied the antimicrobial activity of chitosan, casein and carboxymethyl cellulose films, alone as well as enriched with oleoresins (olive, rosemary, onion, capsicum, cranberry, garlic, oreganum and oreganum + carvacrol 5%), on the microflora of butternut squash and on *L. monocytogenes*. It was found that the only oleoresins showing antimicrobial activity against both squash native microflora and *L. monocytogenes* were olive and rosemary. Capsicum showed activity against *L. monocytogenes* and oreganum+carvacrol against the native microflora. Effect of thyme, clove and cinnamon essential oils at 0.5, 1 and 1.5% v/v on antimicrobial properties of chitosan-based films was examined (Hosseini, et al. 2008). Chitosan-based films incorporated with thyme essential oil produced a larger inhibition zone than those of incorporated with clove and cinnamon essential oils. Films were more effective against Gram-positive bacteria than Gram-negative bacteria due to the differences of cell wall structures of Gram-positive and Gram-negative bacteria.

Edible films based on fish-skin gelatin incorporated with chitosan and/or clove essential oil were produced and their antimicrobial activity was tested on *L. acidophilus, P. fluorescens, L. innocua*, and *E. coli* (Gomez-Estaca, et al. 2009). The single gelatin film and the complex gelatin-chitosan film showed any antibacterial

activity while the films incorporating the clove essential oil showed measurable antimicrobial activity against the four microorganisms tested. Emiroğlu et al. (2010) evaluated the antibacterial activity of soy protein edible films containing 1, 2, 3, 4 and 5% thyme and oregano essential oils against *E. coli, E. coli O157:H7, S. aureus, P. aeruginosa* and *L. plantarum* and effects of these films on microbiological characteristics of fresh ground beef during refrigerated storage. It was found that thyme and oregano essential oils inhibited all test organisms even at minimum concentration (1%). Although no significant differences in total viable counts was observed between the ground beef samples in general. Du et al. (2008) reported the antibacterial activity of edible films made from tomatoes containing carvacrol against *E. coli O157:H7.* They observed no growth around the film discs containing 0.75% or 1.0% carvacrol.

Raybaudi-Massilia et al. (2008) prepared alginate-based edible coating containing malic acid and essential oils of cinnamon, palmarosa and lemongrass and their main active compounds eugenol, geraniol and citral to improve the shelf-life of fresh-cut melon. The incorporation of essential oils or their active compounds into the edible coating prolonged the microbiological shelf-life by more than 21 days. In the study of Maizura et al. (2007), the incorporation of lemongrass oil (0.1% to 0.4%,v/w) into edible films prepared from a mixture of partially hydrolyzed sago starch and alginate was found effective against *E. coli O157:H7.* It is known that the antimicrobial activity of lemongrass oil is related to high amounts of 1,8-cincole (>30%) and citral isomers (geranial, >30%, neral, >20%). Antimicrobial activity of apple-based edible films containing different concentrations (0.5%, 1.5%, and 3%) of cinnamaldehyde or carvacrol was evaluated against *S. enterica* and *E. coli O157:H7* on chicken breasts and *L. monocytogenes* on ham (Ravishankar, et al. 2009). It was reported that the antimicrobial containing films showed a strong concentration-dependent inhibition of both *E. coli* O157:H7 and *S. enterica* on chicken breast samples after 72 h storage at both room and refrigeration temperatures. Carvacrol containing films exhibited stronger activity than did films containing cinnamaldehyde against *L. monocytogenes* on cooked ham. Antibacterial activities of soy protein isolate edible films incorporated with 1, 2, 3, 4, 5 and 6 % cinnamaldehyde, eugenol and cinnamaldehyde-eugenol (1:1) were evaluated against *E.coli* (Jiang 2012). It was found that the addition of essential oil monomers in the edible films resulted in an increase of antibacterial activity with an increasing concentration of essential oil monomers. The synergistic antimicrobial

activity of eugenol incorporated polyhydroxybutyrate (PHB) film (AM) along with pediocin (concentrated and lyophilized) was investigated using culture broth systems against spiked food spoilage bacteria and fungi by Narayanan et al. (2012). Results showed that eugenol–PHB films in conjunction with crude preparations of pediocin can be used to control the growth of foodborne pathogens, food spoilage microorganisms, as well as food contaminating molds effectively. In another study, cinnamaldehyde-incorporated and eugenol-incorporated methyl cellulose films were prepared to obtain active antimicrobial packaging materials (Sanla-Ead, et al., 2012). These antimicrobial cellulose-based packaging films were investigated for antimicrobial activity against target microorganisms using both an agar-disc diffusion technique and a vapour diffusion technique. At a concentration of 50 µl/ml, cinnamaldehyde and eugenol revealed antimicrobial activity against all test strains. The antimicrobial activity of the films consisting of ethylene-vinyl alcohol copolymer structures with oregano essential oil and citral against the pathogenic microorganisms *E. coli, S. enterica* and *L. monocytogenes* and natural microflora was investigated "in vitro" and also on the food itself (Galet, et al. 2012). As a result, the "in vitro" assay with ethylene-vinyl alcohol copolymer films with oregano essential oil and citral produced excellent results against *E. coli, S. enterica* and *L. monocytogenes*, although the antimicrobial effects observed on salad were lower due to agent/food matrix interactions.

Table 5.5. Essential oils and plant extracts used in films and coatings showing antimicrobial activity

Essential oils and plant extracts	**Edible film types**	**Microorganisms**	**References**
Oregano, rosemary and garlic essential oil	whey protein isolate film	*S. aureus* , *S. enteritidis* , *L. monocytogenes* , *E. coli* and *L. plantarum*	Seydim et al. (2006)
Hydrocinnamaldehyde, oregano essential oil, cinnamaldehyde, thymol, and carvacrol	polypropylene film	*E. faecalis, L. monocytogenes, B. cereus, S.aureus, S. choleraesuis, Y. enterocolitica, E. Coli, yeasts and molds*	Gutierrez et al. (2009)
Cinnamon, oregano and clove essential oil	polypropylene and polyethylene/ethylene vinyl alcohol copolymer film	Gram-positive and Gram-negative bacteria	Lopez et al. (2007)
Carvacrol and eugenol	zein film	food pathogens	Armendariz et al. (2010)
Cinnamaldehyde and eugenol	Cellulose-based film	pathogenic and spoilage bacteria	Sanla-Ead et al. (2012)
Citral	ethylene-vinyl alcohol copolymer	*E. coli, S. enterica and L. monocytogenes*	Galet et al. (2012)
Thyme oil	chitosan film	*E. coli, K. pneumoniae, P. aeruginosa* and *S. aureus*	Altıok et al. (2010)
Grapefruit seed extract	polyethylene film	*S. aureus, E. coli, B. Subtilis*	Ha et al. (2001)
Phayom wood extract and kiam wood extract	hydroxylpropylmethyl cellulose	*E. coli* O175:H7, *S. aureus* and *L. monocytogenes*	Jutaporn et al. (2011)
Cinnamaldehyde-eugenol	soy protein isolate film	*Escherichia coli*	Jiang et al. (2012)
Eugenol	polyhydroxybutyrate film	food spoilage microorganisms	Narayanan et al. (2012)

Table 5.5 (continued). Essential oils and plant extracts used in films and coatings showing antimicrobial activity

Essential oils and plant extracts	**Edible film types**	**Microorganisms**	**References**
Oregano, cinnamon, and lemongrass oils	apple puree film	*E. coli* O157:H7	Rojas-Grau et al. (2006)
Clove oil	hydroxylpropyl methylcellulose film	*E. coli* O175:H7, *S. aureus* and *L. monocytogenes*	Nonsee et al. (2011)
Olive, rosemary, onion, capsicum, cranberry, garlic, oreganum and oreganum + carvacrol 5%	chitosan, casein and carboxymethyl cellulose films	*L. monocytogenes*	Ponce et al. (2008)
Thyme, clove and cinnamon essential oils	chitosan film	Gram-positive and Gram-negative	Hosseini et al. (2008)
Clove essential oil	fish-skin gelatin with chitosan	*L. acidophilus, P. fluorescens, L. innocua*, and *E. coli*	Gomez-Estaca et al. (2009)
Thyme and oregano essential oils	soy protein edible film	*E. coli, E. coli O157:H7, S. aureus, P. aeruginosa* and *L. plantarum*	Emiroğlu et al. (2010)
Carvacrol	tomatoes edible film	*E. coli O157:H7*	Du et al. (2008)
Malic acid, essential oils of cinnamon, palmarosa and lemongrass, eugenol, geraniol and citral	alginate film	*Salmonella Enteritidis*	Raybaudi-Massilia et al. (2008)
Lemongrass oil	sago starch and alginate film	*E. coli O157:H7*	Maizura et al. (2007)
Cinnamaldehyde or carvacrol	apple edible film	*S. enterica, E. coli O157:H7 and L. monocytogenes*	Ravishankar et al. (2009)

CHAPTER 6

MATERIALS AND METHODS

6.1. Materials

6.1.1. Bacterial cultures

The four plant pathogenic bacteria; *E. carotovora* (RK-EC-462), *X. vesicatoria* (RK-XCV-110C), *E. amylovora* (RK-EA-228) and *P. syringae* (P.syr-RK-453) were used in this study. The isolates were kindly provided by Assoc. Prof. Recep Kotan from the culture collection in Department of Agricultural Faculty at Atatürk University, Turkey.

6.1.2. Chemicals

Pure phenolic acids and essential oils used in film making and chemicals used for determination of TPC, antimicrobial activity of films are given in Table 6.1. Nutrient agar used in antimicrobial tests was obtained by adding 1.4% agar (Applichem, Darmstadt, Germany) in nutrient broth prepared according to the user's manual. All other chemicals were reagent grade. Phenol extracts of residues of walnut hull and artichoke, oregano, and clove were obtained by an extraction method.

Table 6.1. Chemicals used in the analysis

NO	CHEMICAL	CODE
	Total Phenolic Content	
1	Gallic acid	Sigma Aldrich-G7384
2	Folin–Ciocalteu reagent	Fluka-47641
3	Sodium carbonate (Na_2CO_3)	Riedel-deHaen
	Film Making	
4	Zein	Sigma Aldrich-Z3625
5	Ethanol	Merck (Darmstadt, Germany)
6	Glycerol	Merck-K32593692
7	Gallic acid	Sigma Aldrich-G7384
8	Cinnamic acid	Sigma Aldrich-C80857
9	Vanillic acid	Sigma Aldrich-S57989-508
10	Carvacrol	Sigma Aldrich-W224502
11	Thymol	Sigma Aldrich-T0501
12	Eugenol	Sigma Aldrich-E51791
13	Citral	Sigma Aldrich-W230316
	Antimicrobial Activity of Films	
14	Peptone water	Merck-VM643428-628
15	Nutrient broth	Merck-VM347843-444
16	Nutrient agar	Merck-VM440650-230
17	Agar	Sigma Aldrich-A7002

6.2. Methods

6.2.1. Extraction of Phenols from Plant Material

Extraction of phenols from plant materials was carried out according to the modified method adapted by Chun et al. (2005). One gram of dry plant materials (oregano, clove, residues of walnut and artichoke) were stirred in 100 ml of ethanol (60%) at room temperature for 24 h. The mixture was centrifuged at 9000 rpm for 14 min. The supernatant was evaporated and lyophilized to obtain plant extract.

6.2.2. Total Phenolic Content (TPC)

Determination of TPC of extracts was carried out described in Singleton and Rossi (1965). The TPC values of plant extracts were determined using the Folin–Ciocalteu reagent. The reaction mixture contained 100 µl of plant extracts, 1 ml of the Folin–Ciocalteu reagent and 0.8 ml of sodium carbonate (20% w/v). The final volume was made up to 2 ml with distilled water. After 2 h of reaction, the absorbance at 765 nm was measured. A reference curve was constructed, using GA as standard. The TPC was expressed as milligrams of GA equivalents (GAE) per gram of the extract.

6.2.3. Determination of Minimum Inhibitory Concentration (MIC)

The MIC of the phenolic acids and plant extracts was determined in liquid medium using 96-well microplates. A stock solution of phenolic acids and plant extracts was prepared in nutrient broth and then two-fold dilutions of these phenols (in a concentration range from 0.01 to 5.12 mg/ml) and extracts (in a concentration range from 0.01 to 40.96 mg/ml) were made. The inoculums of microorganisms were prepared using 48 h cultures and suspensions were adjusted to 2 McFarland standard turbidity. The wells of 96-well plate were filled with 10 µl of inoculants, 90 µl of Nutrient broth, and 100 µl of various concentrations of GA, CA, VA and plant extracts. Three wells were prepared for each concentration of compounds. A positive control (containing inoculums but no phenol or extract) and negative control (containing phenol or extract but no inoculums) were included on each microplate. Plates were incubated at 27°C for 24 h under aerobic conditions in a Varioskan Flash (Thermo).

6.2.4. Film Making

Zein films were produced as described in Padgett et al. (1998). Briefly, 1.4 g corn zein was dissolved with 8.2 mL of ethanol (97 %) by mixing at 200 rpm with a magnetic stirrer for 25 min. 0.4 mL glycerol was then added to the medium and the temperature of the mixture was increased until it started to boil. The mixing was then ceased and the film solution was boiled for 5 min. After cooling to room temperature, different amounts of pure GA, CA, VA, CAR, THY, EUG, and CIT were added into

film forming solutions to obtain 0.25, 0.5, 1, 2 and 4 mg phenol per cm^2 of dried films. Different amounts of GA, CA, and VA was added to film solution to obtain Zein-GA-CA, Zein-GA-VA, and Zein-CA-VA mixture films at different concentrations. Studied film contents are listed in Table 6.2 and 6.3. Phenol extract of clove were added to film solution to obtain 0.25, 0.5, 1, 2, 4, 6 and 8 mg phenol extract per cm^2 of dried films. The mixtures were then homogenized (Heidolph, Germany, rotor Φ=6.6 mm tip) at 10000 rpm for 4 min and their 4.3 g portions were poured into glass templates (W x L x H: 8.5 x 8.5 x 0.4 cm). The films used in release test and antimicrobial test were dried at 25°C for 19 h in an incubator, while films used in mechanical testing were kept at the same temperature for 24 h under 50% relative humidity using a controlled test cabinet (TK 120, Nüve, Turkey) after drying at 25°C for 19 h in an incubator. The dried films were peeled from the glass templates carefully and used in different tests.

Table 6.2. Phenolic acids contents of zein films

	Film composition		
	GA (mg/cm^2)	CA (mg/cm^2)	VA (mg/cm^2)
Zein-GA			
	0.25	−	−
	0.5	−	−
	1	−	−
	2	−	−
	4	−	−
Zein-CA			
	−	0.25	−
	−	0.5	−
	−	1	−
	−	2	−
Zein-VA			
	−	−	0.25
	−	−	0.5
	−	−	1
	−	−	2
	−	−	4
Zein-GA-CA			
	0.25	0.25	−
	0.5	0.5	−
Zein-GA-VA			
	0.25	−	0.25
	0.5	−	0.5
Zein-CA-VA			
	−	0.25	0.25
	−	0.5	0.5

Table 6.3. Essential oils contents of zein films

	Film composition			
	CAR (mg/cm^2)	THY (mg/cm^2)	EUG (mg/cm^2)	CIT (mg/cm^2)
Zein-CAR				
	0.25	–	–	–
	0.5	–	–	–
	1	–	–	–
	2	–	–	–
	4	–	–	–
Zein-THY				
	–	0.25	–	–
	–	0.5	–	–
	–	1	–	–
	–	2	–	–
	–	4	–	–
Zein-EUG				
	–	–	0.25	–
	–	–	0.5	–
	–	–	1	–
	–	–	2	–
	–	–	4	–
Zein-CIT				
	–	–	–	0.25
	–	–	–	0.5
	–	–	–	1
	–	–	–	2
	–	–	–	4

6.2.5. Antimicrobial Activity of Films against Plant Pathogens

Fifteen discs (1.3 cm in diameter) were prepared from films by a cork borer under aseptic conditions. During tests, 3 discs were placed carefully onto each Petri dish containing nutrient agar on which inoculated with different plant pathogens. The inoculums of microorganisms were prepared in peptone water using a 48 h culture of plant pathogens incubated at aerobic conditions at 27°C. Before tests, the cell concentration was set to 0.5 McFarland (corresponded to 150 x 10^6 cfu/ml) and Petri dishes were inoculated by spread plate method by using 0.2 ml of culture diluted 1:10 with peptone water. The inoculated Petri dishes containing film discs were incubated at

aerobic conditions at 27°C for 24h and the diameter of the zones formed was measured by using a caliper. Results were expressed as average zone areas (mm^2).

6.2.6. Release Tests in Distilled Water

The release tests in distilled water were conducted to understand diffusion profiles of GA, and VA from zein films and determine free (untrapped or unbound) phenolic content within the films. The tests were conducted by placing films (4 cm × 4 cm) in distilled water at 4 ºC in shaken (80 rpm) Petri dishes. As a sample, peeled films containing clove extract at different concentrations in glass template is shown in Figure 6.1. The tests last for 13 days, until reaching equilibrium or until an insignificant increase occurred in phenolic concentration by time. For sampling, 300 μL of sample was taken at different time periods and assayed for TPC by a spectrophotometer (Shimadzu, model 2450, Tokyo, Japan) using standard Folin_Ciocalteu method described in Singleton et al. (1965). The reaction mixture contained 100 μl of sample, 1 ml of the Folin–Ciocalteu reagent (1/10) and 0.8 ml of sodium carbonate (7.5% w/v). The final volume was made up to 2 ml with distilled water. After 2 h of reaction, the absorbance at 765 nm was measured. Calibration curves were prepared by using pure GA and VA. The average of three measurements was used in calculations, and results were expressed as mg of GA released per cm^2 of GA (mg/cm^2), mg of VA released per cm^2 of VA films (mg/cm^2).

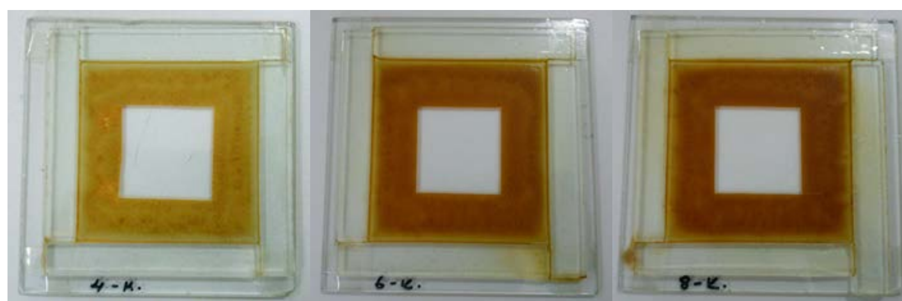

Figure 6.1. Peeled films containing clove extract used in released test (A: 4 mg/cm^2; B: 6 mg/cm^2; C: 8 mg/cm^2)

6.2.7. Mechanical Properties of Films

Tensile strength at break, elongation at break, and Young's modulus were determined using a Texture Analyzer TA-XT2 (Stable Microsystems, Godalming, United Kingdom) according to ASTM Standard Method D 882-02. Films were cut into 8 mm wide and 80 mm length strips. The initial grip distance was 50 mm, and the crosshead speed was 25 mm/min. Five replicates of each film were tested.

6.2.8. Scanning Electron Microscopy (SEM)

The photographs of film cross-sections and film thickness were determined by SEM (Philips XL 30S FEG, FEI Company, Netherlands). The films were prepared for SEM by crashing, following freezing in liquid nitrogen. The thickness of the films was measured from SEM cross-sectional views of films by using Scandium software (Olympus Soft Imaging Solutions).

6.2.9. HPLC Analysis

HPLC analysis was performed using a HPLC System (Perkin Elmer series 200 Shelton, CT USA), consisting of a binary pump and a diode-array detector (DAD) and equipped with a Nucleosil 100-C18 column (5 µm, 250 × 4 mm). Phenolic compounds in the clove extract were analyzed using HPLC method described by Shan et al. (2005). The HPLC method was with the following gradient elution program (solution A, 2.5% formic acid, and solution B, 100% methanol): 0 min, 5% B; 15 min, 30% B; 40 min, 40% B; 60 min, 50% B; 65 min, 55% B; and 90−95 min, 100% B. The flow rate was 0.8 mL/min, and the injection volume was 20 µL. Detection was at 280 nm for phenolic acids. Individual phenolics identified in the clove extract were quantified using HPLC by comparison with an external standard of corresponding GA and expressed as mg/g of extract. Standard curve was made from standard of the GA.

6.2.10. Statistical Analysis

Data including measurements obtained from mechanical properties of films were analyzed by analysis of variance (ANOVA). Fisher's protected least significant difference method was used for pairwise comparison of means. Differences were considered significant if $P < 0.05$.

CHAPTER 7

RESULTS AND DISCUSSION

7.1. Inhibition of Plant Pathogens by Edible Films Containing Phenolic acids

7.1.1. Minimum Inhibitory Concentrations (MIC) of Phenolic Acids

The MIC values of selected phenolic acids, GA, VA and CA, were tested on plant pathogens, *E. carotovora, E. amylovora, P. syringae,* and *X. vesicatoria* before they were used in zein film making. Solutions containing phenolic compounds were added to the culture media in order to reach final concentration ranging from 0.01 mg/mL to 5.12 mg/mL. Photometric readings at 600 nm wavelength for phenolic acids at different concentrations are shown in Appendix A. The MIC values of GA, VA and CA against studied plant pathogens were presented in Table 7.1. The MIC of GA, VA and CA on plant pathogens change between 2,56 and 5,12 mg/mL. The VA is the most effective inhibitor on all plant pathogens. The *E. amylovora* is more sensitive to GA than the other plant pathogen. However, *X. vesicatoria P. syringae E. carotovora* showed similar MIC values against phenolic acids at the test conditions.

Table 7.1. The MIC of phenolic acids against plant pathogens

	MIC (mg/mL)		
Plant pathogen	GA	VA	CA
E. amylovora	2.56	2.56	5.12
E. carotovora	5.12	2.56	5.12
X. vesicatoria	5.12	2.56	5.12
P. syringae	5.12	2.56	5.12

The effect of different concentrations of the phenolic compounds on the inhibition of pathogen growth and lag periods are given in Table 7.2. *E. carotovora* was inhibited by the presence of GA in the growth medium, at a concentration of 5,12 mg/mL. The addition of GA at a concentration of 2,56 mg/mL did not inhibit the growth

of *E. carotovora* but it delayed growth of this organism for 845 min. This result clearly showed the higher sensitivity of *E. carotovora* on *GA* than *X. vesicatoria* and *P. syringae* which have similar MICs against GA. On the other hand, although the CA showed almost similar lag periods on different plant pathogens, *E. amylovora* showed the lowest and *X. vesicatoria* showed the highest lag periods in presence of 2,56 mg/mL of CA. The lowest lag periods against VA were obtained against *E. carotovora* while *X. vesicatoria* showed the highest lag periods against this phenolic acid. These results clearly showed the different resistances of plant pathogens against phenolic acids at sub-inhibitory concentrations

Table 7.2. Resistances of plant pathogens to different concentrations of phenolic compounds by means of lag periods and inhibitions in their growth

		E. carotovora		E. amylovora		P. syringae		X. vesicatoria	
		Lag (min)	Inhibition (%)	Lag (min)	Inhibition (%)	Lag (min)	Inhibition (%)	Lag (min)	Inhibition (%)
GA (mg/mL)	0.01	135 ± 15	-13 ± 1.0	115 ± 9	11 ± 1.9	155 ± 9	-12 ± 3.5	125 ± 23	-10 ± 1.6
	0.02	125 ± 9	-16 ± 0.8	110 ± 9	6 ± 1.9	170 ± 17	-9 ± 2.5	205 ± 48	-10 ± 5.9
	0.04	120 ± 0	-16 ± 1.8	110 ± 9	2 ± 2.8	150 ± 15	-1 ± 5.4	225 ± 15	-12 ± 4.4
	0.08	130 ± 9	-14 ± 1.7	110 ± 9	-1 ± 4.7	150 ± 15	4 ± 1.0	715 ± 38	-45 ± 8.7
	0.16	135 ± 0	-7 ± 2.0	–	-12 ± 2.8	175 ± 23	–	–	-88 ± 1.6
	0.32	–	-7 ± 0.8	–	-7 ± 1.9	30 ± 0	-8 ± 2.9	–	-73 ± 1.6
	0.64	–	-3 ± 1.2	–	-14 ± 2.1	30 ± 0	14 ± 2.5	–	3 ± 0.0
	1.28	260 ± 17	-2 ± 1.0	45 ± 0	-62 ± 1.1	230 ± 38	11 ± 1.9	70 ± 17	32 ± 8.7
	2.56	845 ± 31	-16 ± 3.3	–	-97 ± 1.3	555 ± 15	-27 ± 5.9	580 ± 87	-68 ± 3.3
	5.12	–	-99 ± 0.1	–	-100 ± 0.1	–	-98 ± 0.0	–	-98 ± 0.2
CA (mg/mL)	0.01	65 ± 9	-13 ± 6.0	95 ± 9	3 ± 2.6	160 ± 9	-8 ± 2.6	190 ± 17	16 ± 5.1
	0.02	65 ± 9	-8 ± 5.4	105 ± 15	2 ± 1.7	155 ± 9	-3 ± 1.7	215 ± 9	14 ± 1.9
	0.04	75 ± 0	-16 ± 4.5	85 ± 9	-1 ± 1.0	155 ± 9	-3 ± 1.0	235 ± 31	14 ± 1.9
	0.08	65 ± 9	-16 ± 3.4	105 ± 0	-2 ± 1.7	150 ± 0	-5 ± 4.3	195 ± 30	17 ± 3.3
	0.16	60 ± 0	3 ± 11.9	95 ± 9	–	160 ± 9	–	140 ± 23	8 ± 1.9
	0.32	85 ± 9	6 ± 4.6	105 ± 0	-3 ± 0.0	160 ± 9	2 ± 1.7	185 ± 17	8 ± 1.9
	0.64	80 ± 17	3 ± 4.9	105 ± 0	-8 ± 1.7	165 ± 15	-1 ± 3.5	220 ± 9	3 ± 3.3
	1.28	100 ± 9	-23 ± 9.4	135 ± 0	-33 ± 7.1	175 ± 9	-5 ± 1.7	195 ± 15	-26 ± 13.5
	2.56	225 ± 40	-51 ± 2.7	200 ± 53	-62 ± 8.5	245 ± 9	-14 ± 2.6	280 ± 17	-42 ± 3.8
	5.12	–	-97 ± 0.7	–	-100	–	-100 ± 0.2	–	-100
VA (mg/mL)	0.01	65 ± 17	-5 ± 9.6	110 ± 9	7 ± 1.8	145 ± 9	-14 ± 1.0	190 ± 17	6 ± 2.8
	0.02	75 ± 0	1 ± 2.0	110 ± 9	5 ± 2.1	135 ± 0	-11 ± 2.0	150 ± 15	9 ± 1.6
	0.04	75 ± 0	1 ± 0.0	105 ± 0	3 ± 1.0	125 ± 9	-7 ± 2.0	160 ± 23	6 ± 2.8
	0.08	75 ± 15	-6 ± 6.6	100 ± 9	4 ± 1.8	135 ± 15	-3 ± 4.6	170 ± 9	7 ± 1.6
	0.16	105 ± 40	0 ± 5.6	110 ± 9	8 ± 3.7	120 ± 26	-2 ± 2.0	165 ± 15	2 ± 8.9
	0.32	90 ± 15	1 ± 4.0	115 ± 9	7 ± 1.8	135 ± 15	-4 ± 3.6	195 ± 15	12 ± 4.2
	0.64	90 ± 15	2 ± 3.4	120 ± 0	2 ± 1.0	140 ± 17	-1 ± 1.0	185 ± 17	18 ± 1.6
	1.28	150 ± 40	-17 ± 11.1	200 ± 9	-15 ± 5.7	210 ± 15	-11 ± 1.0	215 ± 31	8 ± 2.8
	2.56	–	-99 ± 1.5	–	-99 ± 2.1	–	-100	–	-100

% values refer the inhibitions in bacterial growth

Recently, Alves et al. (2013) evaluated the antimicrobial activities of sixteen phenolic compounds (phenolic acids, flavonoids and tannins) identified in different wild mushroom on selected Gram-positive and Gram-negative bacteria. Results of that study demonstrated that cinnamic acid derivatives, 2,4-dihydroxybenzoic, protocatechuic, vanillic and p-coumaric acids are potent antimicrobial compounds against tested bacteria. According to Alves et al. (2013), the antimicrobial activities of phenolic acids including benzoic and cinnamic acid derivatives are related to the presence of carboxylic acid (COOH), two hydroxyl (OH) groups in para and ortho positions of the benzene ring and the methoxyl (OCH3) group in the meta position.

In the study of Tafesh et al. (2011), phenolic compounds (tyrosol, protocatechuic acid, vanillic acid, caffeic acid, gallic acid, ferulic acid, *p*-coumaric acid, cinnamic acid, syringic acid, hydroxytyrosol, and 3,4-dihydroxyphenylacetic acid) were tested against Gram-positive reference strains *S. pyogenes* and *S. aureus* and Gram-negative reference strains *E. coli* and *K. pneumoniae.* Caffeic, ferulic, *p*-coumaric, cinnamic, vanillic, protocatechuic, and syringic acid caused no growth inhibition on four isolates up to 1000 μg/mL. Gallic acid at 200 and 400 μg/mL inhibited the growth of *S. pyogenes* and *S. aureus* strains whereas no growth inhibition was observed for the Gram-negative bacteria at these concentraitons.

The minimum inhibitory concentrations of alkyl esters (methyl, ethyl, propyl, butyl and hexyl) of *p*-hydroxybenzoic acid, protocatechuic acid, gentisic acid, vanillic acid, ferulic acid and caffeic acid against different bacteria including *E. coli*, *B. cereus*, *L. monocytogenes*, *F. culmorum*, and *S. cerevisiae* were determined by Merkl et al. (2010). These workers reported that the inhibitory concentrations of phenolic acids butyl esters were below 1.25mM. They generally concluded that the antimicrobial effect of phenolic acid derivatives increases with the increasing length of their alkyl chain. In order to evaluate antilisterial activity of cinnamic, p-coumaric, ferulic and caffeic acid, average minimum inhibitory concentrations of these phenolic acids were determined by Wen et al. (2003). Based on minimum inhibitory concentrations, cinnamic acid exhibited the strongest antilisterial activity, followed by p-coumaric, ferulic and caffeic acids. These results clearly proved the high antimicrobial potential of phenolic acids on different bacteria.

7.1.2. Antimicrobial Activity of Edible Films Containing Phenolic Acids

On the basis of MIC values of pure phenolic acids, antimicrobial zein films containing different concentrations of GA, VA, and CA were produced. The antimicrobial effect of GA, VA, and CA on *E. carotovora* was investigated by using 0.25, 0.5, 1, 2, and 4 mg phenolic compounds per cm^2 of films (Figure 7.1). The use of GA at 0.25 and 0.5 mg/cm^2 did not give measurable zones on *E. carotovora*. However, GA between 1 and 4 mg/cm^2 showed antimicrobial activity on the plant pathogen at a concentration dependent manner. In case of zein films containing VA, inhibition zones on *E. carotovora* were measured at each concentration. As the VA concentration increased, the zone of inhibition also increased significantly for *E. carotovora.* The greatest zone of inhibition was observed by using VA at 4 mg/cm^2. CA at 0.25 mg/cm^2 did not show any inhibitory effect against *E. carotovora*. Films containing 0.5, 1, and 2 mg/cm^2 concentrations of CA were effective against *E. carotovora.* Because of solubility problem, zein film incorporated with CA at 4 mg/cm^2 could not be tested. The results showed that zein film containing VA was the most effective (inhibition zone at each concentration) against *E. carotovora*. Inhibitory effect of CA at 0.5, 1, and 2 mg/cm^2 on *E. carotovora* was higher than effect of GA at the same concentrations.

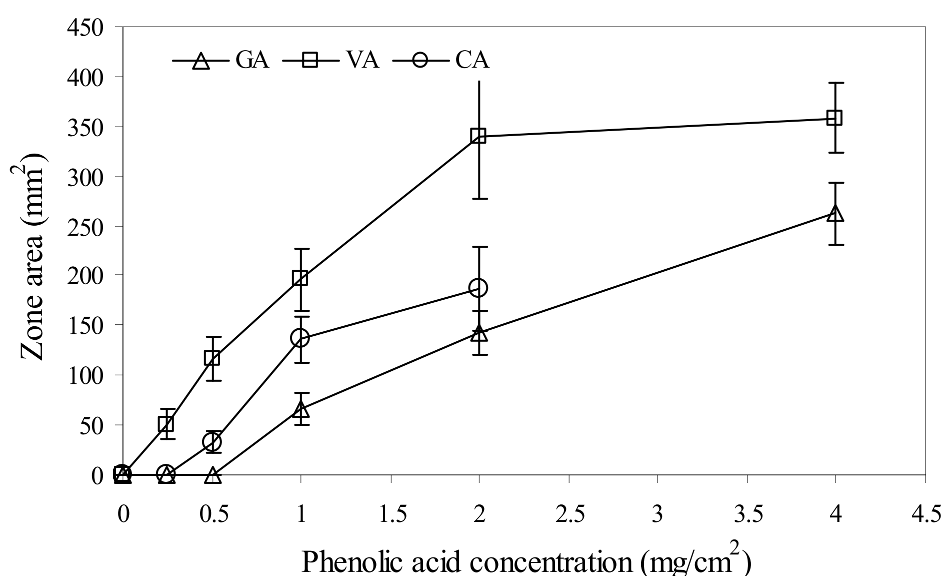

Figure 7.1. Antimicrobial activity of GA, VA, and CA containing zein films on *E. carotovora*

The antimicrobial activities based on zone areas of zein films containing different concentrations of VA, CA, and GA against *E. amylovora* are presented in Figure 7.2. As a result of antimicrobial test, it was observed that zein films with all of the three phenolic acids were highly effective against *E. amylovora*. The films containing 0.25 mg/cm^2 VA and CA did not show any antimicrobial activity on *E. amylovora*. In contrast, films containing GA at this same concentration showed good antimicrobial activity on this microorganism and formed 697.53 ± 69.96 mm^2 clear zones around tested discs. At the studied concentration range, a 2-fold increase in VA and CA concentration caused almost 1.5-2.1 fold increase in antimicrobial activity of films. Growth inhibition of films containing CA, GA and VA are shown in Figure 7.3, Figure 7.4, and Figure 7.5. GA inhibited the growth of *E. amylovora* in a concentration-dependent manner. As can be seen in Figure 7.2 and Figure 7.4, GA at all studied concentrations formed large inhibition zones (from 697.53 ± 69.96 mm^2 to 1551.78 ± 189.10 mm^2) around tested discs. This result agrees with MIC studies that showed the inhibitory effect of GA was more effective on *E. amylovora* than other phenols. However, inhibitory effect of VA at 0.5, 1, 2, and 4 mg/cm^2 on *E. amylovora* was lower than effect of VA at the same concentrations on *E. carotovora*.

In literature, Arcan and Yemenicioğlu (2011) studied the antimicrobial behaviour of zein films containing different phenolic acids (gallic acid, p-hydroxy benzoic acid, ferulic acid), flavonoids (catechin, flavone, quercetin) against *L. monocytogenes* and *C. jejuni*. They found that films containing 3 mg/cm^2 gallic acid showed good antimicrobial activity on both *L. monocytogenes* and *C. jejuni* and formed 2.07 ± 0.34 cm^2 and 1.66 ± 0.36 cm^2 clear zones around tested discs, respectively. Comparison of our zone areas with those of Arcan and Yemenicioğlu (2011) showed the extreme susceptibility of *E. amylovora* on GA (zone area in same unit: 14.53 cm^2 at 2 mg/cm^2).

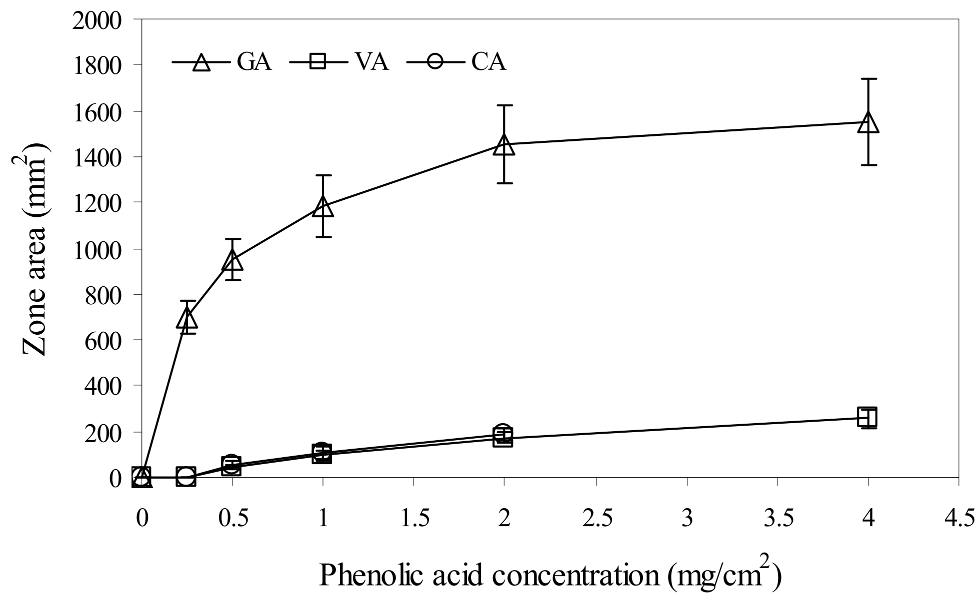

Figure 7.2. Antimicrobial activity of GA, VA and CA containing zein films on *E. amylovora*

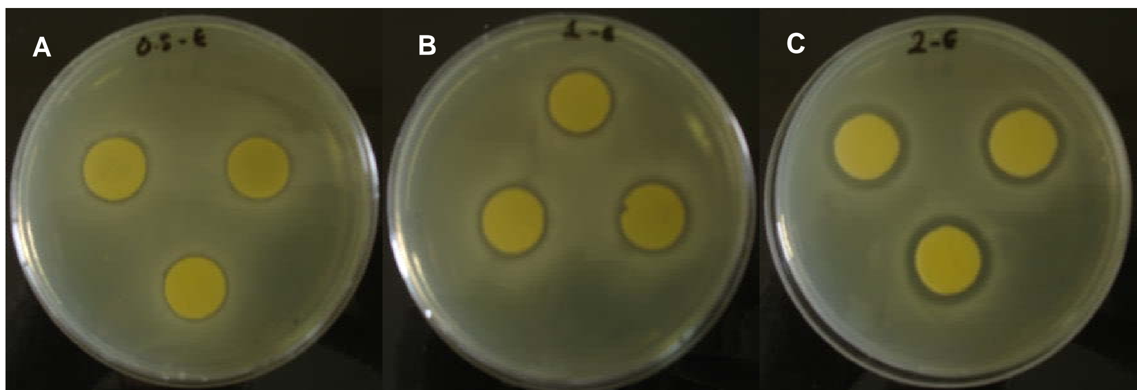

Figure 7.3. Growth inhibition of E. *amylovora* by zein film containing CA (A: 0.5 mg/cm^2; B: 1 mg/cm2; C: 2 mg/cm^2)

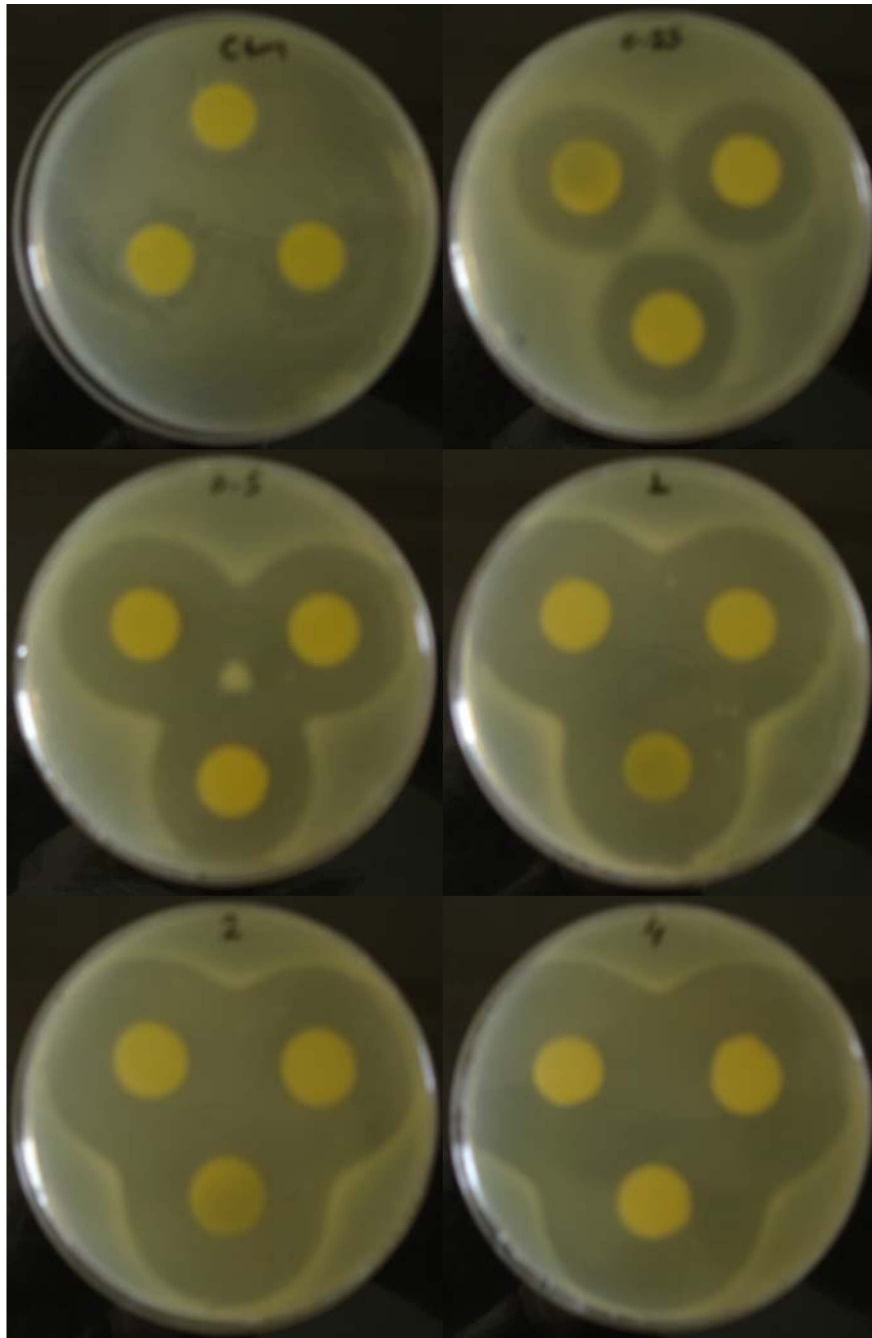

Figure 7.4. Growth inhibition of E. *amylovora* by zein film containing GA (A: Control; B: 0.25 mg/cm^2; C: 0.5 mg/cm^2; D: 1 mg/cm^2; E: 2 mg/cm^2; F: 4 mg/cm^2)

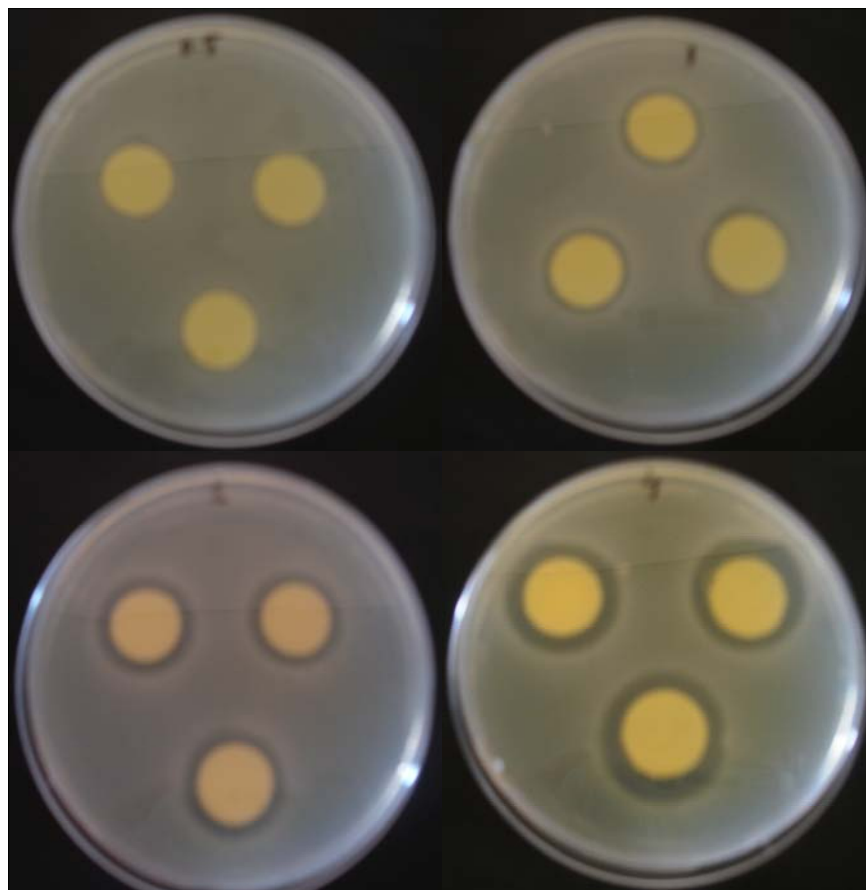

Figure 7.5. Growth inhibition of E. *amylovora* by zein film containing VA (A: 0.5 mg/cm^2; B: 1 mg/cm^2; C: 2 mg/cm^2; D: 4 mg/cm^2*)*

Antimicrobial effects of discs obtained from zein films incorporated with various concentrations of GA, CA, and VA on *P. syringae* are shown in Figure 7.6. The use of GA, CA, and VA at 0.25 and 0.5 mg/cm^2 did not give measurable zones on *P. syringae*. However, GA and VA between 1 and 4 mg/cm^2 and CA at 1 and 2 mg/cm^2 showed antimicrobial activity on *P. syringae* at a concentration dependent manner. As the concentration of phenolic compounds increased with 2-fold, the zone of inhibition also increased with 1.4-2.4 fold for *P. syringae.* However, the results of antimicrobial tests showed that *P. syringae* was the most resistant bacteria against antimicrobial films incorporated with phenolic acids. Measured inhibition zone photos for CA, GA, and VA are shown in Figure 7.7, Figure 7.8, and Figure 7.9.

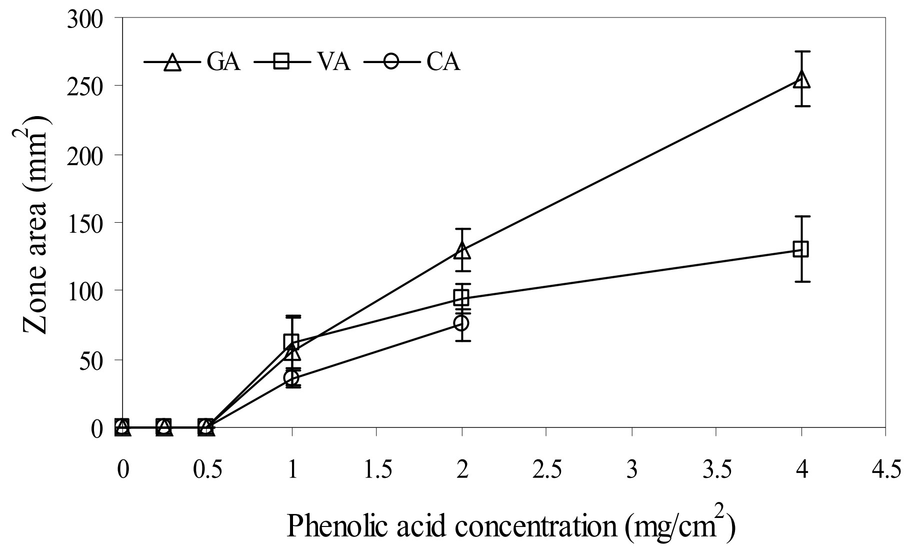

Figure 7.6. Antimicrobial activity of GA, VA, and CA containing zein films on *P. syringae*

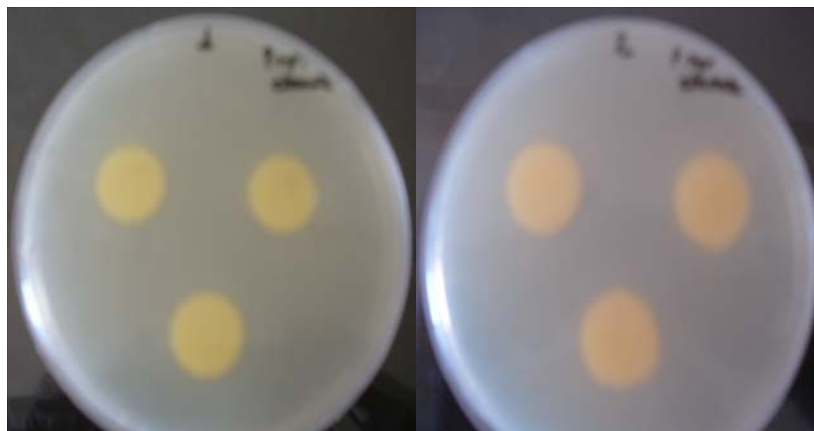

Figure 7.7. Growth inhibition of *P. syringae* by zein film containing CA (A: 1 mg/cm^2; B; 2 mg/cm^2)

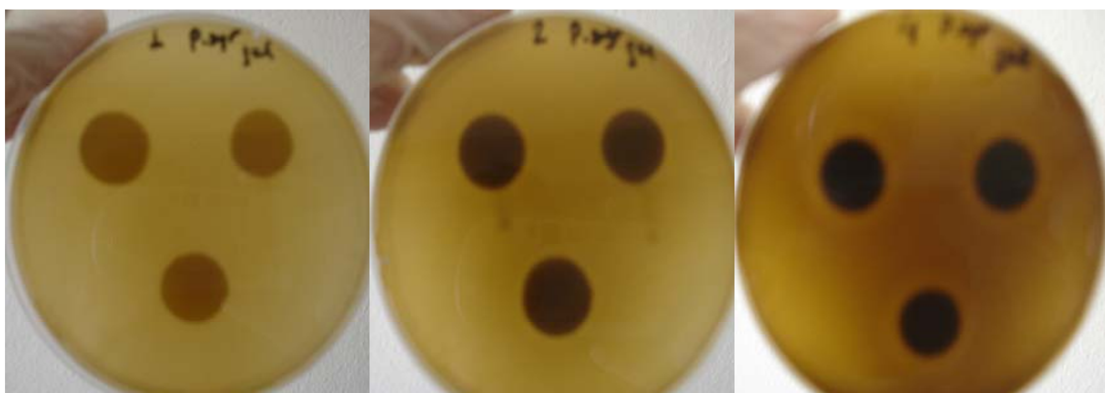

Figure 7.8. Growth inhibition of *P. syringae* by zein film containing GA (A: 1 mg/cm^2; B: 2 mg/cm^2; C: 4 mg/cm^2)

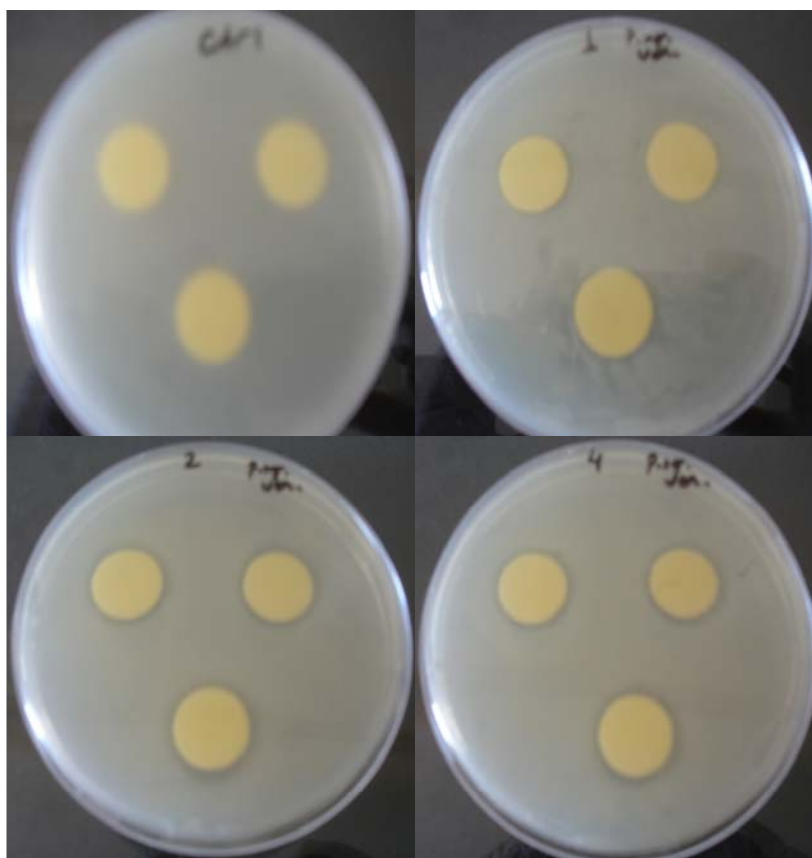

Figure 7.9. Growth inhibition of *P. syringae* by zein film containing VA (A: Control; B: 1 mg/cm^2; C: 2 mg/cm^2; D: 4 mg/cm^2)

The same antimicrobial tests were also applied to *X. vesicatoria* by using the films containing GA, VA, and CA at 0.25, 0.5, 1, 2, and 4 mg phenolic acids per cm^2 of films. Figure 7.10 shows the antimicrobial profiles of different phenolic compounds at different concentrations. Films containing GA at 0.25 and 0.5 mg/cm^2 was not effective against *X. vesicatoria*. However films incorporated with GA between 1 and 4 mg/cm^2 gave measurable zone on *X. vesicatoria*. The use of VA at 0.25 mg/cm^2 did not give inhibition zones on *X. vesicatoria*. However, when the concentration of VA in zein films was increased, significant inhibition was observed against *X. vesicatoria*. Films containing CA at 0.5, 1, 2 mg/cm^2 showed the highest antimicrobial activity against *X. vesicatoria*. At the studied concentration range, a 2-fold increase in GA, VA and CA concentration caused almost 1.5-2.4 fold increase in antimicrobial activity of films against *X. vesicatoria.*

The evaluation of overall antimicrobial test results on *E. carotovora, E. amylovora, P. syringae,* and *X. vesicatoria* showed that the inhibitory effects of phenolic acids on plant pathogens were in the following order: GA > VA > CA. The

higher antimicrobial activity of GA than VA in film studies contradicts with results of MIC conducted with phenolic solutions in culture medium. It appeared that the differences in solubilities of phenolic compounds and interactions of phenolic compounds with the film matrix affected their solubility and release profiles and resulting antimicrobial properties. The GA is highly water soluble and it seemed that this phenolic compound readily solubilized from films to show its antimicrobial activity.

Antibacterial and antifungal effects of different phenolic compounds have been investigated in the literature. Our results for gallic acid and plant pathogens are not in agreement with the study reported by Rauha et al. (2000) since they reported that gallic acid had no effect on eukaryotic microbes and its activity was restricted to only *Pseudomonas aeruginosa.* Alkan, et al. (2011) reported that the use of GA at 1.25 mg/cm^2 did not give measurable zones on *C. jejuni,* but higher concentrations of GA were found highly effective on inhibiting this bacterium. In this study, GA at 1 mg/cm^2 caused measurable inhibition zones on all studied plant pathogens. Thus, it seemed that the plant pathogens are more susceptible to GA than *C. jejuni* a major human pathogen.

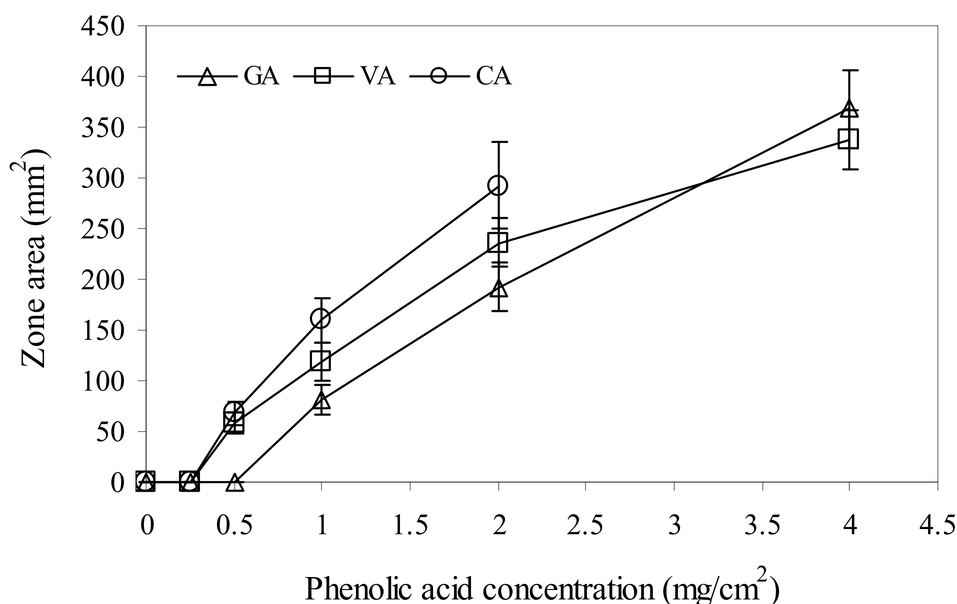

Figure 7.10. Antimicrobial activity of GA, VA, and CA containing zein films on *X. vesicatoria*

7.1.3. Antimicrobial Activity of Edible Films Containing Mixture of Different Phenolic Acids

Following antimicrobial test of films incorporated with only one type of phenolic compounds, antimicrobial activities of films containing mixture of different phenolic compounds were also determined. The use of mixtures of phenolic compound aimed obtaining a synergetic effect between phenolic compounds or reducing the individual concentrations of each phenolic compound within the films. Figure 7.11 shows the inhibition zone areas of discs obtained from zein films containing different combinations of GA, CA, and VA. *E. amylovora* was more sensitive to various combinations of GA, CA, and VA than other three pathogens. The combinations of phenolic compounds effectively inhibited *E. carotovora* and *E. amylovora* but they are not very effective on *P. syringae* and *X. vesicatoria*. GA alone was not effective on *E. carotovora* at a concentration of 0.25 mg/cm^2 but the incorporation of 0.25 mg/cm^2 CA or VA with 0.25 mg/cm^2 GA inhibited the growth of this microorganism. Moreover, although zein films incorporated with only 0.25 mg/cm^2 CA or 0.25 mg/cm^2 VA were not inhibitory against *E. amylovora*, combination of 0.25 mg/cm^2 CA and 0.25 mg/cm^2 VA showed antimicrobial activity on this bacterium. In contrast, the combination of GA and CA, and GA and VA at a concentration of 0.25 mg/cm^2 did not show any considerable inhibitory effect against *P. syringae* and *X.vesicatoria.* On the other hand, the combination of 0.5 mg/cm^2 CA and 0.5 mg/cm^2 VA or combination of 0.5 mg/cm^2 GA and 0.5 mg/cm^2 CA increased the antimicrobial activity of these films against *E. carotovora, E. amylovora* and *P. syringae.* However, combination of 0.5 mg/cm^2 GA and 0.5 mg/cm^2 VA was not effective on *X.vesicatoria.* Thus, it is worth to note that to obtain antimicrobial activity against *X.vesicatoria* the presence of 0.5 mg/cm^2 CA is essential in the combinations. The results of antimicrobial tests for films incorporated with combinations of phenolic compounds showed that *P. syringae and X. vesicatoria* was more resistance to various combinations of GA, CA, and VA than other two pathogens. Figure 7.12, Figure 7.13, and Figure 7.14 present the inhibitory effects of combinations of GA, CA, and VA on *E. amylovora, E. carotovora,* and *P. syringae.*. In the literature, synergistic antimicrobial effects of phenolic compounds have been also shown by Tafesh et al. (2011). These workers tested different mixtures of olive mill wastewater with hydroxytyrosol, gallic acid and ascorbic acid and achieved complete

inhibition of the Gram-positive (*S. pyogenes* and *S. aureus*) and Gram-negative bacteria (*E. coli* and *K. pneumoniae*).

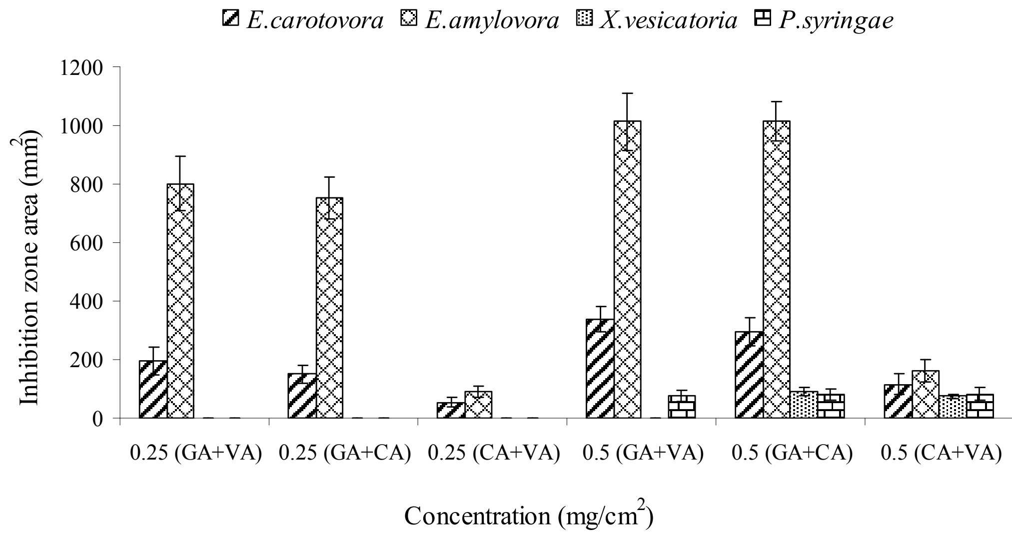

Figure 7.11. Effect of GA, CA, and VA combinations incorporated zein film against plant pathogens

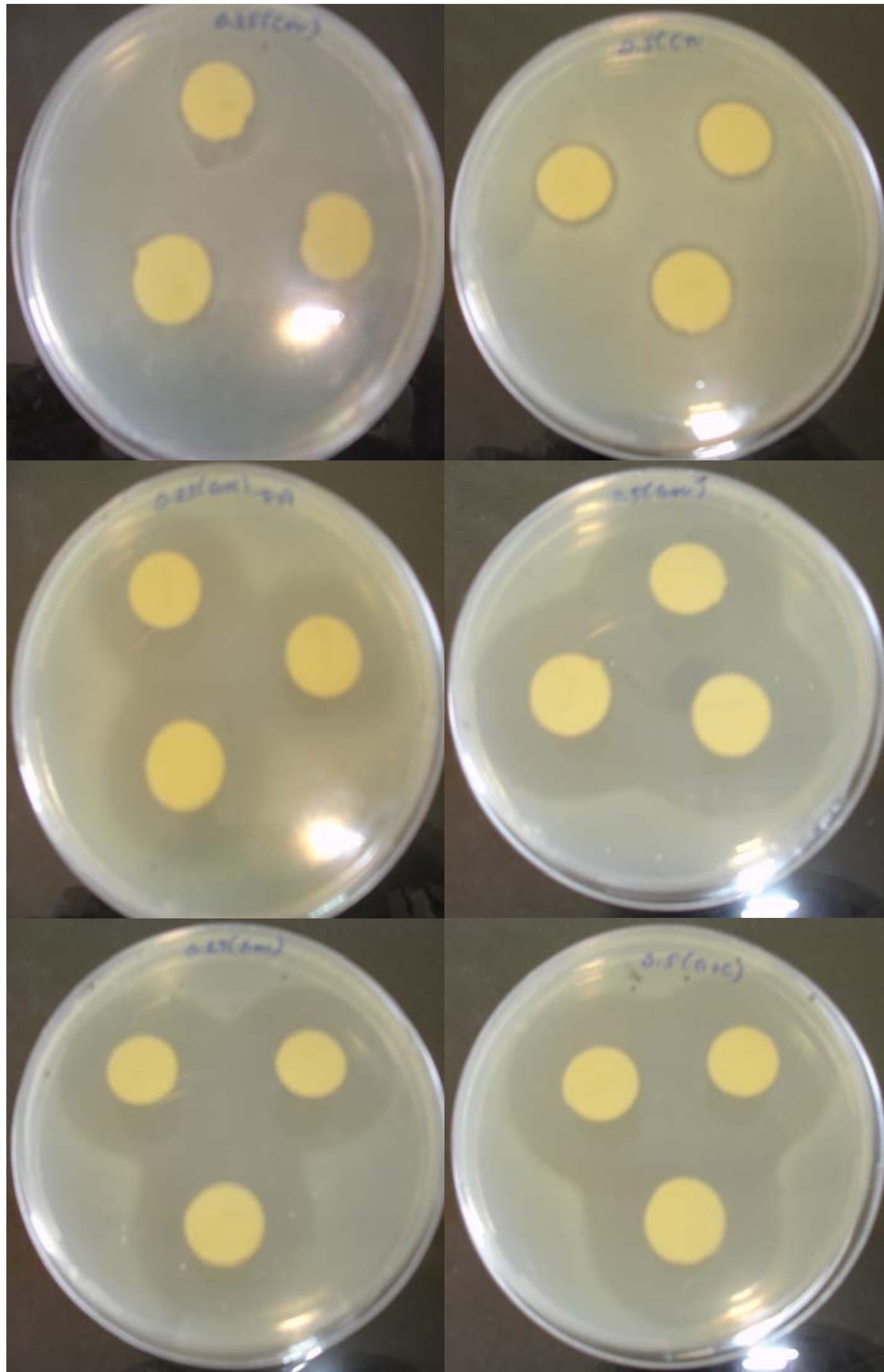

Figure 7.12. Growth inhibition of *E. amylovora* by zein film containing combinations of phenolic acids (A: 0.25 mg/cm^2 C+V; B: 0.5 mg/cm^2 C+V; C: 0.25 mg/cm^2 G+V; D: 0.5 mg/cm^2 G+V; E: 0.25 mg/cm^2 G+C; F: 0.5 mg/cm^2 G+C)

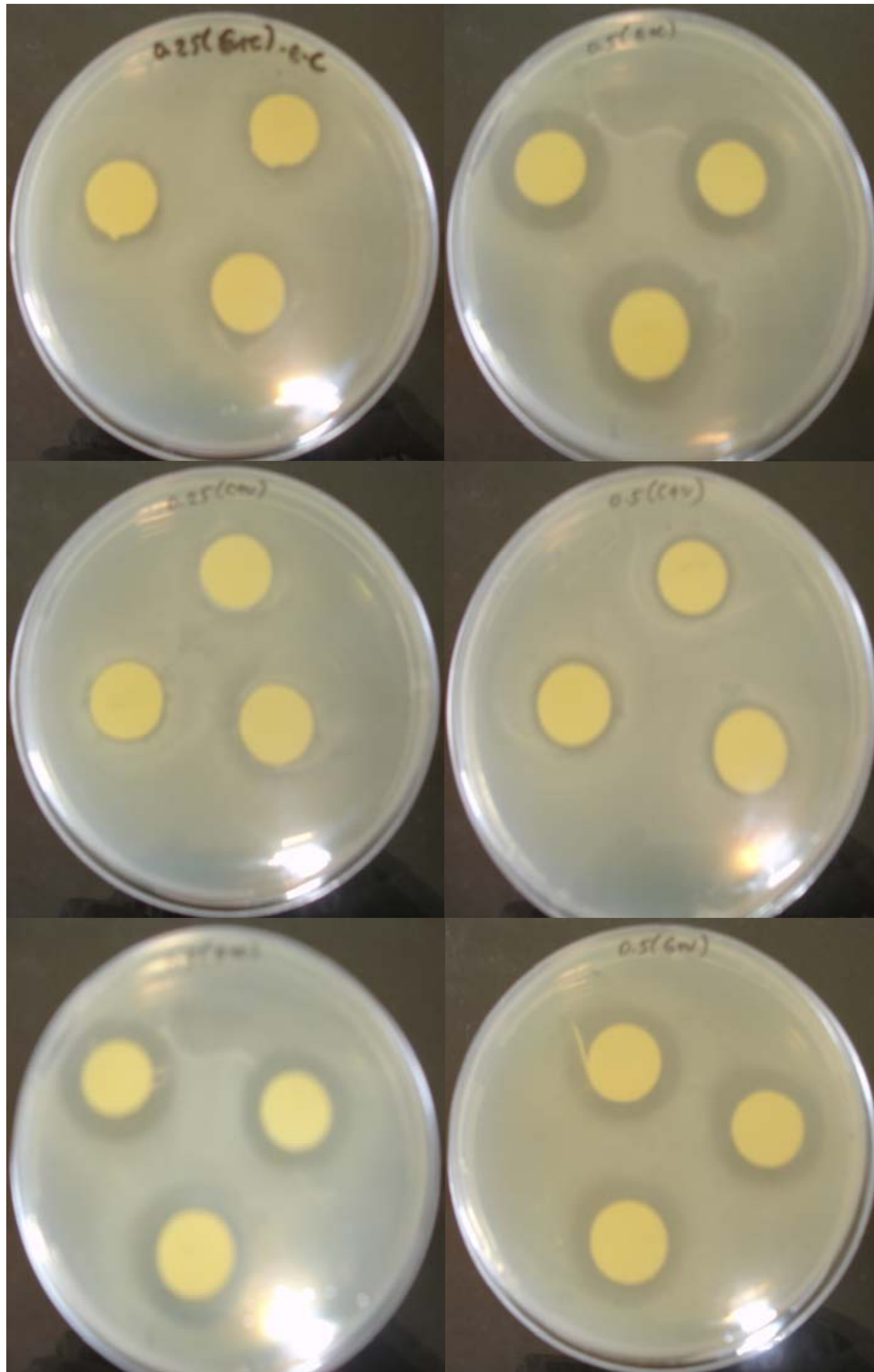

Figure 7.13. Growth inhibition of *E. carotovora* by zein film containing combinations of phenolic acids (A: 0.25 mg/cm^2 G+C; B: 0.5 mg/cm^2 G+C; C: 0.25 mg/cm^2 C+V; D: 0.5 mg/cm^2 C+V; E: 0.25 mg/cm^2 G+V; F: 0.5 mg/cm^2 G+V)

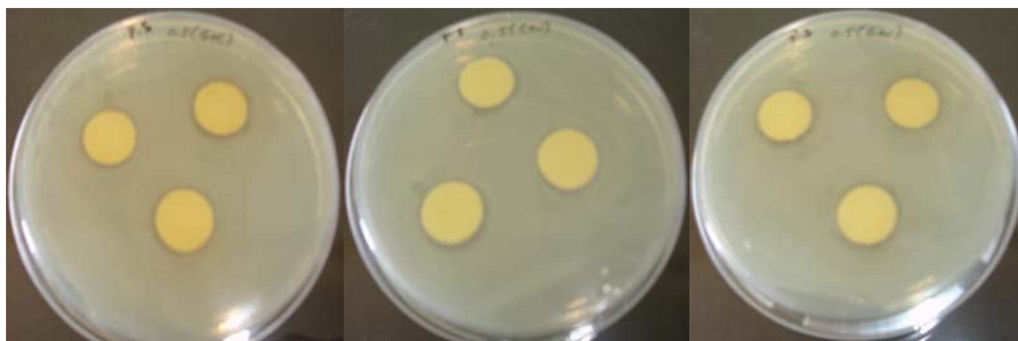

Figure 7.14. Growth inhibition of *P. syringae* by zein film containing combinations of phenolic acids (A: 0.5 mg/cm^2 G+C; B: 0.5 mg/cm^2 C+V; C: 0.5 mg/cm^2 G+V)

7.1.4. Release Tests of Edible Films Containing Phenolic Acids

In order to determine the soluble phenolic contents of films release tests were conducted by placing films into distilled water. The release tests were conducted at 4ºC to simulate the average storage temperature of fruits and vegetables to be coated with the developed edible films. Figure 7.15 shows the release profiles of 1 mg/cm^2 concentration of GA and VA containing zein films incubated in distilled water at 4 ºC. No release curves were obtained for CA containing films since this phenolic compound did not release from films incubated in distilled water. The results show that the GA and VA in the films were water soluble and release from the films with quite similar release profiles. At 4 ºC, the maximum amounts of the GA and VA released from the zein film reached to approximately 68% and 63% of the total GA and VA incorporated into films, respectively. Thus, it is clear that almost 30 % of GA and almost 40% of VA incorporated into films is entrapped within the films or bound onto film matrix due to the capacity of phenolic compounds to form H-bonding with carbonyl groups of zein protein. The results obtained from the release tests of zein films supported our previous findings that showed the release of 60-75% of incorporated GA from zein films (Alkan, et al. 2011).

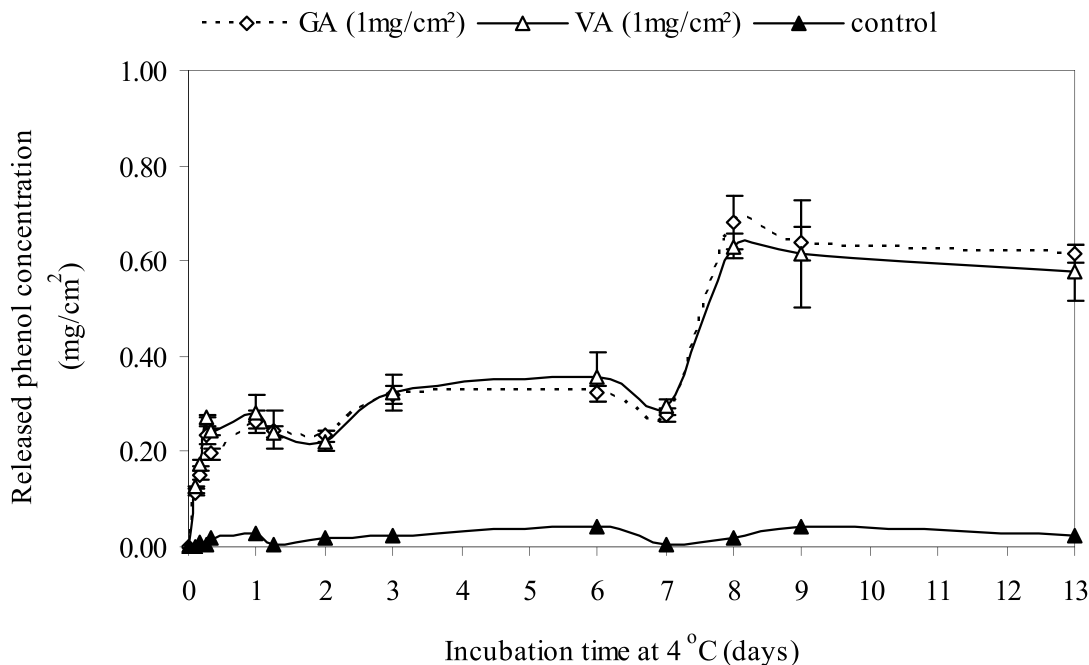

Figure 7.15. Release profiles of GA and VA from zein films incubated in distilled water at 4 ºC

7.1.5. Mechanical Properties of Edible Films Containing Phenolic Acids

To characterize the mechanical properties of films Tensile strength, elongation, and Young's modulus of films were determined. Table 7.3 lists the mechanical properties of the zein films incorporated with GA, CA, VA, and their combinations. The results show that the addition of phenolic acids into zein film did not cause considerable changes in the average thicknesses of the films (except films containing 0.5 mg/cm^2 CA). The control zein films showed highly brittle structures with poor elongation. Addition of low concentrations of GA, CA, VA (0.25-1 mg/cm^2) and combinations of phenolic compounds at 0.25-0.5 mg/ cm^2 did not cause any statistically significant change in film elongation ($P > 0.05$). On the other hand, the incorporation of GA at 2 or 4 mg/cm^2, CA at 2 mg/cm^2, and VA at 2 or 4 mg/cm^2 into films increased the elongation and resulting flexibility of films. VA containing film showed higher elongation than GA and CA containing films at 2 mg/cm^2 phenolic concentration, but addition of VA at 4 mg/cm^2 into film caused lower elongation than film containing similar amounts of GA. The use of VA at 2 mg/cm^2 achieve highly flexible films with 146 % elongation, but a further increase in VA concentration (to 4 mg/cm^2) reduced

elongations of films (12.2 %). It seems that addition of high amount of VA into zein film reduced its plasticizing effect due to polymerization of this phenolic compound.

Statistically significant differences were determined between tensile strength and Young's modulus of zein films containing different phenolic compounds ($P > 0.05$). The tensile strength and Young's modulus of zein films reduced as phenolic concentration in the films was increased. However, films containing combination of GA, CA, and VA showed lower tensile strength and Young's modulus values than films containing GA, CA, and VA alone. The low flexibility of films containing mixture of phenolic compounds could be related to degree of compatibility of each compound in the mixture. It is thought that the high degree of polymerization among mixture of different phenolic compounds lead to an extensive network formation and resulting reduction in film flexibility. Positive effects of phenolic compounds on mechanical properties of zein films have been investigated in our earlier study (Alkan, et al. 2011). In that study, it was reported that GA at 2.5 and 5 mg/cm^2 caused a considerable increase in elongation (57-280%) of zein films and eliminated their classical flexibility problems. Arcan and Yemenicioğlu (2011) reported that tensile strength and Young's modulus of zein films reduced and flexibility of films increased by addition of gallic acid and catechin at 3 mg/cm^2 concentration. However, the effects of mixture of different pure phenolic compounds on mechanical properties of films have not been investigated before in the literature. Further studies are needed to understand potential interactions among phenolic compounds and zein.

Table 7.3. Mechanical properties of zein films containing phenolic acids (mean ± SD)

Film composition						
GA (mg/cm^2)	CA (mg/cm^2)	VA (mg/cm^2)	Tensile strength (MPa)	Young's modulus (MPa)	Elongation (%)	Film thickness (μm)
–	–	–	10.73 ± 0.59^{a}	648.28 ± 19.78^{b}	3.69 ± 0.44^{d}	115.4^{b} ± 1.06
0.25	–	–	8.99 ± 0.69^{ab}	465.35 ± 11.92^{d}	3.36 ± 0.81^{d}	115.86^{b} ± 0.74
0.5	–	–	8.90 ± 0.28^{ab}	459.83 ± 15.28^{d}	3.56 ± 0.68^{d}	130.73^{b} ± 1.30
1	–	–	8.59 ± 0.42^{b}	428.50 ± 27.10^{d}	3.52 ± 0.23^{d}	115.66^{b} ± 1.77
2	–	–	4.30 ± 0.45^{c}	230.27 ± 7.18^{f}	31.50 ± 9.43^{d}	99.92^{b} ± 0.95
4	–	–	0.87 ± 0.09^{d}	28.89 ± 7.37^{h}	276.60 ± 37.16^{a}	86.40^{b} ± 1.02
–	0.25	–	9.15 ± 1.62^{ab}	559.23 ± 34.99^{c}	1.95 ± 0.48^{d}	121.84^{b} ± 0.77
–	0.5	–	5.16 ± 0.61^{c}	364.56 ± 30.45^{e}	1.58 ± 0.35^{d}	200.75^{a} ± 1.38
–	1	–	7.47 ± 1.19^{b}	517.37 ± 29.50^{c}	4.15 ± 1.68^{d}	130.17^{b} ± 0.78
–	2	–	1.07 ± 0.16^{d}	168.73 ± 4.74^{g}	95.08 ± 44.80^{c}	137.60^{b} ± 0.99
–	–	0.25	8.44 ± 1.52^{b}	708.69 ± 31.19^{a}	2.05 ± 1.33^{d}	108.78^{b} ± 0.93
–	–	0.5	8.33 ± 0.91^{b}	574.06 ± 9.10^{c}	1.68 ± 0.50^{d}	126.27^{b} ± 0.85
–	–	1	6.99 ± 2.38^{b}	445.49 ± 28.11^{d}	2.75 ± 1.41^{d}	124.98^{b} ± 0.97
–	–	2	1.64 ± 0.17^{d}	159.55 ± 28.75^{g}	146.26 ± 90.36^{b}	142.53^{b} ± 0.67
–	–	4	0.33 ± 0.16^{d}	90.05 ± 6.26^{g}	12.15 ± 3.04^{d}	142.50^{b} ± 4.33
–	0.25	0.25	2.95 ± 0.66^{c}	147.77 ±12.01^{g}	1.71 ± 0.33^{d}	141.54^{b} ± 1.89
0.25	0.25	–	4.29 ± 0.36^{c}	187.66 ± 6.16^{g}	2.21 ± 0.11^{d}	126.7^{b} ± 3.49
0.25	–	0.25	3.1 ± 0.49^{c}	160.10 ± 15.62^{g}	1.61 ± 0.21^{d}	114.06^{b} ± 0.89
–	0.5	0.5	2.20 ± 0.20^{cd}	125.95 ± 9.48^{g}	1.56 ± 0.14^{d}	154.80^{b} ± 0.89
0.5	0.5	–	3.47 ± 0.41^{c}	159.02 ± 11.14^{g}	1.86 ± 0.21^{d}	124.66^{b} ± 1.45
0.5	–	0.5	3.14 ± 0.47^{c}	139.28 ± 11.07^{g}	2.01 ± 0.29^{d}	140.20^{b} ± 1.26

a-h. Different letters within a column indicate significant difference ($p<0.05$).

7.1.6. Scanning Electron Microscopy (SEM) of Edible Films Containing Phenolic Acids

The SEM images of control zein films and zein films containing GA, CA, VA and their combinations at different concentrations are given in Figure 7.16, A-F; 7.17A-D; 7.18A-E and 7.19A-F. It was observed that the addition of GA at low concentration did not cause any morphological changes in zein films. However, the addition of GA at a concentration of 2 mg/cm^2 and 4 mg/cm^2 increased the film porosity (Figure 7.16A-F). Arcan and Yemenicioğlu (2011) investigated the effect of different phenolic compound on the structure of zein film and showed the increase of film porosity by addition of GA at a concentration of 3 mg/cm^2. As seen in Figure 7.17A-D, the addition of CA also caused increased porosity of zein films at a concentration dependent manner. However, the morphological changes caused by CA are considerably different than those of GA. It was clear that the CA containing films contained higher number of large pores than GA containing films. Figure 7.18A-E illustrates the SEM photographs of zein films containing various concentrations of VA. The photographs show that the addition of VA also increased the pore size of zein films considerably.

The SEM images of zein films containing the mixture of GA-CA, GA-VA, and CA-VA are given in Figure 7.19A-F. It was interesting to note that the addition of mixture of phenolic compound into zein films caused significantly larger pores than addition of GA, CA, and VA alone into zein films. In fact, the films containing mixture of phenolic compounds contain also very large cracks and cavities.

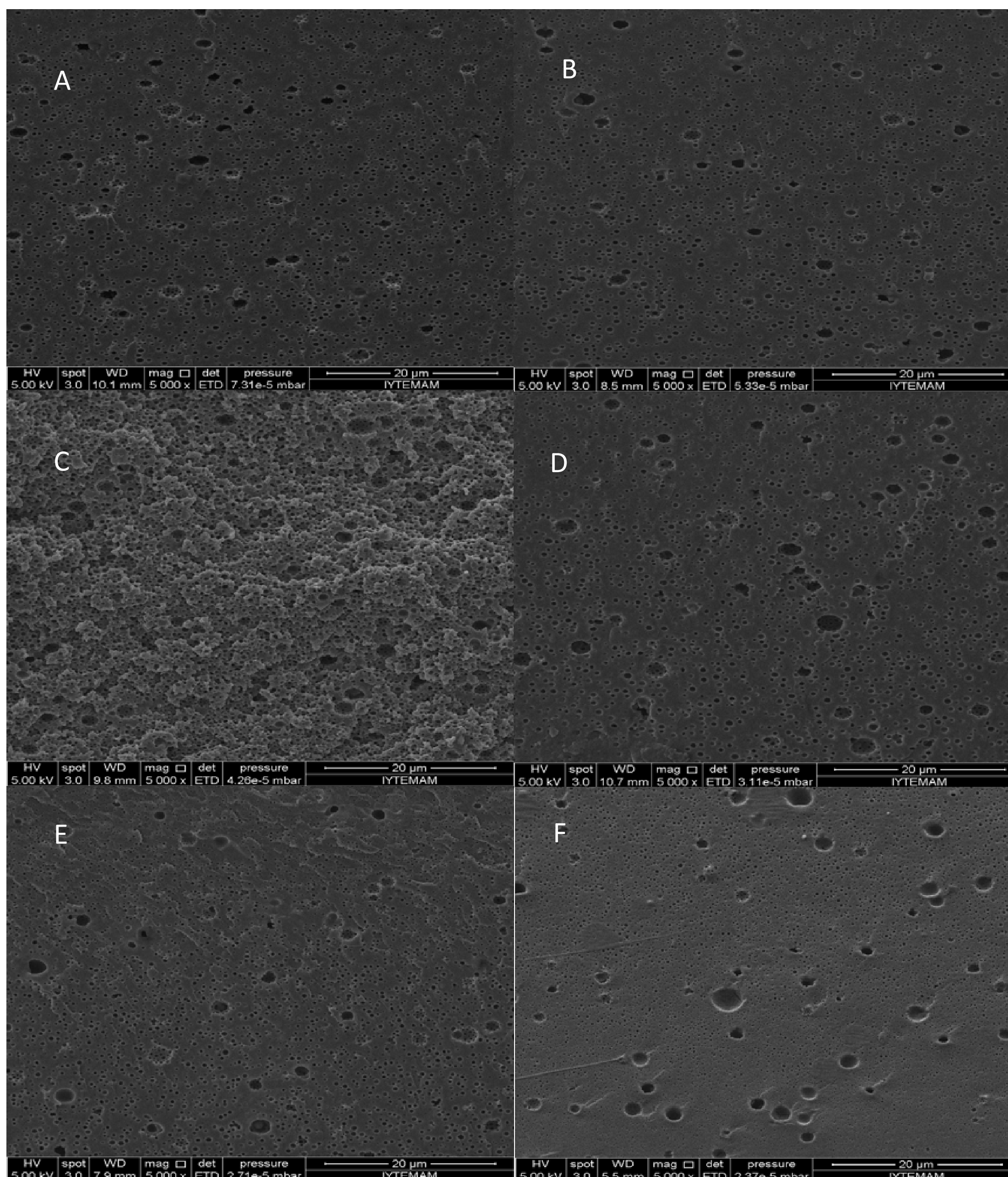

Figure 7.16. SEM photographs of different film cross-sections. (A) Control zein film, (B) 0.25 mg/cm^2 GA containing zein film, (C) 0.5 mg/cm^2 GA containing zein film, (D) 1 mg/cm^2 GA containing zein film, (E) 2 mg/cm^2 GA containing zein film, (F) 4 mg/cm^2 GA containing zein film. Magnifications were 5000× for A-F.

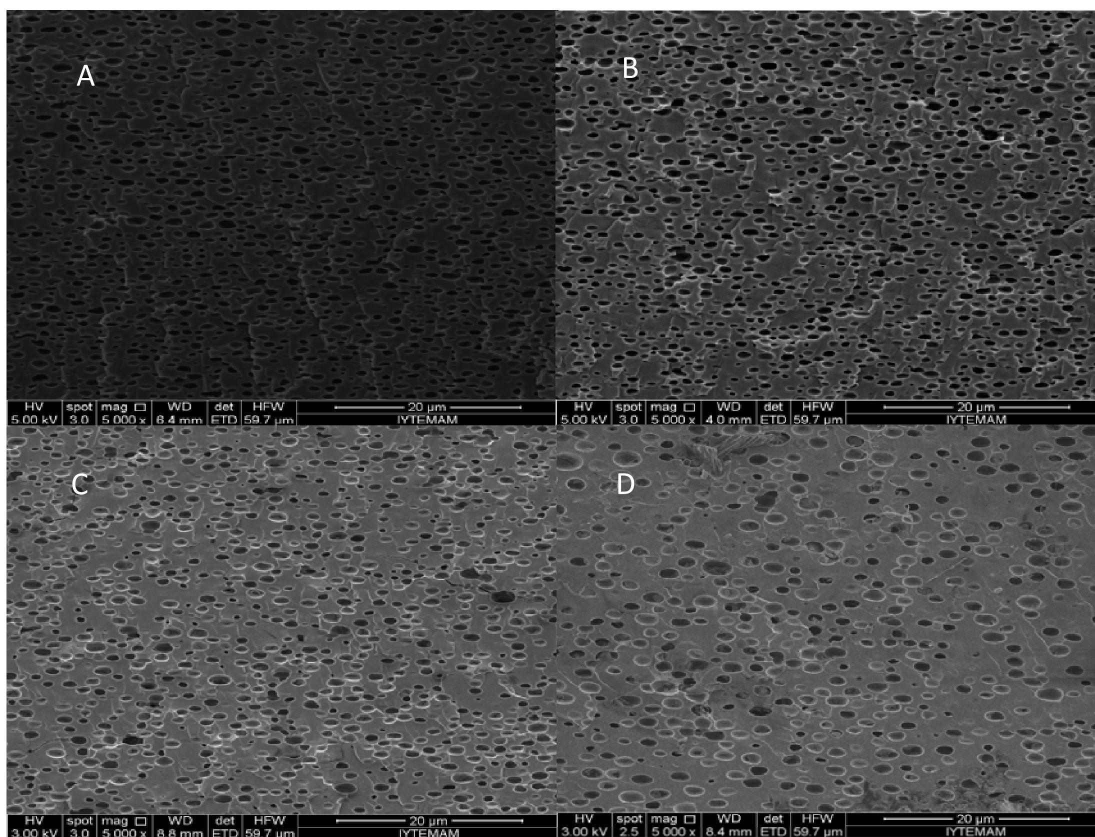

Figure 7.17. SEM photographs of different film cross-sections. (A) 0.25 mg/cm^2 CA containing zein film, (B) 0.5 mg/cm^2 CA containing zein film, (C) 1 mg/cm^2 CA containing zein film, (D) 2 mg/cm^2 CA containing zein film. Magnifications were 5000× for A-D.

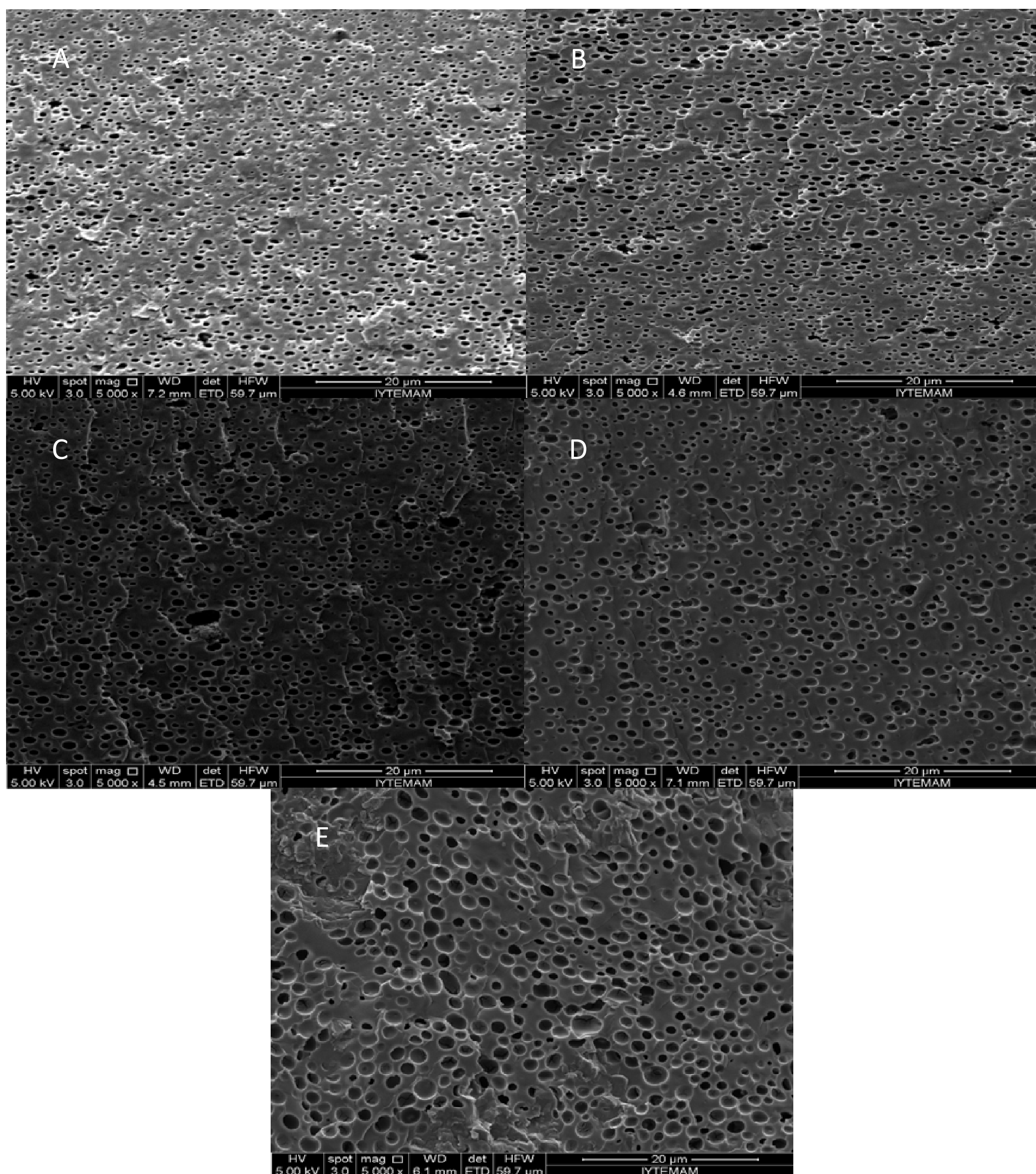

Figure 7.18. SEM photographs of different film cross-sections. (A) 0.25 mg/cm^2 VA containing zein film, (B) 0.5 mg/cm^2 VA containing zein film, (C) 1 mg/cm^2 VA containing zein film, (D) 2 mg/cm^2 VA containing zein film, (E) 4 mg/cm^2 VA containing zein film. Magnifications were 5000× for A-E.

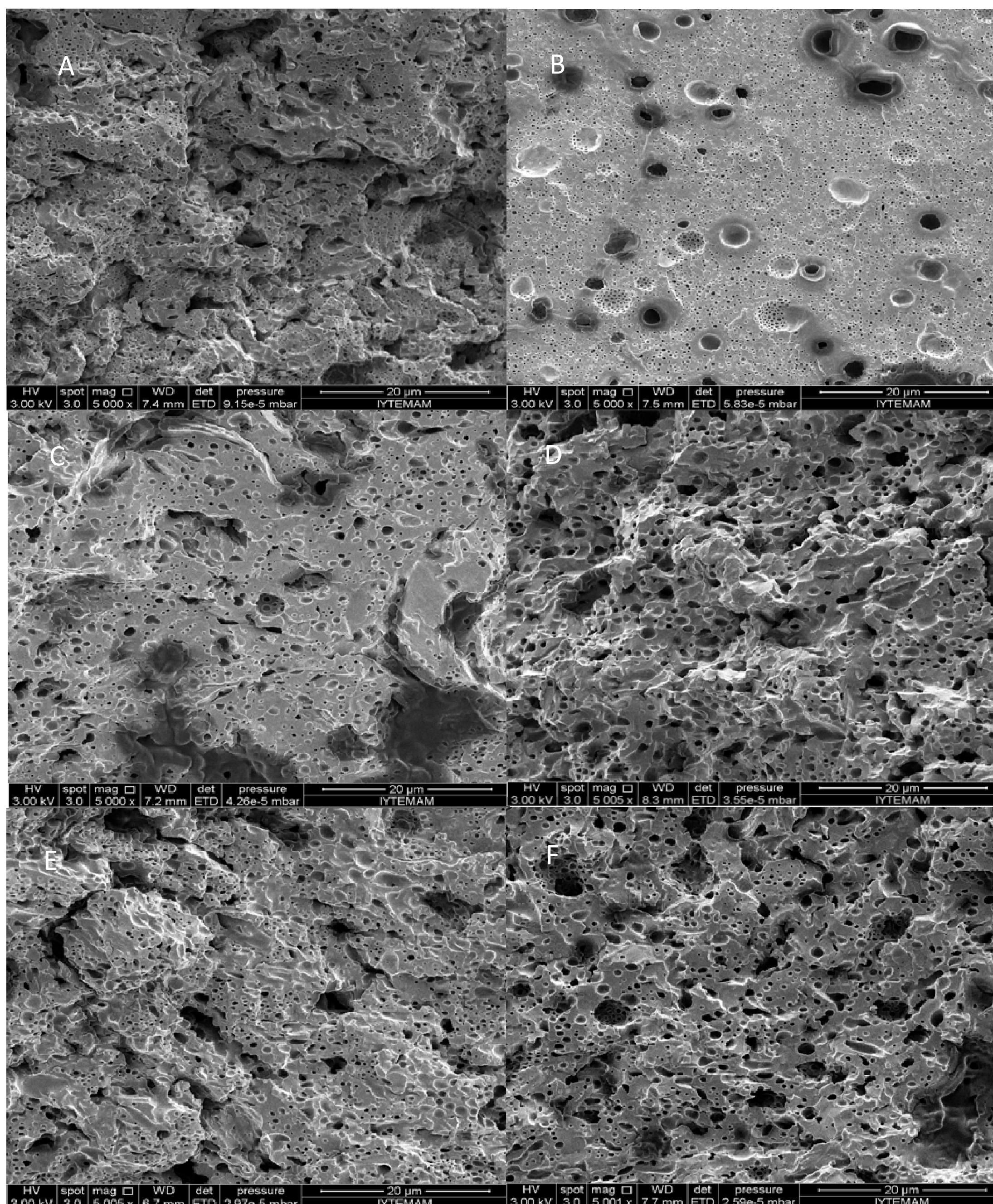

Figure 7.19. SEM photographs of different film cross-sections. (A) 0.25 mg/cm^2 VA+0.25 mg/cm^2 GA containing zein film, (B) 0.25 mg/cm^2 GA+0.25 mg/cm^2 CA containing zein film, (C) 0.25 mg/cm^2 VA+0.25 mg/cm^2 CA containing zein film, (D) 0.5 mg/cm^2 VA+0.25 mg/cm^2 CA containing zein film, (E) 0.5 mg/cm^2 GA+0.25 mg/cm^2 CA containing zein film, (F) 0.5 mg/cm^2 VA+0.25 mg/cm^2 GA containing zein film. Magnifications were 5000× for A-F.

7.2. Inhibition of Plant Pathogens by Edible Films Containing Essential Oils

7.2.1. Antimicrobial Activity of Edible Films Containing Essential Oils

THY, CAR, EUG and CIT are insoluble in water, but they are extremely soluble in alcohols and organic solvents. Because of the solubility problem of essential oils in water, MIC of all essential oils could not determined by using 96-well microplates. For this reason, antimicrobial zein films containing different concentrations of THY, CAR, EUG and CIT were directly tested on *E. amylovora, E. carotovora, X. vesicatoria* and *P. syringae* applying 0.25, 0.5, 1, 2, and 4 mg essential oils per cm^2 of films. The results of antimicrobial tests showed that the films containing 0.25, 0.5, 1, 2 and 4 mg/cm^2 concentrations of THY, CAR and CIT did not show any antimicrobial activity against *P. syringae.* In contrast, films containing EUG AT 4 mg/cm^2 showed antimicrobial activity against *P. syringae*.

Figure 7.20 shows the antimicrobial effect of films containing 0.25, 0.5, 1, 2, and 4 mg of THY, CAR, EUG and CIT on *E. amylovora.* The use of all essential oils at 0.25 and 0.5 mg/cm^2 did not give measurable zones on this pathogen. However, the THY, CAR and EUG between 1 and 4 mg/cm^2 inhibited the growth of *E. amylovora* at a concentration-dependent manner. *E. amylovora* was inhibited by use of THY at 1, 2 and 4 mg/cm^2 concentrations with 140.01±24.20, 695.41±77.59 and 1219.66±128.23 mm^2 inhibition areas, respectively. Similar to THY, CAR showed antimicrobial activity on *E. amylovora* at 1, 2 and 4 mg/cm^2 concentrations with 205.04±33.17, 504.52±61.16 and 1045.36±159.58 mm^2 inhibition areas, respectively. Inhibition zone areas in Petri dish obtained from addition of CAR at 1, 2 and 4 mg/cm^2 concentrations into films are presented in Figure 7.21. Antimicrobial effect of EUG at 1, 2 and 4 mg/cm^2 concentrations (respective zone areas: 61.02±11.58, 204.14±21.21, 611.48±143.48) was weaker than that of THY and CAR (Figure 7.22). *E. amylovora* was not inhibited by CIT at 0.25, 0.5 and 1 mg/cm^2 concentrations but the use of CIT at 2 mg/cm^2 gave 137.53±26.38 mm^2 inhibition area. Interestingly, the use of CIT at 4 mg/cm^2 concentration showed an extremely high antimicrobial activity and totally inhibited the growth of this organism in a Petri dish (Figure 7.23).

In the literature, data about effect of essential oil containing edible films on plant pathogens were scarce. However, it is worth to report the effectiveness of essential oils on important human pathogens studied by different workers. For example, strong antibacterial activity of citral against the pathogenic microorganisms *E. coli, S. enterica* and *L. monocytogenes* has been shown by Galet et al. (2012). They reported that the use of 3 mg of citral produced a zone of inhibition against *E. coli* and *L. monocytogenes* while 5 mg of citral was necessary for inhibition of *S. enterica.* They achieved total inhibition of pathogens by use of 7 mg of citral.(agents added in DMSO solution).

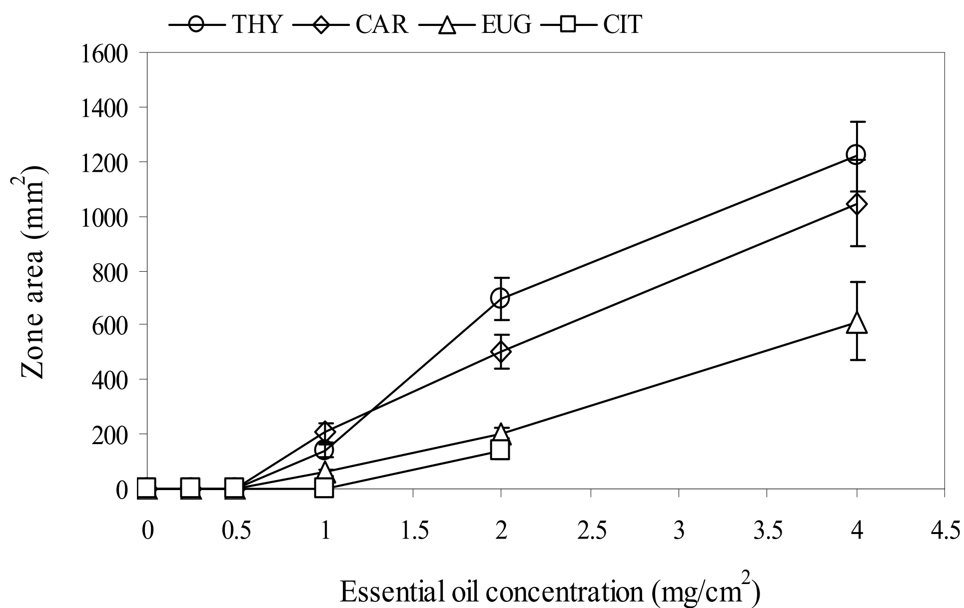

Figure 7.20. Antimicrobial activities of essential oils containing zein films on *E. amylovora*

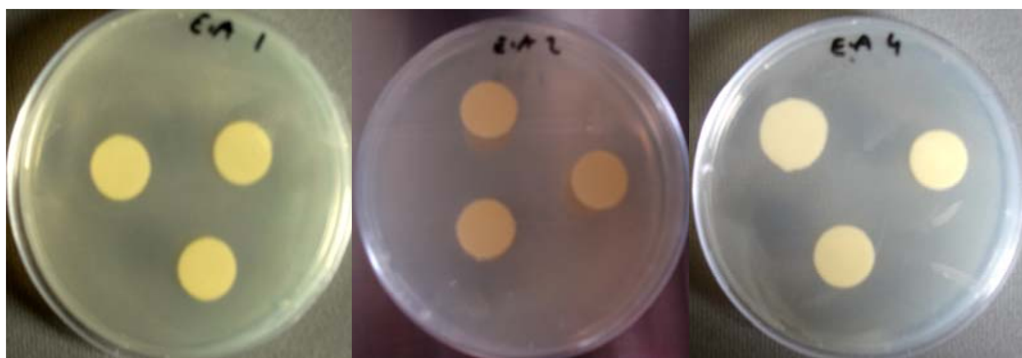

Figure 7.21. Growth inhibition of *E. amylovora* by zein film containing CAR (A: 1 mg/cm^2; B: 2 mg/cm2; C: 4 mg/cm^2)

Figure 7.22. Growth inhibition of *E. amylovora* by zein film containing EUG (A: 1 mg/cm^2; B: 2 mg/cm^2; C: 4 mg/cm^2)

Kim et al. (1995) tested antibacterial activity of 11 essential oil constituents (including carvacrol and citral) against *Escherichia coli, E. coli* 0157:H7, *Salmonella typhimurium, Listeria monocytogenes,* and *Vibrio vulnificus*. They concluded that carvacrol at 250 µg/mL showed bactericidal activity against *S. typhimurium* and *V. vulnificus* while citral had minimum bactericidal concentration of 100 µg/mL against *V. vulnificus*. Moreover, in that study, citral at 500 µg/mL completely inhibited *E. coli, E. coli* 0157:H7, *S. typhimurium.* Du et al. (2008) developed novel edible films made from tomatoes puree containing carvacrol and antimicrobial behavior of these films was tested against *E. coli* 0157:H7. As a result of that study, no growth was observed on the plates around the tomato puree film discs containing 0.75% or 1.0% carvacrol (w/w) whereas films prepared with 0.5% carvacrol did not inhibit growth of *E. coli* 0157:H7.

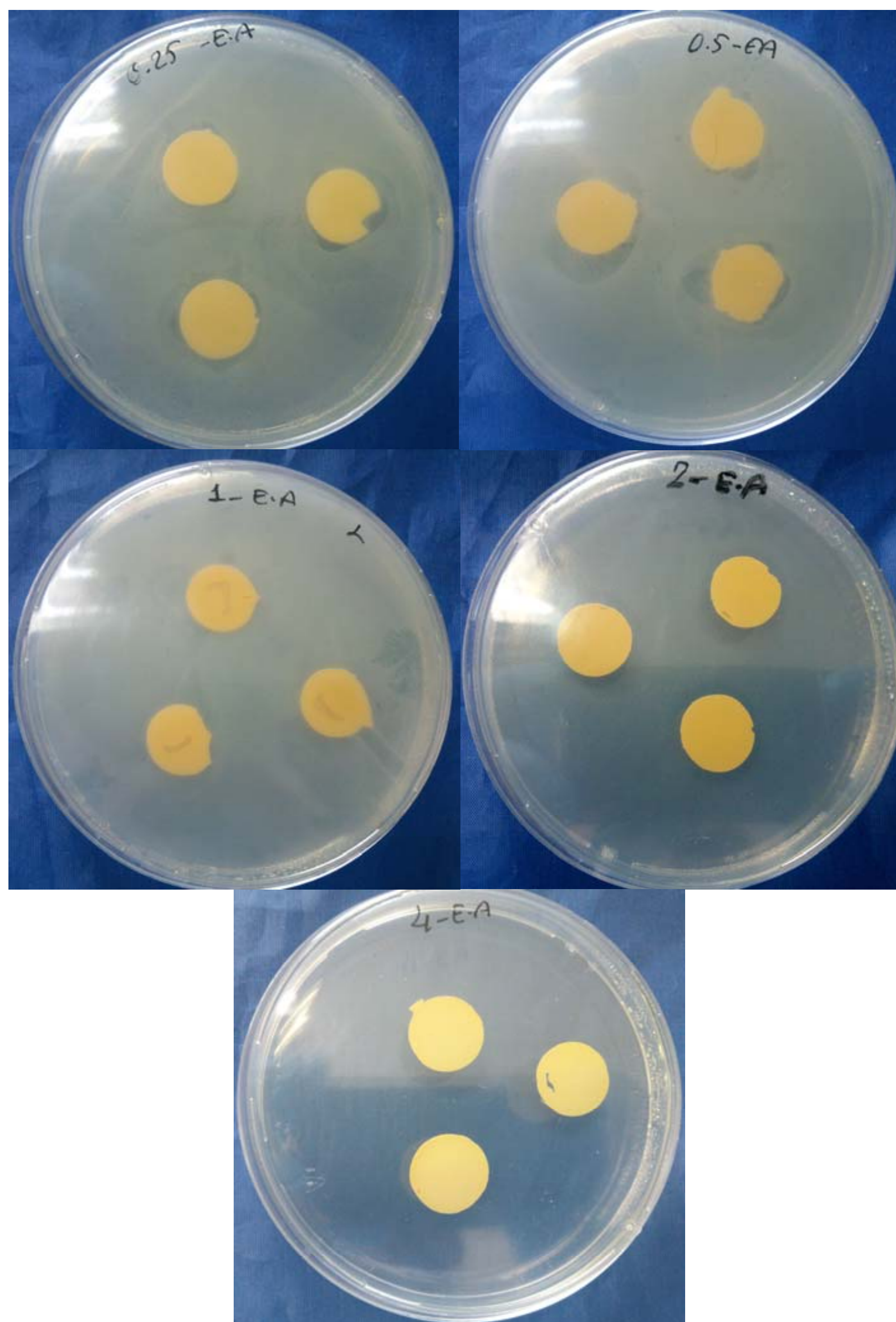

Figure 7.23. Growth inhibition of *E. amylovora* by zein film containing CIT (A: 0.25 mg/cm^2; B: 0.5 mg/cm^2; C: 1 mg/cm^2; D: 2 mg/cm^2; E: 4 mg/cm^2)

The antimicrobial activity based on zone areas of zein films containing different concentrations of THY, CAR, EUG and CIT against *E. carotovora* is presented in Figure 7.24. THY between 0.5 and 4 mg/cm^2 showed antimicrobial activity on *E. carotovora*. The use of THY at 0.5, 1 and 2 mg/cm^2 gave measurable zones on *E. carotovora* (114.54±29.82, 120.49±19.77 and 525.55±82.05 mm^2, respectively). Film containing THY at a concentration of 4 mg/cm^2 showed very high antimicrobial activity

on this microorganism and no growth was observed in Petri dish surface (Figure 7.25). CAR at 0.25 and 0.5 mg/cm^2 did not show any inhibitory effect against *E. carotovora.* However, CAR between 1 and 4 mg/cm^2 inhibited the growth of *E. carotovora* in a concentration-dependent manner and gave measurable zones changing between 151.58±28.61 and 913.88±102.27 mm^2 (Figure 7.26). Results showed that the inhibitory effect of THY on *E. carotovora* was higher than that of CAR. Figure 7.24 show that *E. carotovora* was more resistant against EUG at low concentrations, by high concentration of EUG (4 mg/cm^2) is quite effective on this bacteria (zone area: 744.42±63.29 mm^2). Photos of Petri dishes showing antimicrobial effects of EUG at different concentrations are shown in Figure 7.27. Similar to THY, CIT at high concentration (4 mg/cm^2) also totally inhibited the growth of *E. carotovora* (Figure 7.28).

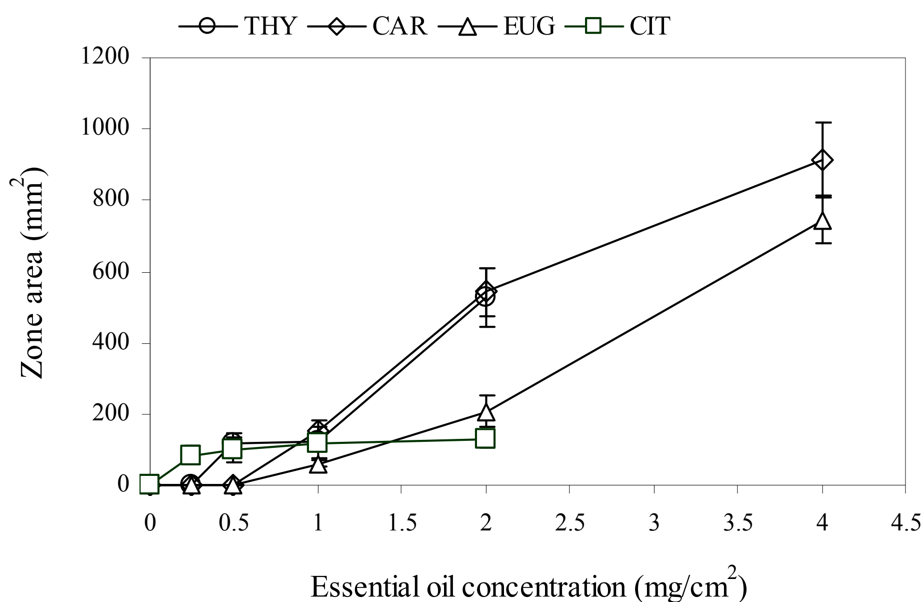

Figure 7.24. Antimicrobial activities of essential oils containing zein films on *E. carotovora*

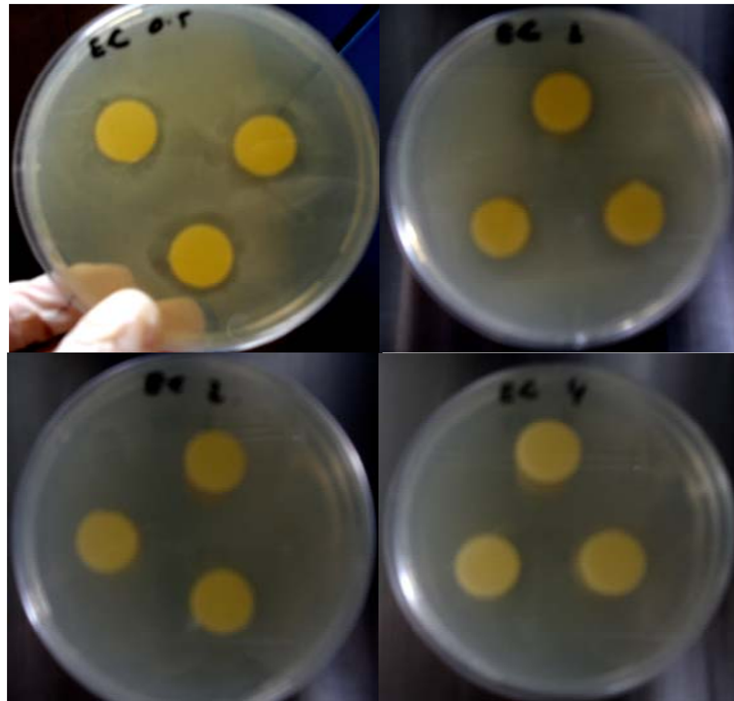

Figure 7.25. Growth inhibition of *E. carotov*ora by zein film containing THY (A: 0.5 mg/cm^2; B: 1 mg/cm^2; C: 2 mg/cm^2; D: 4 mg/cm^2)

Figure 7.26. Growth inhibition of *E. carotovora* by zein film containing CAR (A: 1 mg/cm^2; B: 2 mg/cm^2; C: 4 mg/cm^2)

Figure 7.27. Growth inhibition of *E. carotovora* by zein film containing EUG (A: 1 mg/cm^2; B: 2 mg/cm^2; C: 4 mg/cm^2)

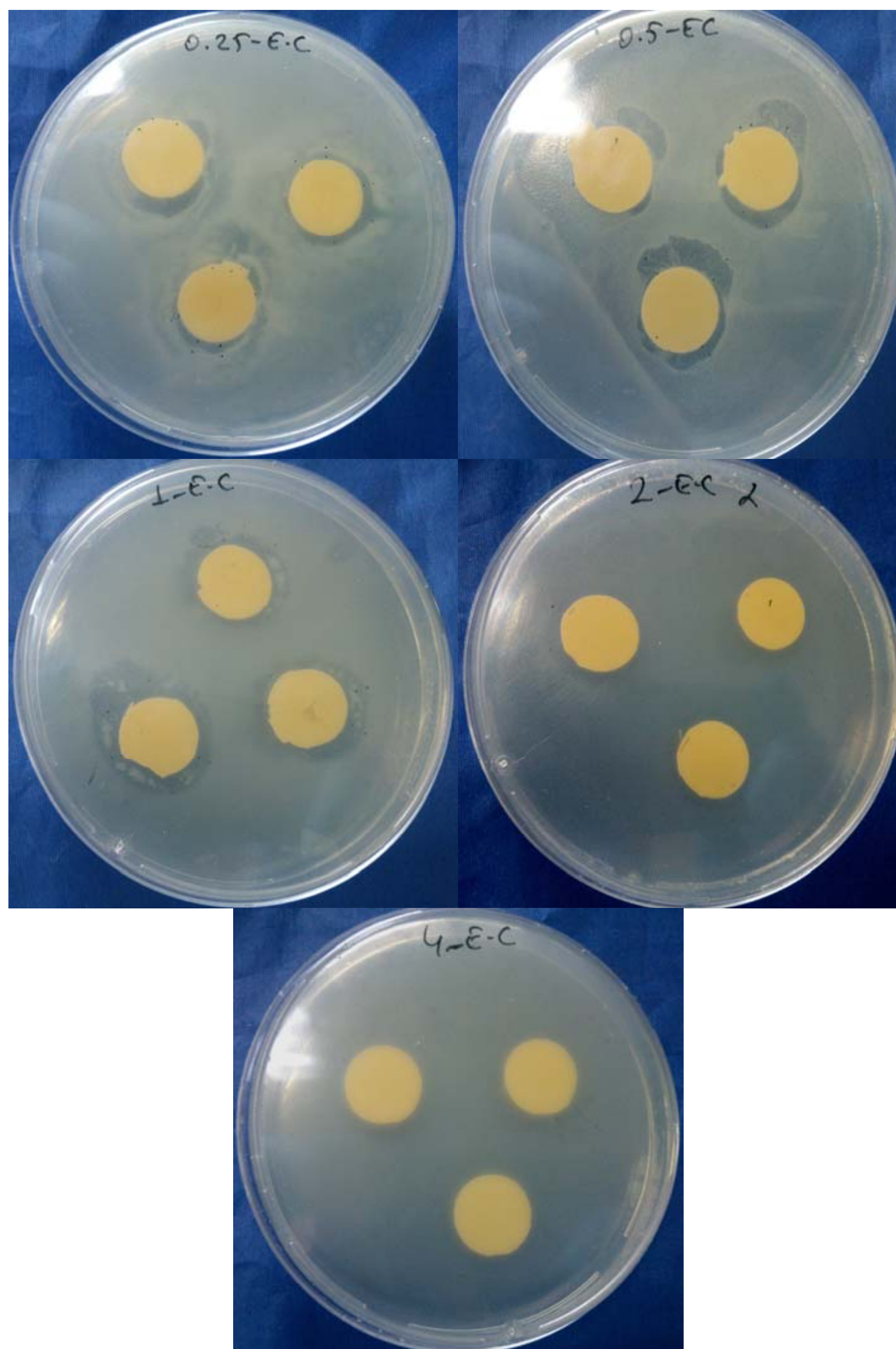

Figure 7.28. Growth inhibition of *E. carotovora* by zein film containing CIT (A: 0.25 mg/cm^2; B: 0.5 mg/cm^2; C: 1 mg/cm^2; D: 2 mg/cm^2; E: 4 mg/cm^2)

The results of further antimicrobial tests showed that zein films with THY, CAR and CIT were highly effective against *X. vesicatoria* (Figure 7.29). The use of THY at 2 and 4 mg/cm^2 concentrations, CAR and CIT at 4 mg/cm^2 achieve total inhibition in the Petri dishes (Figure 7.30 and Figure 7.31). The films containing 0.25 mg/cm^2 concentration of THY showed no antimicrobial activity on *X. vesicatoria* whereas 1, 2 and 4 mg/cm^2 concentrations of THY inhibited the growth of this pathogen effectively.

As can be seen in Figure 7.29, THY at 1 mg/cm^2 concentration formed large inhibition zone (811.24 ± 143.35 mm^2) around tested discs. In case of zein films containing CAR, inhibition zones on *X. vesicatoria* were measured at every concentration. As the concentration increased, the zone area of inhibition also increased significantly on *X. vesicatoria.* CAR at 0.25 and 0.5 mg/cm^2 concentrations gave inhibition zones with 86.08±24.67 and 140.11±33.24 mm^2 whereas CAR at 1 and 2 mg/cm^2 formed significantly larger inhibition zones, 878.47±141.68 and 1326.24±170.86 mm^2, respectively. Antimicrobial behavior of EUG and CIT at 1 and 2 mg/cm^2 concentrations is similar whereas 4 mg/cm^2 concentration of CIT was more effective than EUG against *X. vesicatoria* (Figure 7.32).

In the literature, the antibacterial properties of thymol have been investigated against many spoilage bacteria and food borne pathogens (Nobile, et al. 2008). Armendariz et al. (2010) investigated the antimicrobial activity of different films containing oregano and clove essential oils which contain carvacrol and eugenol as major phenolic compounds, respectively. These authors reported that films containing 40% (w/w) concentration of carvacrol and eugenol were effective against *L. innocua*, *S. aureus, S. enteritidis.*

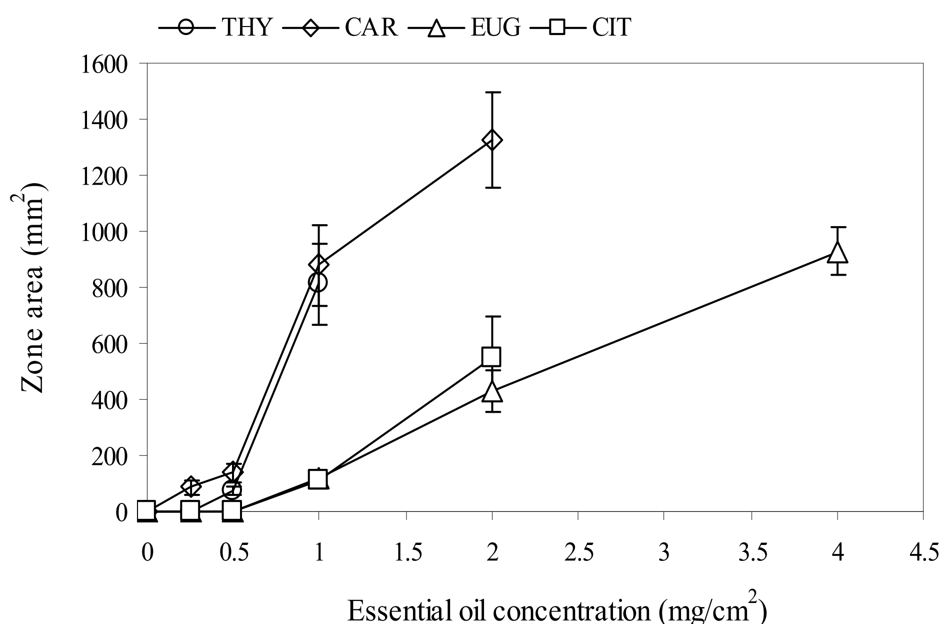

Figure 7.29. Antimicrobial activities of essential oils containing zein films on *X. vesicatoria*

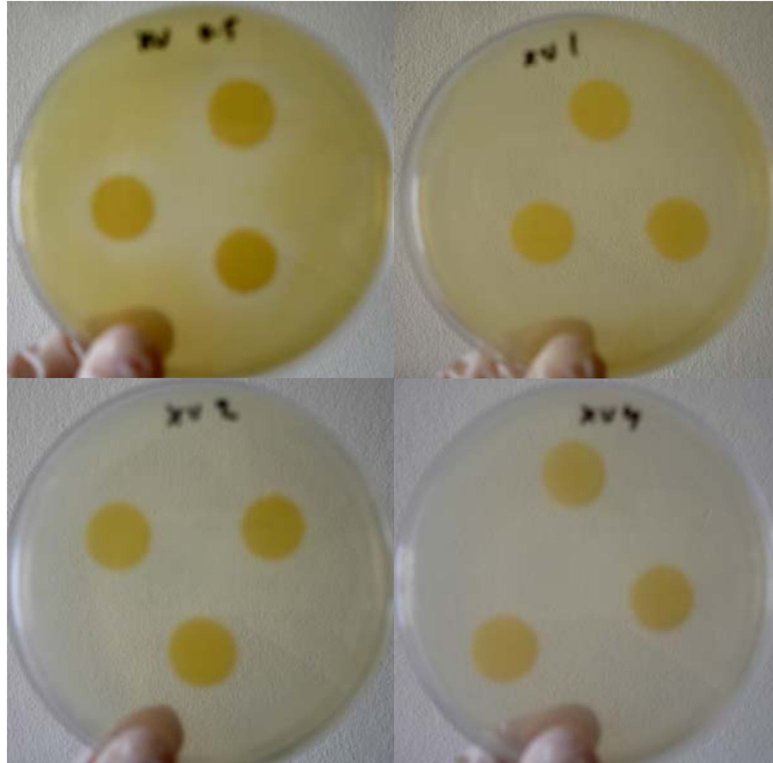

Figure 7.30. Growth inhibition of *X. vesicatoria* by zein film containing THY (A: 0.5 mg/cm^2; B: 1 mg/cm^2; C: 2 mg/cm^2; D: 4 mg/cm^2)

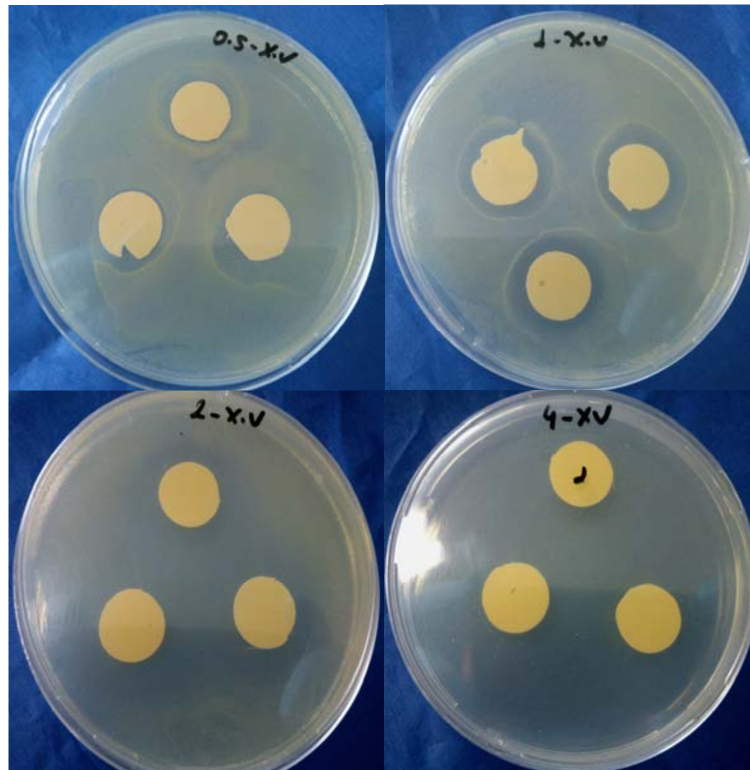

Figure 7.31. Growth inhibition of *X. vesicatoria* by zein film containing CIT (A: 0.5 mg/cm^2; B: 1 mg/cm^2; C: 2 mg/cm^2; D: 4 mg/cm^2)

Figure 7.32. Growth inhibition of *X. vesicatoria* by zein film containing EUG (A: 1 mg/cm^2; B: 2 mg/cm^2; C: 4 mg/cm^2)

Figure 7.33 shows the antimicrobial effect of films containing 0.25, 0.5, 1, 2, and 4 mg THY, CAR, EUG and CIT on *P. syringae.* The films containing THY, CAR, and CIT did not show any antimicrobial activity on *P. syringae* whereas EUG at 4 mg/cm^2 concentration inhibited the growth of this pathogen (Figure 7.34).

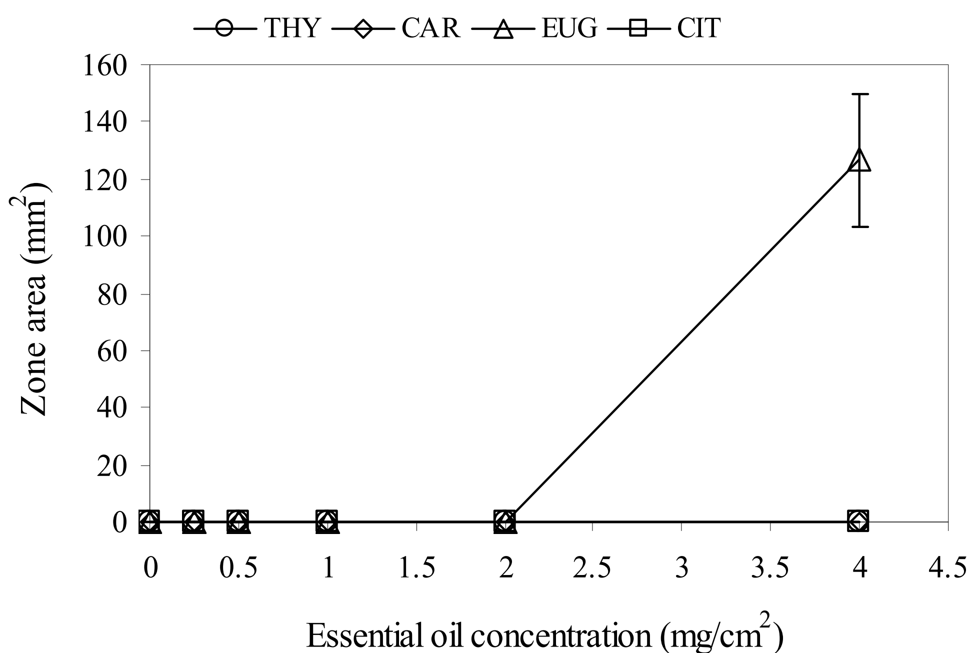

Figure 7.33. Antimicrobial activities of essential oils containing zein films on *P.syringae*

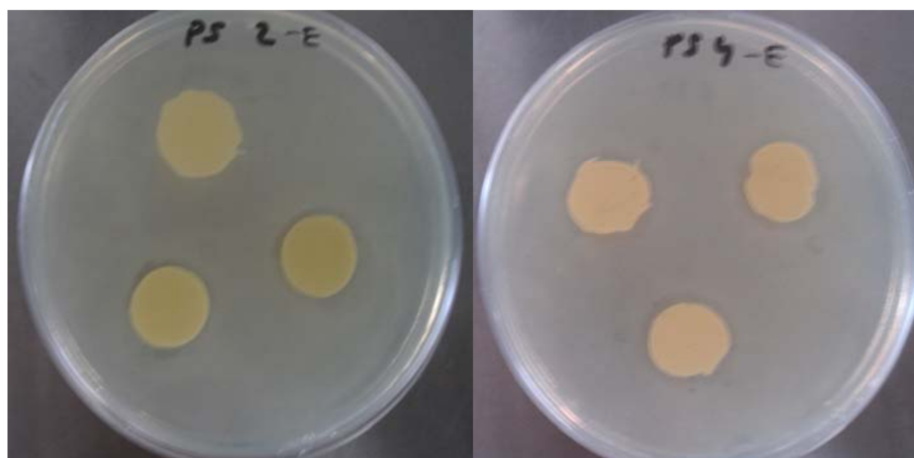

Figure 7.34. Growth inhibition of *P. syringae* by zein film containing EUG (A: 2 mg/cm^2; B: 4 mg/cm^2)

7.2.2. Mechanical Properties of Edible Films Containing Essential Oils

The thicknesses of the zein films containing different essential oils showed that only zein film incorporated with 2 mg/cm^2 concentration of EUG was statistically different from other films. Incorporation of THY, CAR, and EUG at the concentration of 2 or 4 mg/cm^2 caused a significant reduction in tensile strength of the films (Table 7.4). However, no reduction was observed in the tensile strength of the films by addition of the CIT into films. It was also observed that films containing EUG at a concentration of 0.25, 0.5, and 1 mg/cm^2 had higher values of tensile strength (11.75 ± 1.22, 11.10 ± 1.17, and 7.56 ± 0.81 MPa, respectively) than the other films.

On the other hand, the presence of essential oils at low concentrations did not significantly change the elongation of most films. However, at concentrations of 2 and 4 mg/cm^2, THY, CAR, and EUG significantly increased the elongation at break value. Compared to the control films lacking essential oils, incorporation of CIT at low concentrations caused inconsiderable reduction in elongation of the films. In contrast, the percent elongation of zein film reached a maximum value of 394.02 % with EUG. Young's modulus of zein films containing THY, EUG and CIT at low concentrations were also higher than control films. However, significant reductions in the Young's modulus of the films was observed at concentrations of 2 and 4 mg/cm^2 of THY, CAR, and EUG. No significant differences were noted in the Young's modulus among different concentrations of the films incorporated with CIT.

In the literature effect of essential oils on mechanical properties of edible films was investigated by Rojas-Graü et al. (2007). In that study, authors studied incorporation of essential oils into alginate based composite films and reported that the tensile strength and elastic modulus of films reduced while elongation of films increased by addition of carvacrol. Thus, although Rojas-Graü et al. (2007) worked in a different film system their result are in agreement with those obtained in this study using carvacrol.

Table 7.4. Mechanical properties of zein films containing essential oils (mean ± SD)

	Film composition						
THY (mg/cm^2)	CAR (mg/cm^2)	EUG (mg/cm^2)	CIT (mg/cm^2)	Tensile strength (MPa)	Young's modulus (MPa)	Elongation (%)	Film thickness (μm)
–	–	–	–	5.60 ± 0.24^c	376.53 ± 11.93^c	1.58 ± 0.24^a	$126.79^b \pm 3.13$
0.25	–	–	–	5.38 ± 0.67^c	470.33 ± 7.62^d	1.08 ± 0.15^a	$160.30^b \pm 1.81$
0.5	–	–	–	4.58 ± 1.84^c	454.22 ± 104.39^d	0.99 ± 0.47^a	$134.28^b \pm 1.21$
1	–	–	–	5.09 ± 0.92c	345.79 ± 62.59^c	1.55 ± 0.08^a	$128.25^b \pm 1.22$
2	–	–	–	2.71 ± 0.36^b	180.33 ± 24.80^b	33.61 ± 17.26^a	$144.83^b \pm 1.00$
4	–	–	–	1.24 ± 0.09^a	53.25 ± 7.62^a	366.67 ± 19.45^c	$140.08^b \pm 1.57$
–	0.25	–	–	4.72 ± 0.75^c	314.79 ± 5.60^c	1.67 ± 0.24^a	$126.47^b \pm 3.61$
–	0.5	–	–	5.89 ± 0.25^c	351.16 ± 14.10^c	1.84 ± 0.21^a	$144.07^b \pm 1.39$
–	1	–	–	4.68 ± 0.95^c	226.82 ± 30.92^b	8.79 ± 1.12^a	$129.27^b \pm 1.07$
–	2	–	–	2.85 ± 0.22^b	183.92 ± 18.63^b	52.74 ± 15.90^a	$137.84^b \pm 2.60$
–	4	–	–	1.11 ± 0.53^a	37.36 ± 15.03^a	220.50 ± 128.19^b	$158.67^b \pm 1.52$
–	–	0.25	–	11.75 ± 1.22^e	567.56 ± 50.07^e	2.49 ± 0.23^a	$124.31^b \pm 0.91$
–	–	0.5	–	11.10 ± 1.17^e	507.34 ± 15.61^{de}	3.53 ± 1.16^a	$121.78^b \pm 2.45$
–	–	1	–	7.56 ± 0.81^d	344.05 ± 23.07^c	7.83 ± 3.94^a	$134.63^b \pm 0.73$
–	–	2	–	2.15 ± 0.07^{ab}	163.16 ± 6.50^b	238.61 ± 86.00^b	$171.08^a \pm 1.44$
–	–	4	–	1.31 ± 0.07^a	52.57 ± 2.07^a	394.02 ± 11.61^c	$151.59^b \pm 2.41$
–	–	–	0.25	2.82 ± 0.61^b	451.47 ± 27.78^d	0.64 ± 0.16^a	$136.18^b \pm 2.22$
–	–	–	0.5	4.66 ± 0.65^c	554.35 ± 35.56^e	0.88 ± 0.10^a	$118.98^b \pm 3.54$
–	–	–	1	4.32 ± 0.25^c	412.16 ± 47.39^{cd}	1.21 ± 0.17^a	$157.80^b \pm 3.22$
–	–	–	2	4.52 ± 0.38^c	383.80 ± 20.94^c	1.84 ± 0.22^a	$122.07^b \pm 1.70$
–	–	–	4	4.51 ± 0.29^c	370.88 ± 23.65^c	3.59 ± 0.69^a	$138.71^b \pm 3.33$

a-e. Different letters within a column indicate significant difference ($p<0.05$).

7.2.3. Scanning Electron Microscopy (SEM) of Edible Films Containing Essential Oils

The morphologies of the antimicrobial zein films incorporated with THY, CAR, EUG, and CIT were identified by SEM. Figures 7.35A-F, 7.36A-F, 7.37A-F, and 7.38A-F show the cross-section images of the control zein film and zein films incorporated with essential oils at 0.25, 0.5, 1, 2, and 4 mg/cm^2. These cross-section images of films gave information about the change of porosity of zein film by addition of essential oils at different concentration. The incorporation of different essential oils caused formation of different film structures. The control film (without essential oil) had a porous structure. Some large pores were also observed within control zein film (Fig. 7.35-A). The addition of low concentrations of THY did not cause significant changes in zein film morphology. On the other hand, the incorporation of 2 and 4 mg/cm^2 of THY into films caused formation of great numbers of pores at different sizes.

The cross-section images also showed the increased film porosity by the addition of CAR at high concentrations (Figure. 7.36). In particular, 4 mg/cm^2 CAR containing zein film had larger pores and more cracks than zein films incorporated low concentrations of CAR. Figure 7.37 are the SEM of films containing different concentrations of EUG. Low concentrations of EUG did not significantly affect morphology of films, but increased EUG concentrations caused dramatic increases in sizes and number of pores.

Figure 7.38 illustrates the scanning electron micrograph of zein films containing various concentrations of CIT. The incorporation of CIT even at low concentrations caused formation of large pores and numerous cavities within the films. It is worth to note that the CIT caused the most drastic changes in film morphologies and formed largest pores. The increase in CIT concentration caused an increase in film pore sizes. However, the extremely large pores formed with CIT suggest the presence of some solubility problems with this essential oil.

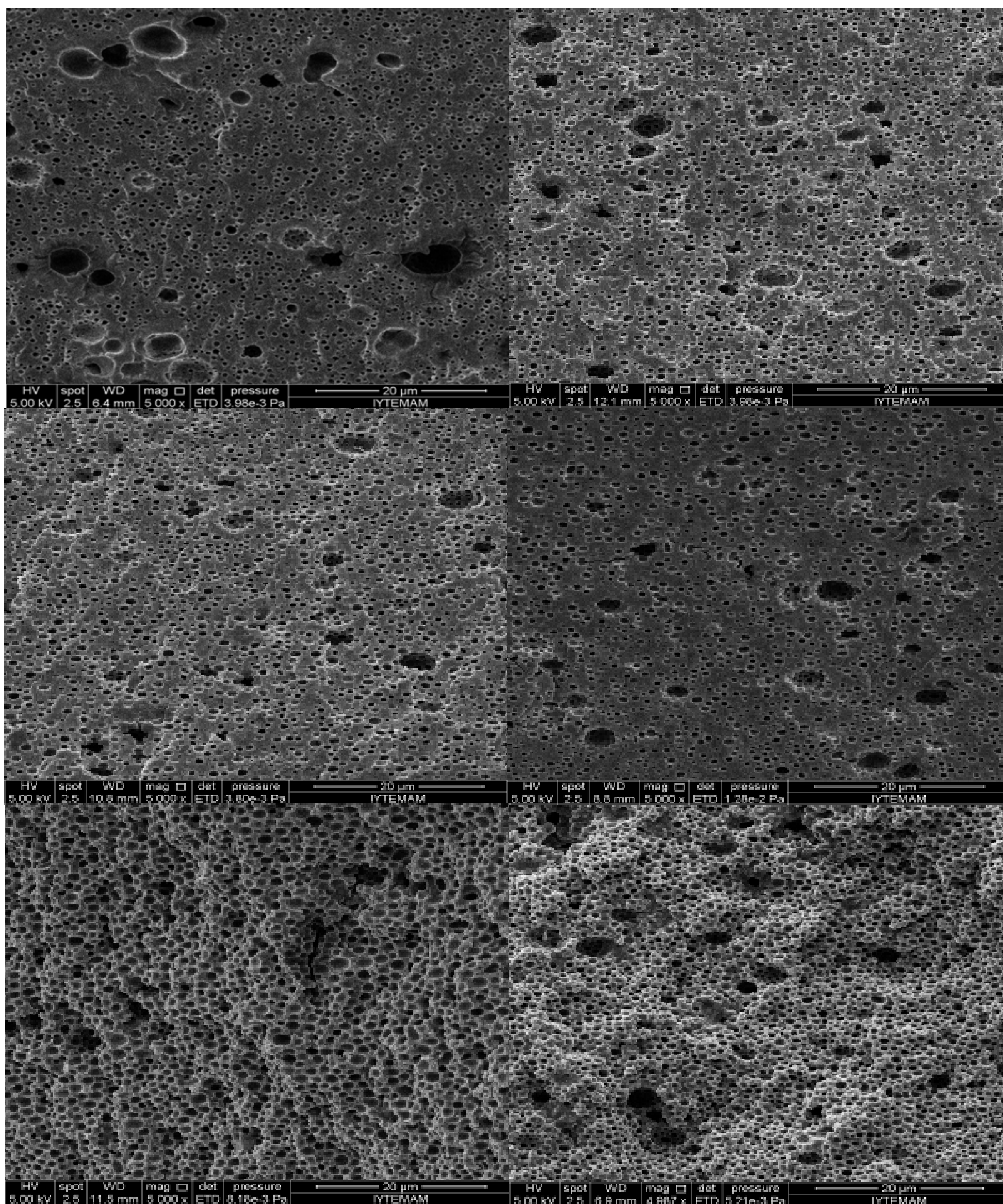

Figure 7.35. SEM photographs of different film cross-sections. (A) Control zein film, (B) 0.25 mg/cm^2 THY containing zein film, (C) 0.5 mg/cm^2 THY containing zein film, (D) 1 mg/cm^2 THY containing zein film, (E) 2 mg/cm^2 THY containing zein film, (F) 4 mg/cm^2 THY containing zein film. Magnifications were 5000× for A-F.

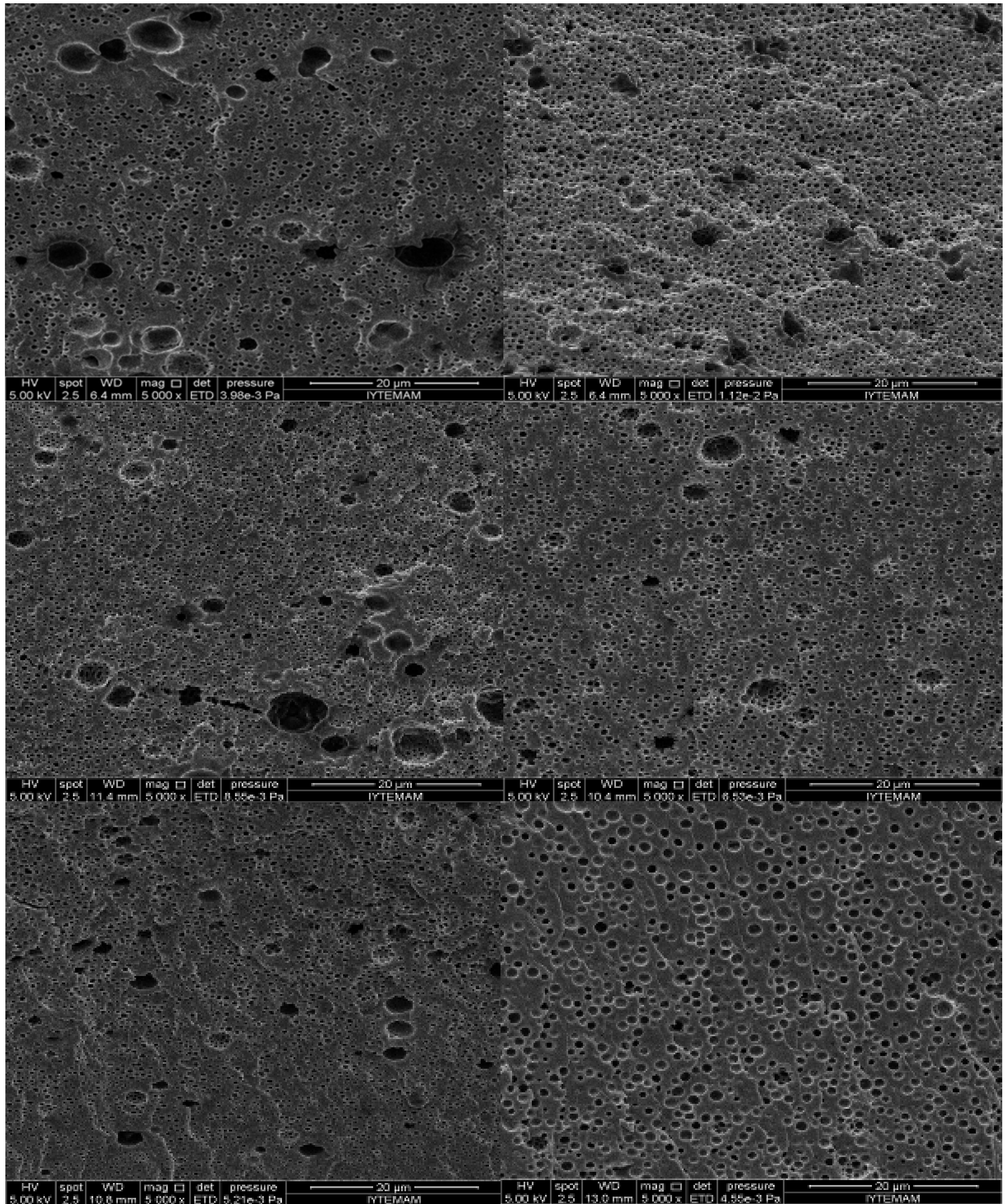

Figure 7.36. SEM photographs of different film cross-sections. (A) Control zein film, (B) 0.25 mg/cm^2 CAR containing zein film, (C) 0.5 mg/cm^2 CAR containing zein film, (D) 1 mg/cm^2 CAR containing zein film, (E) 2 mg/cm^2 CAR containing zein film, (F) 4 mg/cm^2 CAR containing zein film. Magnifications were 5000× for A-F.

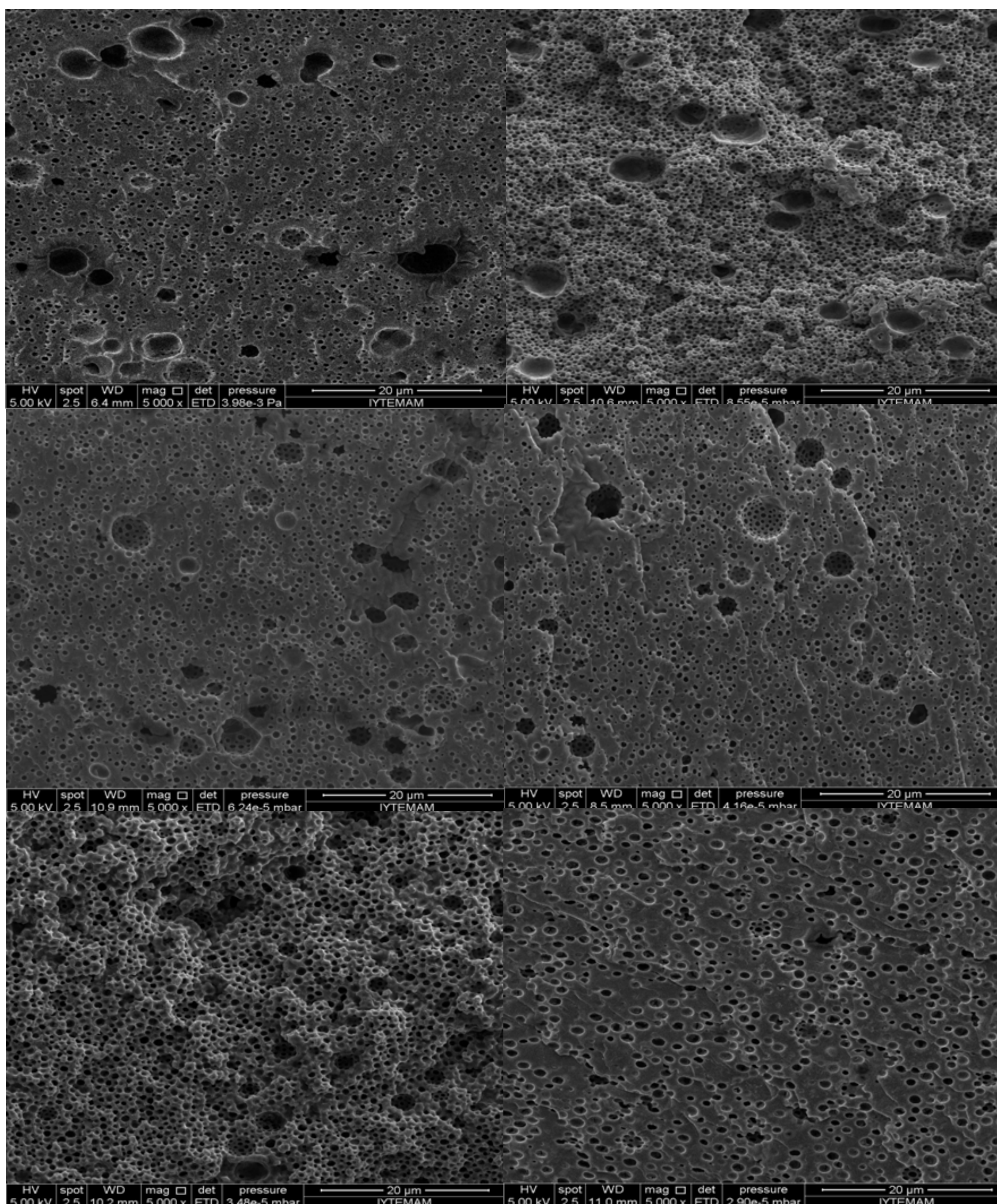

Figure 7.37. SEM photographs of different film cross-sections. (A) Control zein film, (B) 0.25 mg/cm^2 EUG containing zein film, (C) 0.5 mg/cm^2 EUG containing zein film, (D) 1 mg/cm^2 EUG containing zein film, (E) 2 mg/cm^2 EUG containing zein film, (F) 4 mg/cm^2 EUG containing zein film. Magnifications were 5000× for A-F.

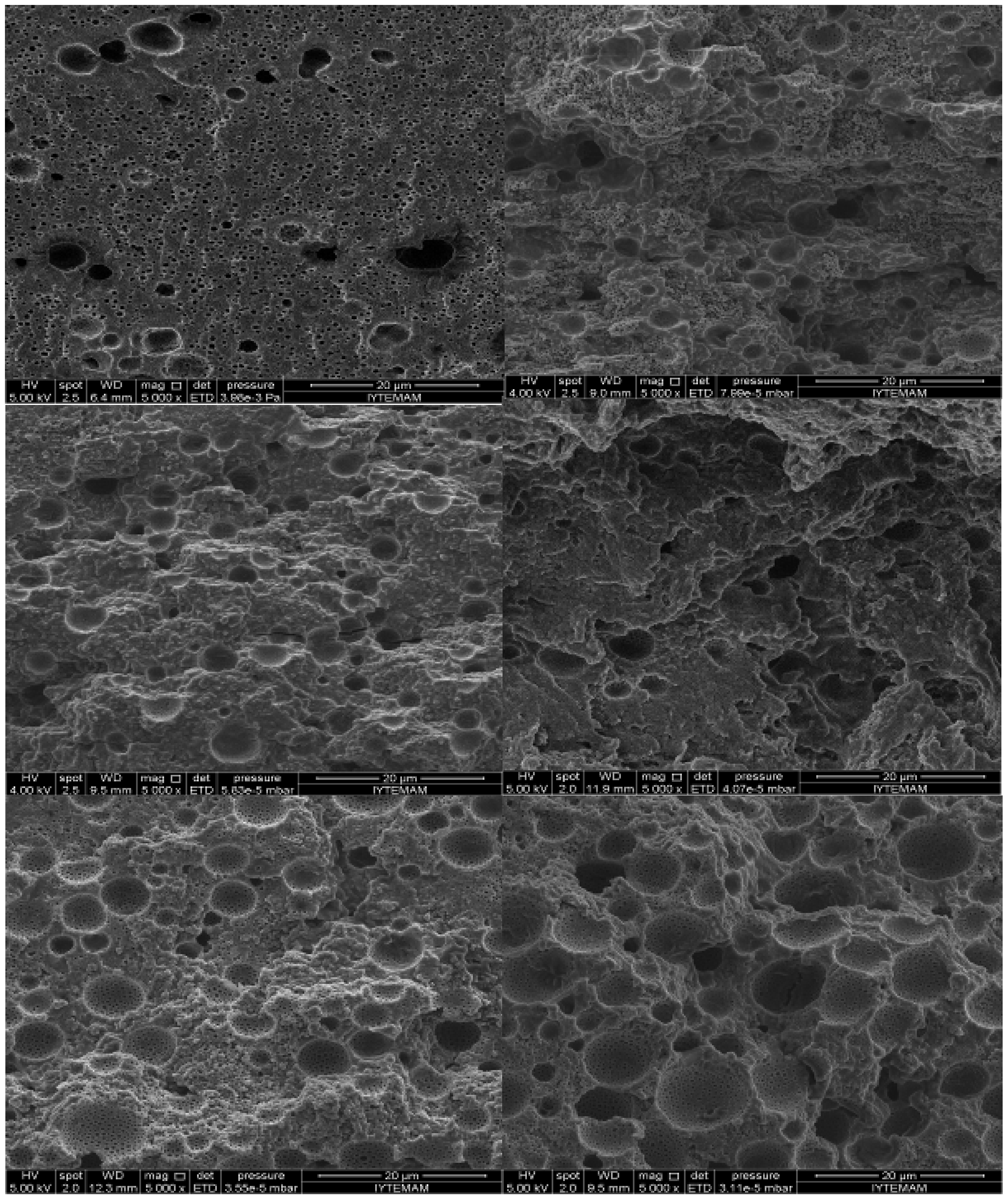

Figure 7.38. SEM photographs of different film cross-sections. (A) Control zein film, (B) 0.25 mg/cm^2 CIT containing zein film, (C) 0.5 mg/cm^2 CIT containing zein film, (D) 1 mg/cm^2 CIT containing zein film, (E) 2 mg/cm^2 CIT containing zein film, (F) 4 mg/cm^2 CIT containing zein film. Magnifications were 5000× for A-F.

7.3. Inhibition of Plant Pathogens by Edible Films Containing Plant Extracts

7.3.1. Minimum Inhibitory Concentrations of Plant Extracts

Phenolic extracts of oregano, walnut shells, artichoke stems and clove were screened for antimicrobial activity using 96-well microplates against *E. amylovora, E. carotovora, X. vesicatoria* and *P. syringae*. Antimicrobial effects of plant extracts at different concentrations on the growth of tested microorganisms are presented in Table 7.5. The MIC of oregano, artichoke, walnut and clove was evaluated in a concentration range from 0.01 to 40.96 mg/ml. Walnut extract did not show any antimicrobial activity against four pathogens at studied concentrations. In contrast, the growth of *E. carotovora* was inhibited by 100% and 44% at 0.24 mg/ml and 5.12 mg/ml of clove extract, respectively. 40.96 mg/ml of oregano and artichoke extract produced a considerable reduction (about 47%) in *E. carotovora* growth.

E. amylovora was inhibited by 91% and 100% in the presence of oregano extract and clove extract at the concentrations of 40.96 mg/ml and 10.24 mg/ml, respectively. Also, 2,56 mg/ml of clove extract reduced bacterial growth with 42 % inhibition. In addition to this, 40.96 mg/ml of artichoke extract achieved 49% inhibition in *E. amylovora* growth.

It was observed that *P. syringae* was more resistance to all plant extracts. Total inhibition of *P. syringae* was only obtained by addition of 10.24 mg/ml of clove extract.

The growth of *X. vesicatoria* was inactivated by 74 % and 90 % by using a concentration of 20.48 and 40.96 mg/ml of oregano extract, respectively. Among all microorganisms, *X. vesicatoria* was found as the most sensitive bacteria to artichoke extract. Artichoke extract produced best inhibition profiles on *X. vesicatoria*, with 34%, 67% and 68% inhibitions at concentrations of 10.24, 20.48 and 40.96 mg/ml, respectively. On the other hand, similar to *E. amylovora*, *X. vesicatoria* was inhibited with clove extract at a concentration of 10.24 mg/ml. The results showed that clove extract had a relatively stronger antimicrobial activity than other plant extracts against all tested pathogens.

Table 7.5. Antimicrobial effects of plant extracts at different concentrations on the growth of plant pathogens

	Concentrations of the phenolic compounds (mg/ml)												
	0.01	0.02	0.04	0.08	0.16	0.32	0.64	1.28	2.56	5.12	10.24	20.48	40.96
E. carotovora													
oregano	+	+	+	+	+	+	+	+	+	+	+	+	47%
walnut shell	+	+	+	+	+	+	+	+	+	+			
artichoke stem	+	+	+	+	+	+	+	+	+	+	+	+	48%
clove	+	+	+	+	+	+	+	+	+	44%	100%	100%	100%
E. amylovora													
oregano	+	+	+	+	+	+	+	+	+	+	+	+	91%
walnut shell	+	+	+	+	+	+	+	+	+	+			
artichoke stem	+	+	+	+	+	+	+	+	+	+	+	+	48%
clove	+	+	+	+	+	+	+	+	42%	82%	100%	100%	100%
P. syringae													
oregano	+	+	+	+	+	+	+	+	+	+	+	+	+
walnut shell	+	+	+	+	+	+	+	+	+	+			
artichoke stem	+	+	+	+	+	+	+	+	+	+	+	+	+
clove	+	+	+	+	+	+	+	+	+	+	100%	100%	100%
X. vesicatoria													
oregano	+	+	+	+	+	+	+	+	+	+	+	73%	90%
walnut shell	+	+	+	+	+	+	+	+	+	+			
artichoke stem	+	+	+	+	+	+	+	+	+	+	39%	67%	68%
clove	+	+	+	+	+	+	+	+	57%	77%	100%	100%	100%

% values refer the inhibitions in bacterial growth

7.3.2 Antimicrobial Activity of Edible Films Containing Clove Extract

Due to the fact that clove presented the strongest antibacterial properties in broth medium on all microorganisms, antimicrobial zein films were obtained by using this extract. The antimicrobial effect of clove extract on all plant pathogens was investigated by using 0.25, 0.5, 1, 2, 4, 6 and 8 mg phenolic extract per cm^2 of films. As a result of antimicrobial test, clove extract did not show any antimicrobial effect on *P. syringae.* The use of clove extract at 0.25, 0.5, 1 and 2 mg/cm^2 did not also give measurable zones on other three plant pathogens. However, the films containing clove extract at 4 mg/cm^2 effectively inhibit plant pathogens except *P. syringae.* Clove extract at 4, 6 and 8 mg/cm^2 concentrations showed antimicrobial activity on *X. vesicatoria* at a concentration dependent manner (Figure 7.39). A 1.5-fold increase in clove extract concentration caused almost 1.5 fold increase in antimicrobial activity of films against *X. vesicatoria* . Among three plant pathogens, *E. carovotora* was found more resistance to clove extract than the others (Figure 7.39). Clove extract showed weak antimicrobial activity on *E. carovotora* at 6 and 8 mg/cm^2 concentrations (zone areas: 55.19±19.54 and 90.87±22.76 mm^2, respectively). Films containing clove extract at 4 and 6 mg/cm^2 showed antimicrobial activity on *E. amylovora* and formed 122.65 ± 44.57 and 174.96 ± 43.38 mm^2 zones around tested discs. However, the use of clove extract at 8 mg/ cm^2 caused total inhibition of *E. amylovora* in petry dishes. Growth inhibition of *E. amylovora* and *X. vesicatoria* by zein film containing clove extract is presented in Figure 7.40 and 7.41.

In literature, antimicrobial activity of apple puree film forming solutions containing clove bud oil against *E. coli* O157:H7, *Salmonella enterica,* and *Listeria monocytogenes* was determined by Du et al. (2009). These workers found that clove bud oil at 3% (w/w) in the film was effective against *E. coli* O157:H7 and *S. enterica* while clove bud oil at 1.5% in the film suppressed the growth of *Listeria monocytogenes*. Antimicrobial effect of clove oil was also reported by Gòmez-Estaca et al. (2010). These workers tested essential oils of clove, fennel, cypress, lavender, thyme, pine and rosemary for their antimicrobial activities on some important spoilage bacteria and food pathogen and they found that the highest inhibitory effect was obtained by clove essential oil. Thus, Gòmez-Estaca et al. (2010) developed gelatin–chitosan-based edible

films by incorporating clove essential oil into films and obtained antimicrobial activity against six selected microorganisms.

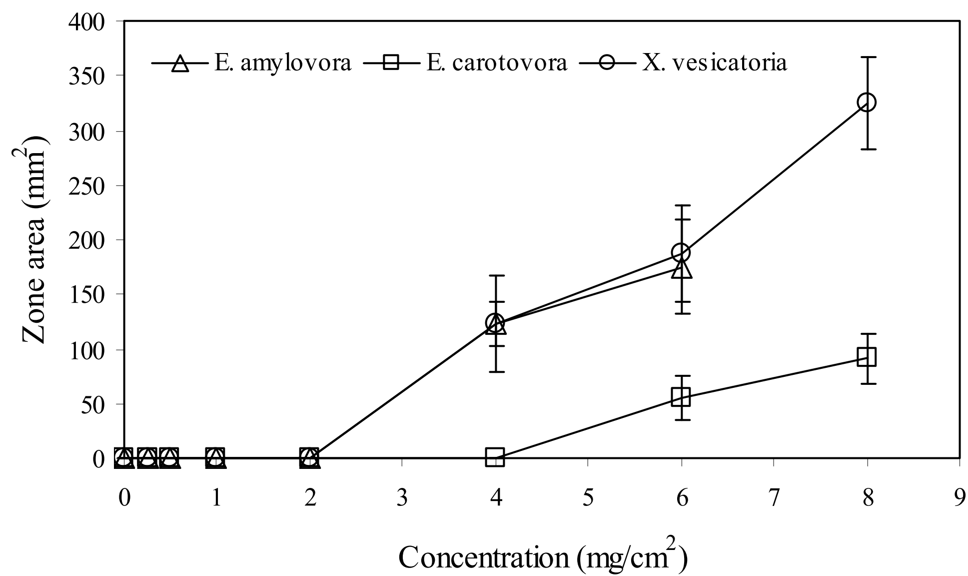

Figure 7.39. Antimicrobial activities of clove extract containing zein film on plant pathogens

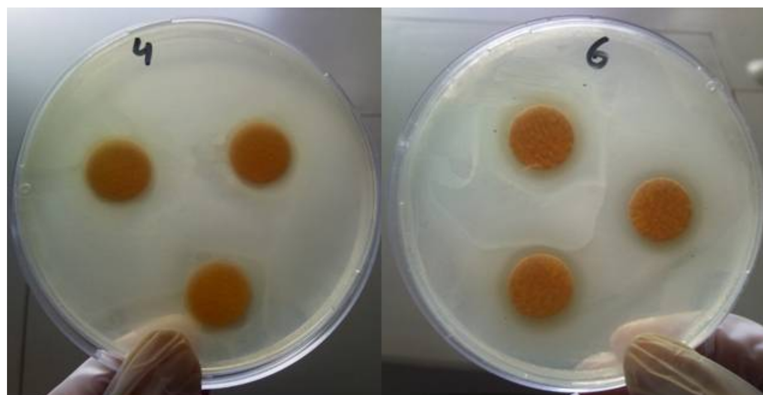

Figure 7.40. Growth inhibition of *E. amylovora* by zein film containing clove extract

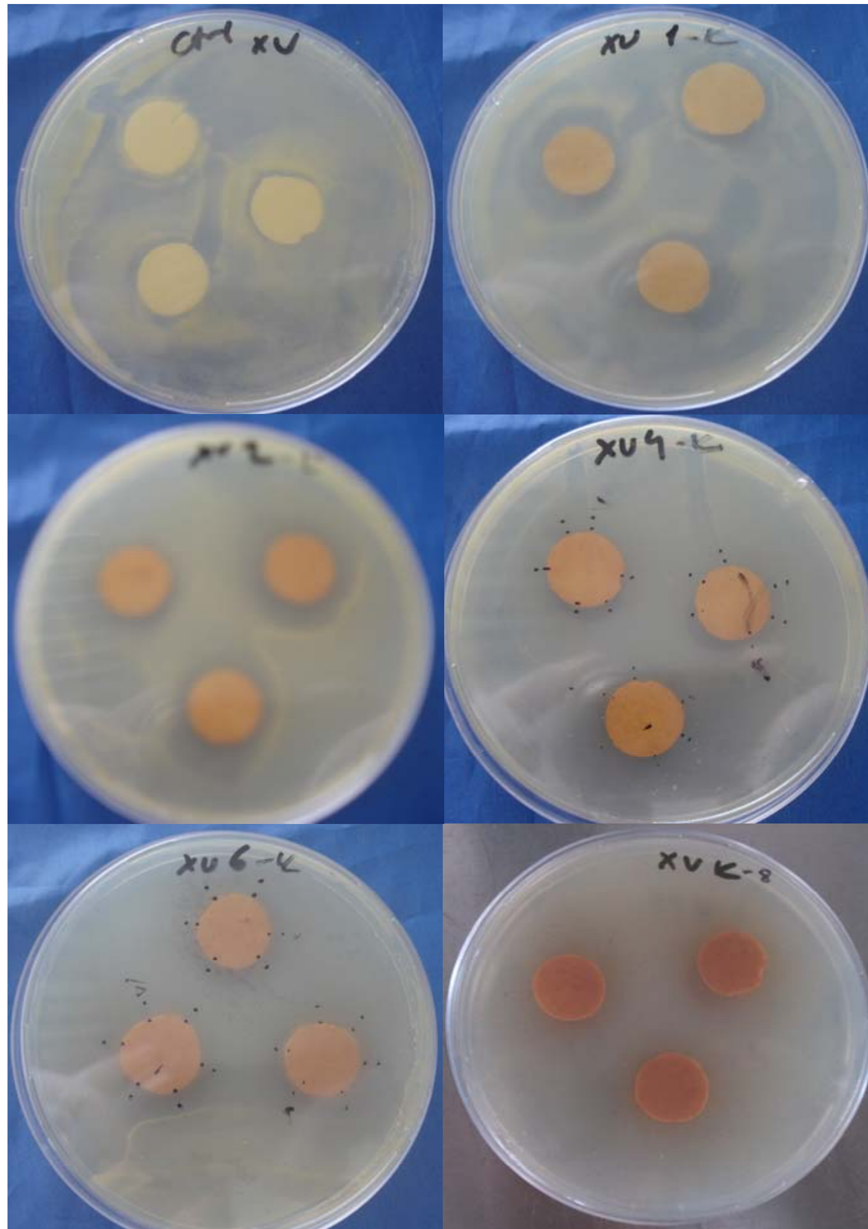

Figure 7.41. Growth inhibition of *X. vesicatoria* by zein film containing clove extract

7.3.3. Release Tests of Edible Films Containing Clove Extract

The release kinetics of an active agent from films has received considerable attention in the last years due to the specific applications of films in the food packaging, As seen in Figure 7.42, for zein films incorporated with 4 mg/cm^2, 6 mg/cm^2, and 8 mg/cm^2 clove extract, the amount of phenolic compound released from the films corresponded 21.25%, 22.83%, and 23.12% of the total amount of clove extract into films, respectively. The films containing different concentrations of clove extract

showed similar release profiles. It appeared that a significant part of the clove extract phenolics were trapped within the zein films. However, it should be specified that the clove extract is not a pure one and it contains all ethanol soluble solids.

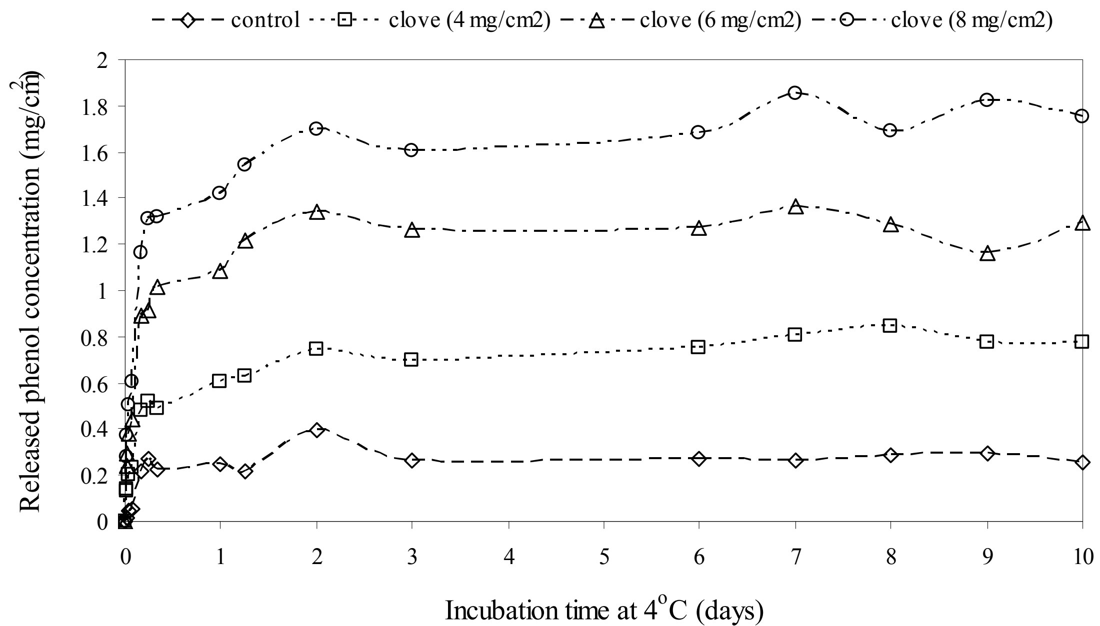

Figure 7.42. Release profiles of clove extract from zein films incubated in distilled water at 4 ºC

7.3.4. Mechanical Properties of Edible Films Contaning Clove Extract

The effect of clove extract on mechanical properties of zein film was tested at different concentrations (Table 7.6). The tensile strength values of zein film containing clove extract at 8 mg/cm^2 concentration were statistically different from control zein film and zein films containing 2, 4, and 6 mg/cm^2 concentration of clove extract (P>0.05). Addition of 8 mg/cm^2 concentration of clove extract into zein film decreased the tensile strength of zein film. In the concentration range studied, the Young's modulus decreased when concentration of clove extract was increased in zein film. Young's modulus value of films at 2 mg/cm^2 concentration of clove extract was not statistically different from that of control zein film. However, incorporation of clove extract at the concentration of 8 mg/cm^2 reduced Young's modulus of zein film significantly.

The elongation of zein film incorporated with 2 mg/cm^2 clove extract was lower than control zein film. Zein films containing 4 and 6 mg/cm^2 clove extract had similar elongation values with controls, but zein films containing 8 mg/cm^2 clove extract showed slightly higher elongation than controls. In the literature, data about effect of ethanolic clove extracts in mechanical properties of edible films scarce, but several reports exist about effects of clove oil. Nonsee et al. (2011) observed increased elongation of edible hydroxypropyl methylcellulose films by increased concentration of encapsulated clove oil in the films. The reduced tensile strength of films by clove oil was reported by Hosseini et al. (2009) for chitosan based edible films. However, these studies did not form a reference for this study since there are significant differences between the phenolic compositions of ethanolic clove extracts (mostly water soluble) and clove oil.

Table 7.6. Mechanical properties of zein films containing clove extract (mean ± SD)

Film composition	Mechanical properties		
Clove extract (mg/cm^2)	Tensile strength (MPa)	Young's modulus (MPa)	Elongation (%)
–	8.79 ± 0.53^c	557.75 ± 19.03^d	1.53 ± 0.14^b
2	5.82 ± 0.86^b	553.92 ± 50.14^d	0.96 ± 0.24^a
4	5.51 ± 0.84^b	431.36 ± 52.27^c	1.30 ± 0.34^{ab}
6	5.92 ± 0.91^b	336.55 ± 45.68^b	2.18 ± 0.32^b
8	3.09 ± 0.26^a	151.27 ± 10.92^a	9.72 ± 0.69^c

a-d. Different letters within a column indicate significant difference ($p<0.05$).

7.3.5. Scanning Electron Microscopy (SEM) of Edible Films Containing Clove Extract

The morphologies of the antimicrobial zein films incorporated with clove extract at 2, 4, 6 and 8 mg/cm^2 concentrations were identified by SEM obtained for cross-sections of these films (Figure 7.43). The incorporation of clove extract into zein film caused the formation of larger pores than the control film. However, the number of pores in control film was greater than the number of pores in zein films containing clove extract. The porosity of films decreased when concentration of clove extract was increased in zein film. Some large crack and cavities were observed within zein films containing clove extract at high concentrations.

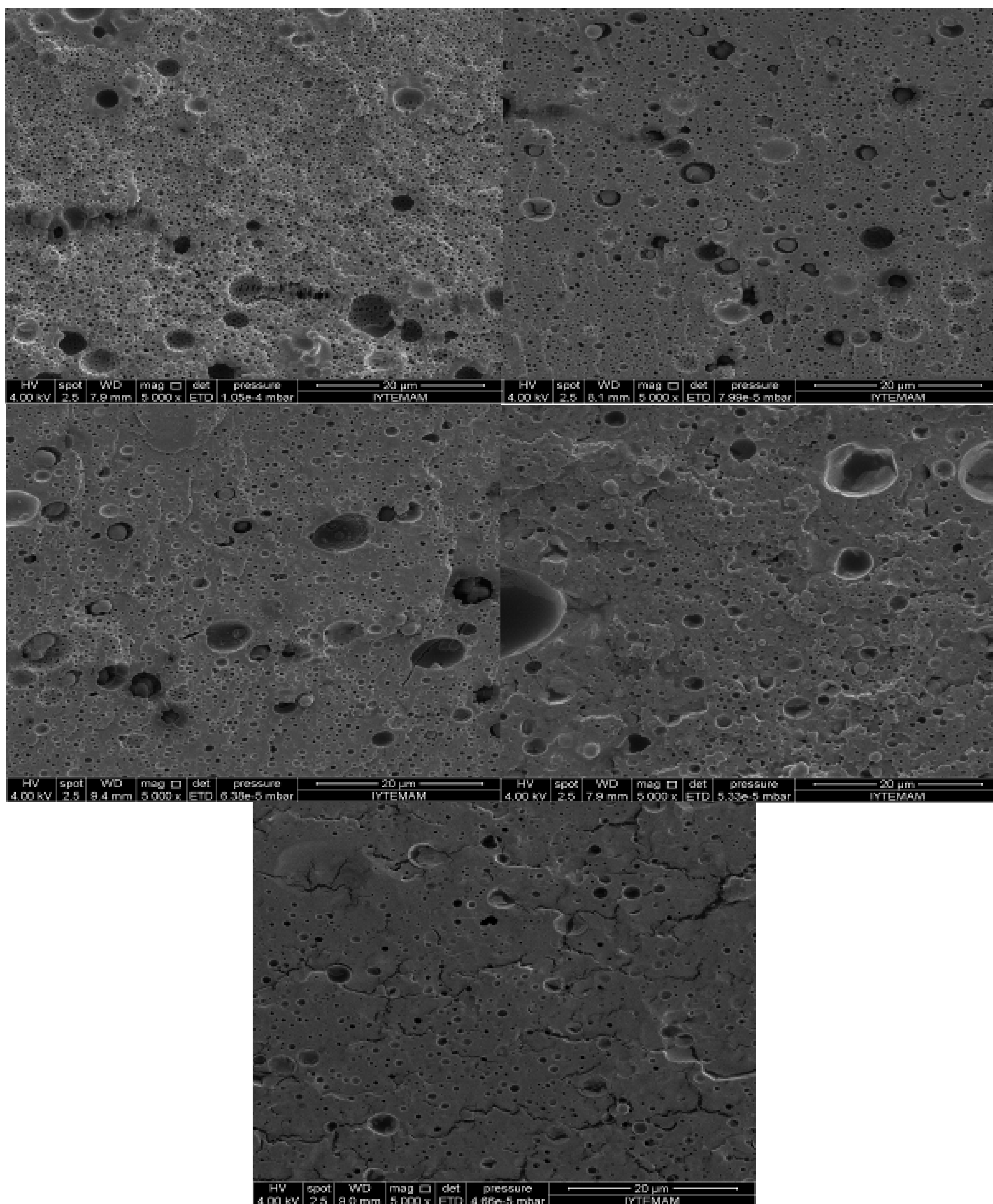

Figure 7.43. SEM photographs of different film cross-sections. (A) Control zein film, (B) 2 mg/cm^2 clove extract containing zein film, (C) 4 mg/cm^2 clove extract containing zein film, (D) 6 mg/cm^2 clove extract containing zein film, (E) 8 mg/cm^2 clove extract containing zein film. Magnifications were 5000× for A-F.

7.3.6. Total Phenol Contents of Plant Extracts

The mean values and standard deviations of TPCs for the natural extracts are presented in Figure 7.44. Clove extract had the highest TPC (274.06±36.46 mg of GA/g of liyophilized extract), while artichoke had the lowest (35.41±2.14 mg of GA/g of liyophilized extract). Kim et al. (2011) evaluated TPC, total flavonoids content and antioxidant activities of the hot water extracts of 13 spices. The results of these authors showed that clove had the highest TPC (108.28 mg catechin equivalent /g) among different spice extracts. It is also worth to note that the clove and oregano extracts obtained in our study had higher total phenol content than those used in the study of Shan et al. (2005). In that study, TPCs of oregano and clove extract were found as 100.17 mg of GAE/g of dry weight and 140.18 mg of GAE/g of dry weight, respectively. Thus, it appears that the high inhibitory effects of clove extract on the growth of plant pathogens is related with the high phenolic content of this plant extract.

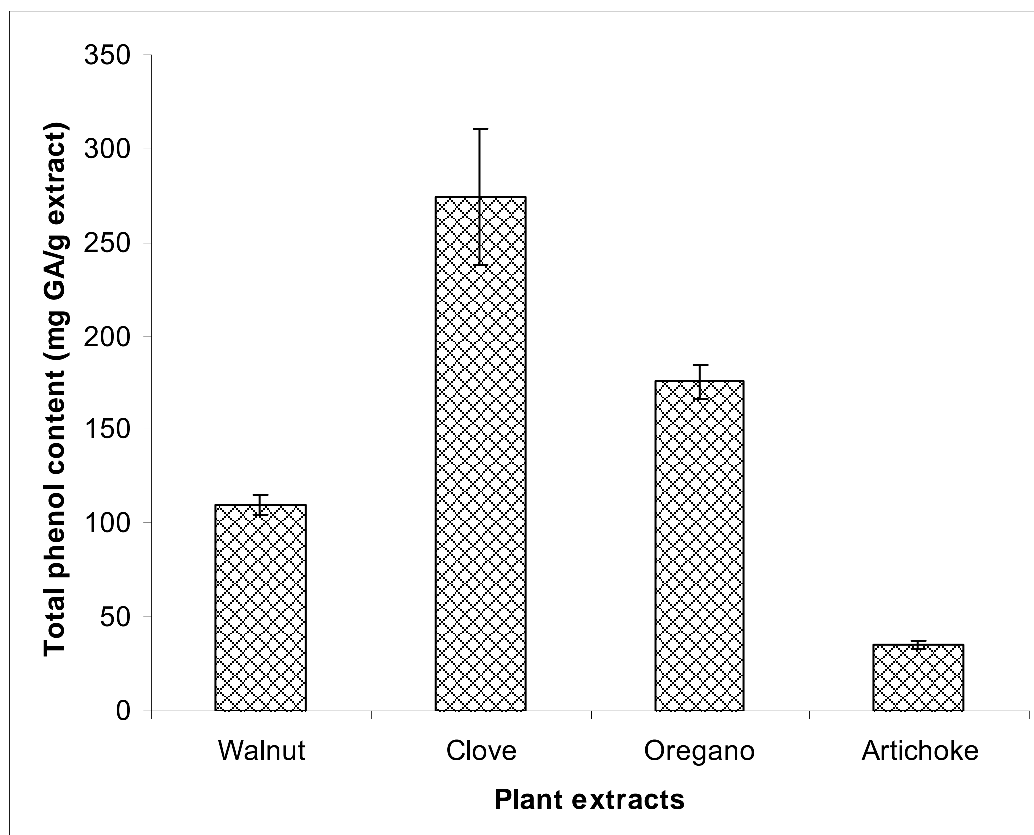

Figure 7.44. TPCs of different plant extracts (mean ± SD)

7.3.7. Phenol Composition of Clove Extract

Because of the fact that all clove constituents are quite volatile, analysis of clove extracts or oil has been generally carried out by means of gas chromatography (GC) or gas chromatography coupled with the mass spectroscopy (GC-MS) in the literature. Different volatile compounds, mainly eugenol (71.56 %) and eugenol acetate (8.99 %), were identified from the n-hexane extract of the buds of clove by GC-MS (Nassar, et al. 2007). Moreover, clove bud oil, which was cultivated in the Mediterranean region of Turkey, were obtained by steam-distillation method and its major constituents were analyzed by GC and GC-MS (Alma, et al. 2007). In that study, it was found that essential oil of the buds of clove contain 87% eugenol, 8.01% eugenyl acetate and 3.56% β-Caryophyllene. As alternative to the GC method, the simultaneous determination of clove constituents (eugenol, eugenol acetate, b-caryophyllene, a-humulene and caryophyllene oxide) by means of HPLC was achieved by Musenga et al. (2006). Rana et al. (2011) analyzed clove oil by HPLC and data confirmed presence of considerable eugenol in clove oil.

In our study, the major constituent of the ethanol extracts of clove was analyzed through a HPLC technique. Chromatogram presented in Figure 7.45 was obtained from clove extract using HPLC coupled with a Diode array detector. The chromatogram obtained shows a peak at 3.5 min corresponding to gallic acid. The HPLC analysis showed that gallic acid was the major compound present in clove extract, with a concentration of 21.95 mg/g of liyophilized extract. In the study of Shan et al. (2005), phenolic content of 26 common spice extracts were identified. In that study, HPLC analysis of clove extract showed that clove bud extracts had high levels of gallic acid (7.84 mg/g of dry weight). In our study, the antimicrobial activity of the clove extract against studied plant pathogens should be partly due to the presence of high level of gallic acid. This result was in agreement to that obtained by antimicrobial test of gallic acid containing films applied to plant pathogens. This phenolic acid inhibited the growth of the four plant pathogens effectively in a dose dependent-manner. Molecules of gallic acid possess three hydroxyl groups and its antimicrobial activity is attributed to its molecular structure.

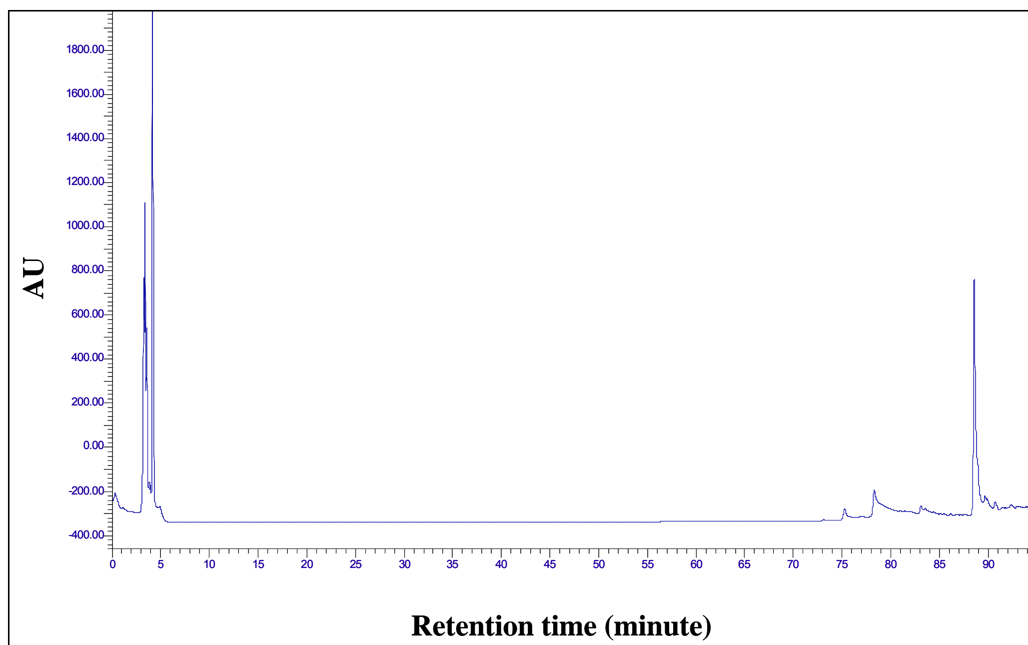

Figure 7.45. Hplc profile of clove extract (retention time of gallic acid: 3.5 min)

CHAPTER 8

CONCLUSION

>To develop a bio-based method to control plant pathogens edible antimicrobial films are developed against major plant pathogenic bacteria including *Pseudomonas syringae, Erwinia amylovora, Xanthomonas vesicatoria* and *Erwinia carotovora.*

>The films were developed by incorporating pure phenolic acids, essential oils and phenolic extracts into edible zein films. The most effective films on plant pathogens are those incorporated by single pure phenolic compounds and mixture of different pure phenolic compounds followed by films incorporated with essential oils and a phenolic extract from cloves.

>The most effective inhibitor on plant pathogens is the pure gallic acid. However, the incorporation of combination of different pure phenolic acids, gallic acid, cinnamic acid, and vanillic acid into zein films showed greater inhibitory activity on plant pathogens than incorporation of a single phenolic acid into films. The inhibitory activity of pure phenolic acids increases as a concentration dependent manner.

>The overall results of antimicrobial tests suggest that the *E. amylovora* was the most sensitive while *P. syringae* was the most resistant pathogen against antimicrobial films containing phenolic acids,

>The incorporation of pure phenolic acids at low concentrations did not cause major changes in the mechanical properties of zein films. However, increased concentration of pure phenolic compounds caused significant reductions in tensile strength and Young's modulus, and significant increases in elongation of films. In contrast, combination of gallic acid, cinnamic acid, and vanillic acid showed considerably different effects than using single pure phenolic compounds and reduced the elongation of film.

>The SEM images obtained for cross sections of films showed that the addition of different phenolic acids caused formation of different film structures. In particular, the combination of gallic acid, cinnamic acid, and vanillic acid caused the formation of large crack and cavities in film structures.

>The essential oils such as thymol, carvacrol, eugenol and citral possess a promising antimicrobial activity against *E. amylovora, X. vesicatoria, E. carotovora*.

However, *P. syringae* could not be effectively inhibited by the essential oils at the studied concentration range.

>The overall results of antimicrobial tests suggest that the *E. amylovora* was the most sensitive while *P. syringae* was the most resistant pathogen against antimicrobial films containing essential oils.

>The incorporation of essential oils (except citral) into zein films at the inhibitory concentrations caused significant reductions in tensile strength and Young's modulus of films and increased film elongation. The eugenol has a particularly high plastisizing effect on films and caused elongation of films up to 394 %.

>The incorporation of essential oils into films caused formation of pores and cracks and cavities in films. The morphological changes became sound by increased essential oil concentration.

>The antimicrobial films against plant pathogens could also be obtained by use of clove extract which contains considerable amounts of gallic acid. However, similar to essential oils films containing clove extract did not inhibit the growth of *P. syringae.*

>The release profiles of films in distilled water showed that 22% of clove extract phenolics are soluble in films while the remaining phenolic compounds exist in bound form.

>The increased clove extract concentration in films decreased the tensile strength and Young's modulus of films while an increase occurred in film elongation.

>The clove extract caused an increase in film porosity.

>The zein coatings lacking natural antimicrobials are already applied for coating of fresh fruits and vegetables to control their respiration rate and prolong their refrigerated storage. This work clearly showed the possibility of incorporating natural antimicrobial compounds in zein coatings and possibility of inhibiting plant pathogens which cause great economic losses during cold storage.

> Further studies are now continuing in the light of this project to apply zein coatings for cold stored cucumbers which suffer greatly from plant pathogens.

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APPENDIX A

PHOTOMETRIC MEASUREMENTS OF VARIOSKAN FLASH PLATE READER AT 600 NM

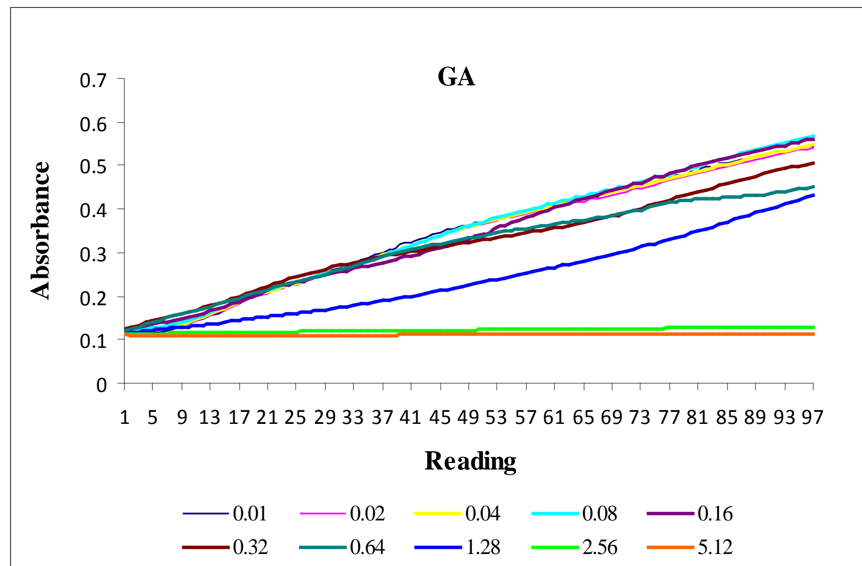

Figure A.1. Growth inhibition of *E. amylovora* by GA at different concentrations

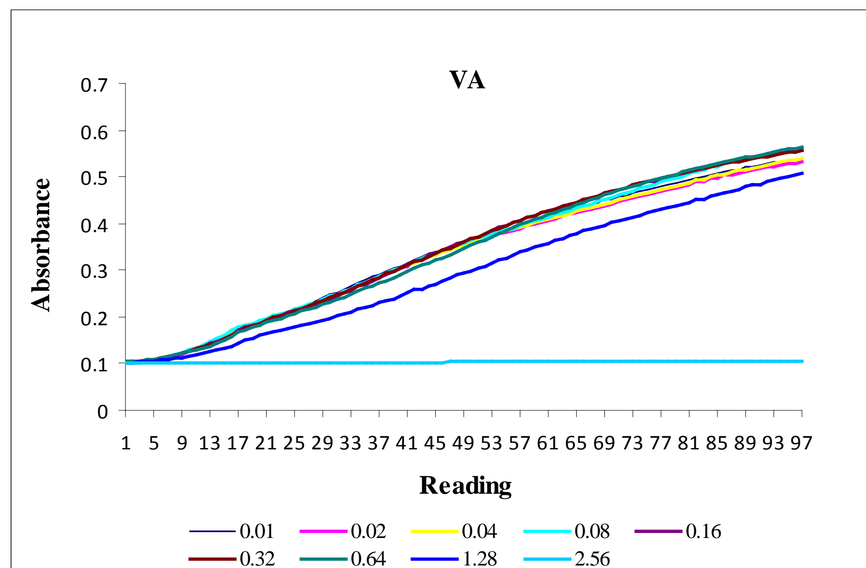

Figure A.2. Growth inhibition of *E. amylovora* by VA at different concentrations

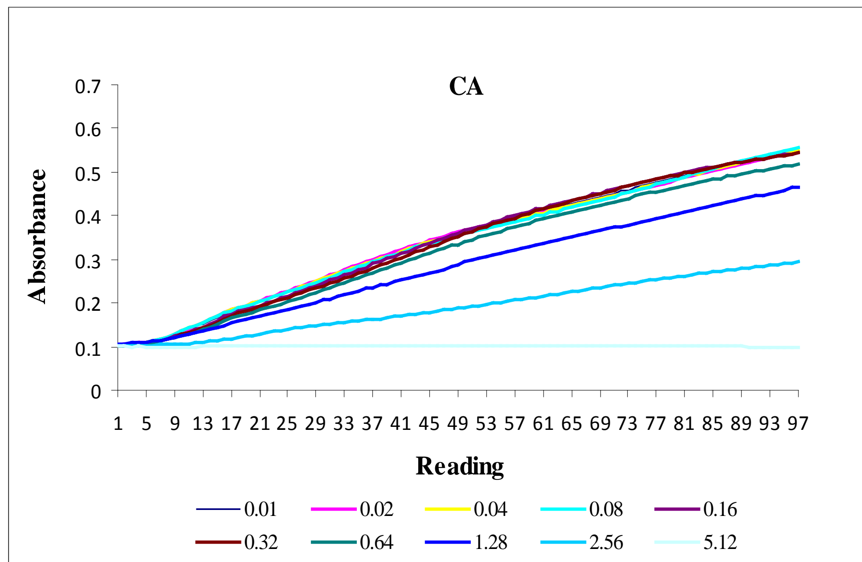

Figure A.3. Growth inhibition of *E. amylovora* by CA at different concentrations

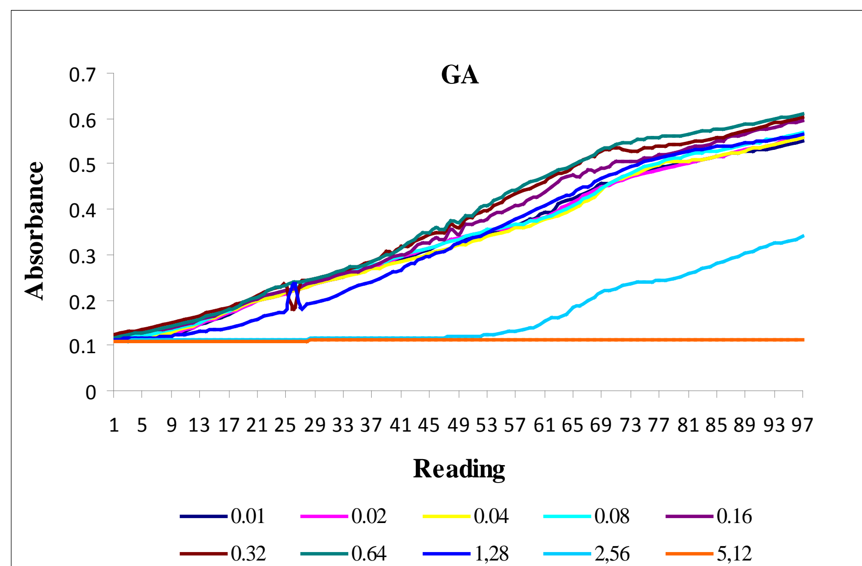

Figure A.4. Growth inhibition of *E. carotovora* by GA at different concentrations

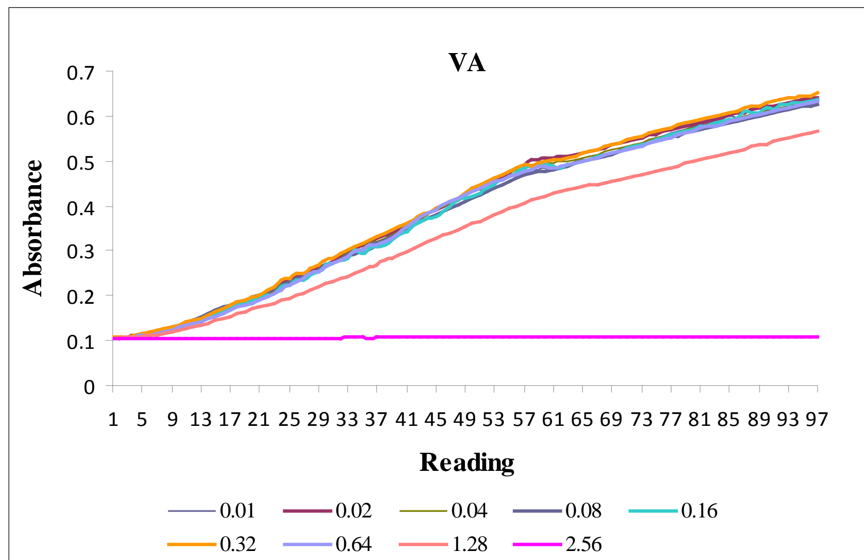

Figure A.5. Growth inhibition of *E. carotovora* by VA at different concentrations

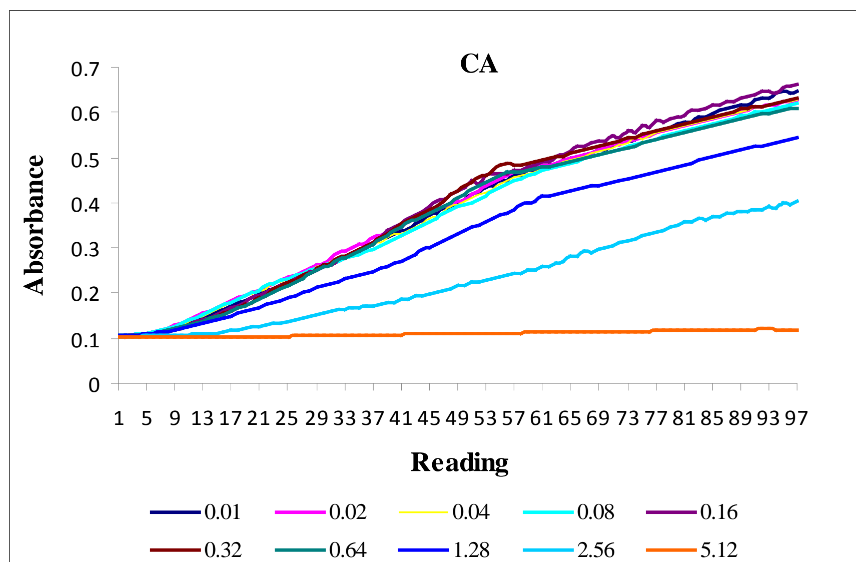

Figure A.6. Growth inhibition of *E. carotovora* by CA at different concentrations

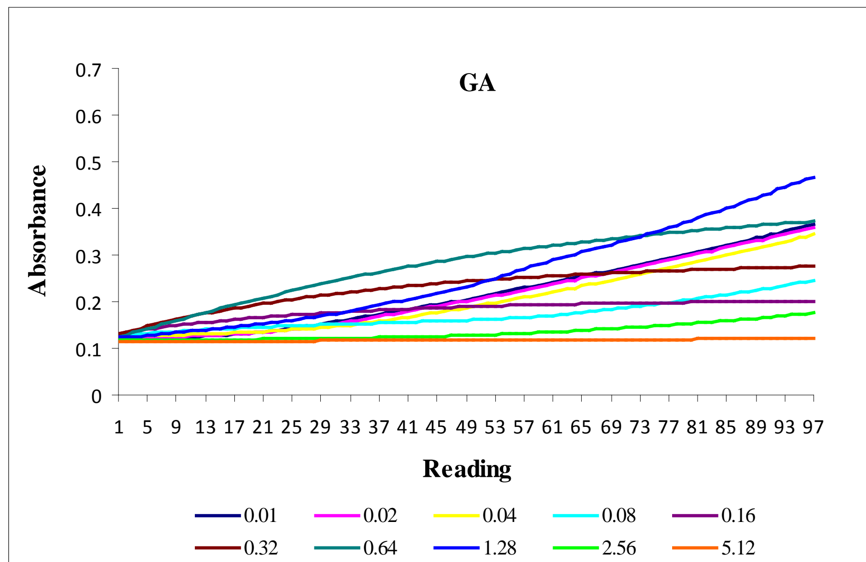

Figure A.7. Growth inhibition of *X. vesicatoria* by GA at different concentrations

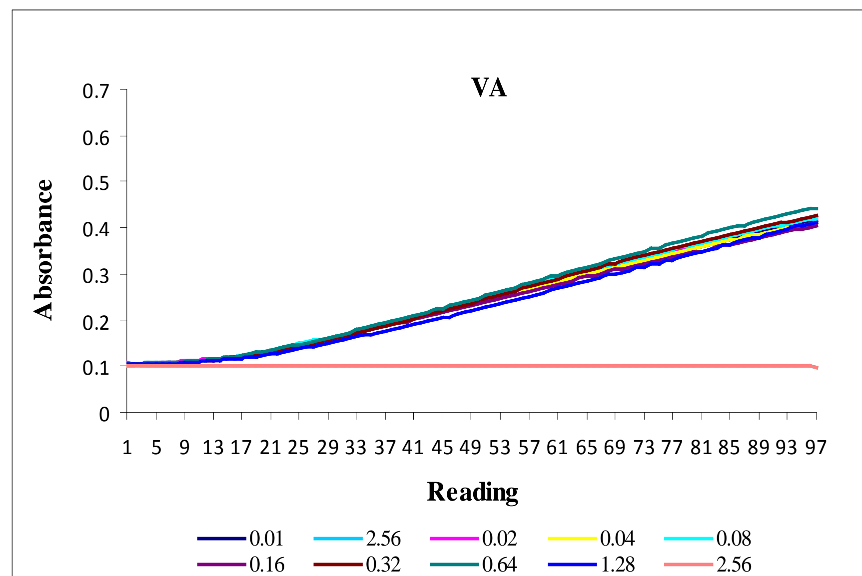

Figure A.8. Growth inhibition of *X. vesicatoria* by VA at different concentrations

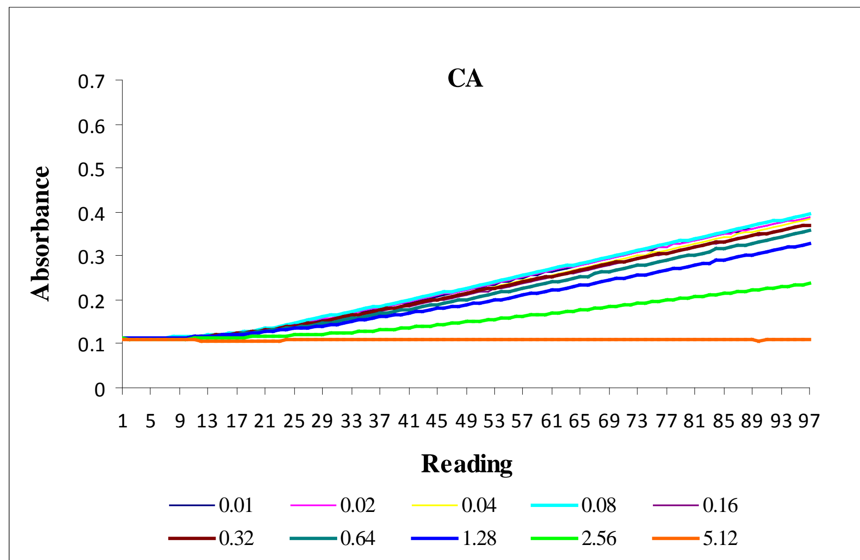

Figure A.9. Growth inhibition of *X. vesicatoria* by CA at different concentrations

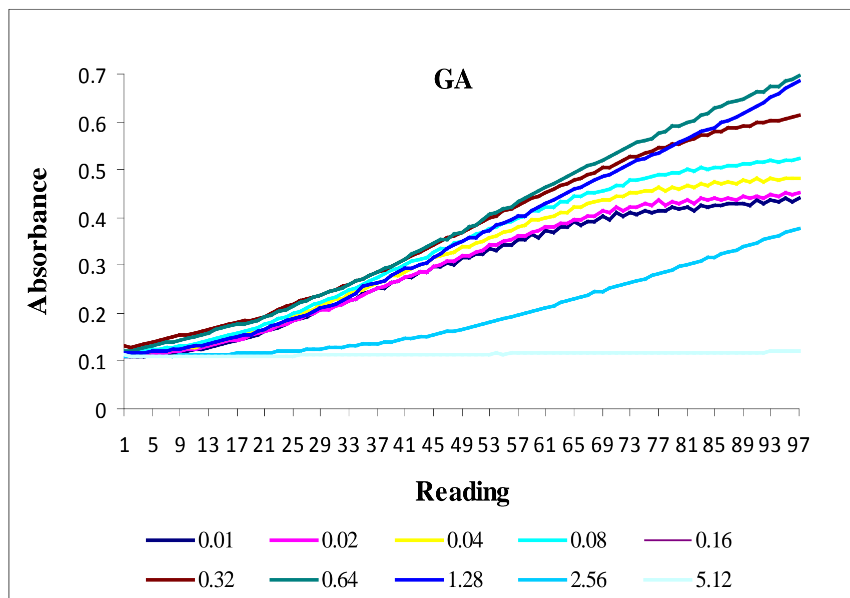

Figure A.10. Growth inhibition of *P. syringae* by GA at different concentrations

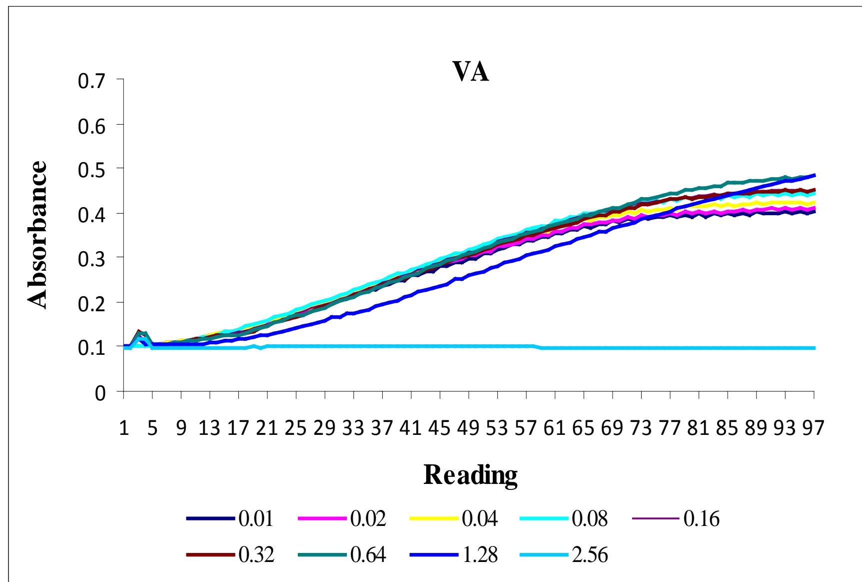

Figure A.11. Growth inhibition of *P. syringae* by VA at different concentrations

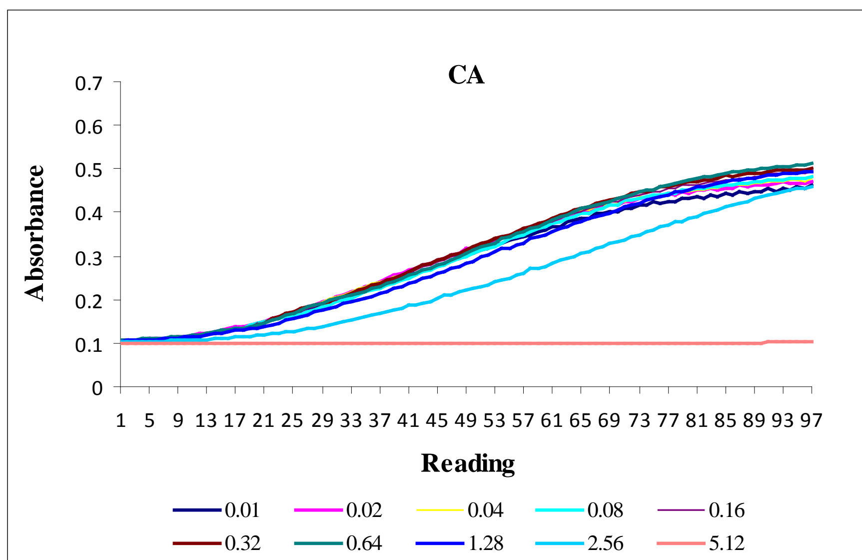

Figure A.12. Growth inhibition of *P. syringae* by CA at different concentrations

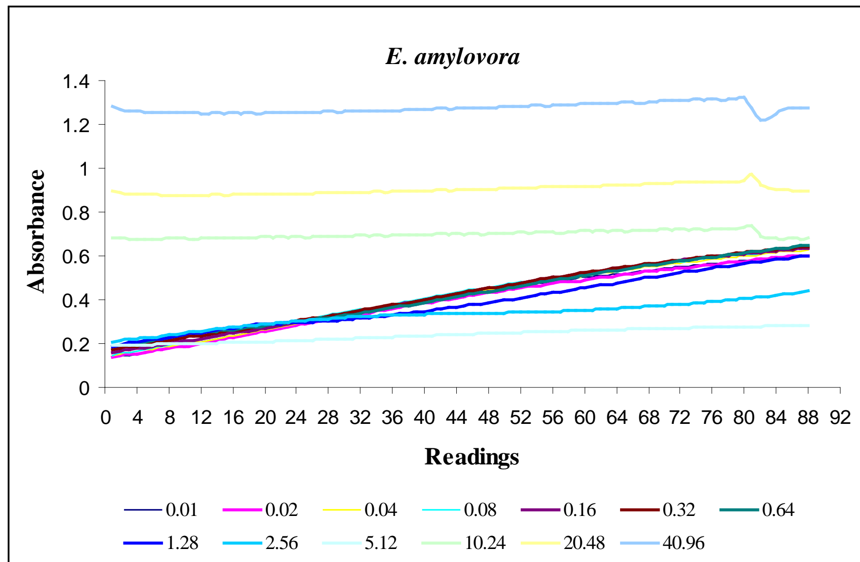

Figure A.13. Growth inhibition of *E. amylovora* by clove extract at different concentrations

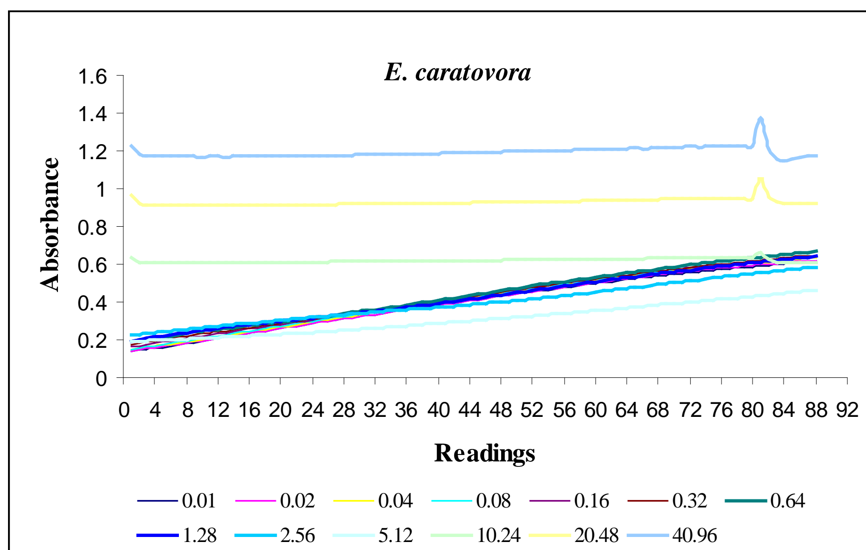

Figure A.14. Growth inhibition of *E. carotovora* by clove extract at different concentrations

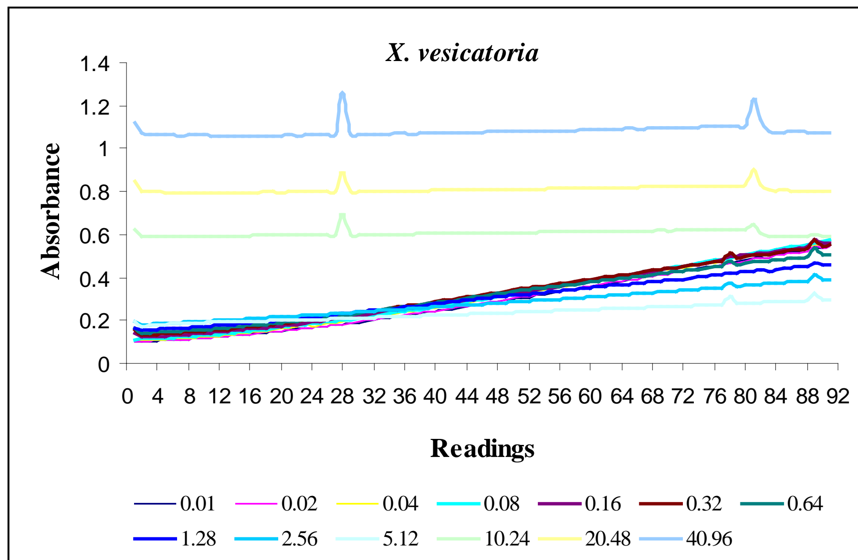

Figure A.15. Growth inhibition of *X. vesicatoria* by clove extract at different concentrations

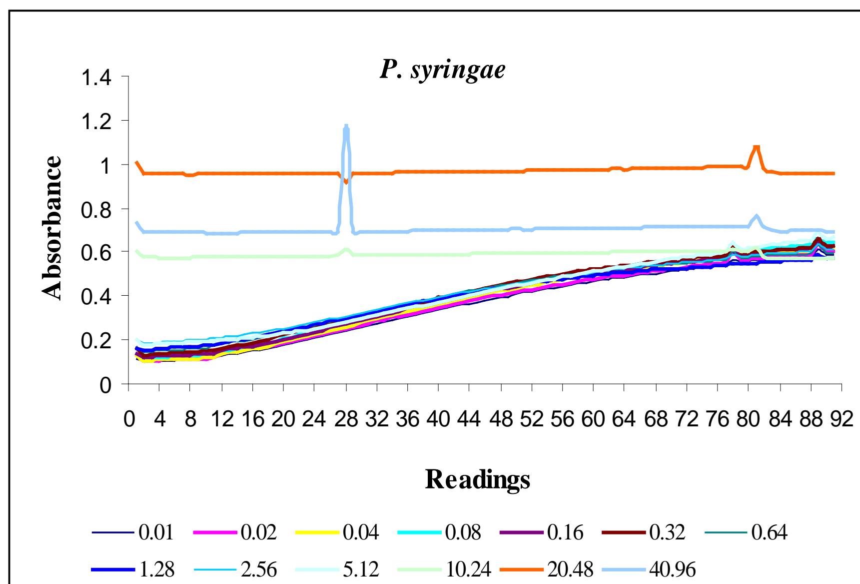

Figure A.16. Growth inhibition of *P. syringae* by clove extract at different concentrations

APPENDIX B

STANDARD CURVES OF GA AND VA FOR RELEASE TEST

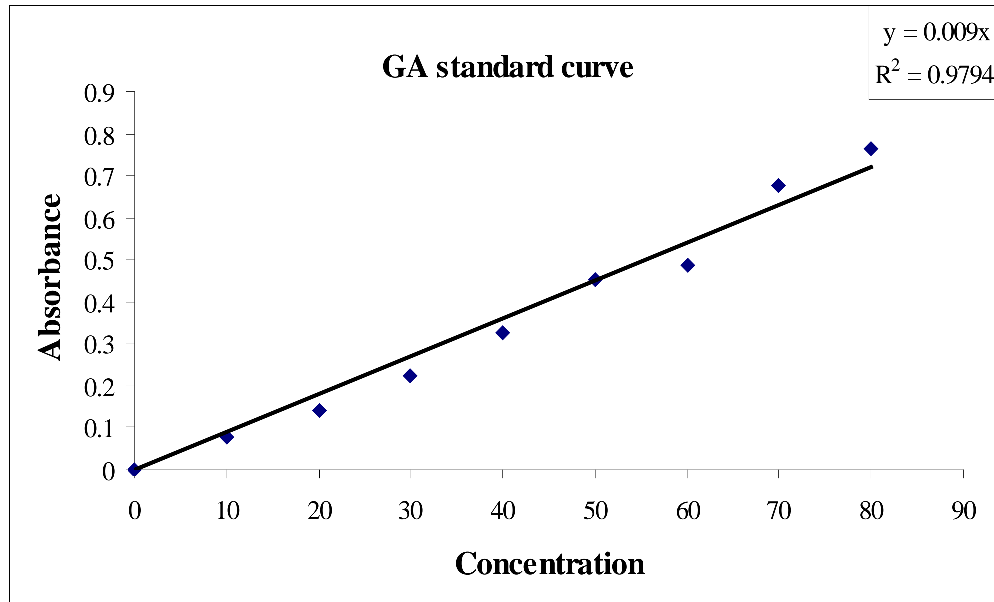

Figure B.1. GA standard curve for release test

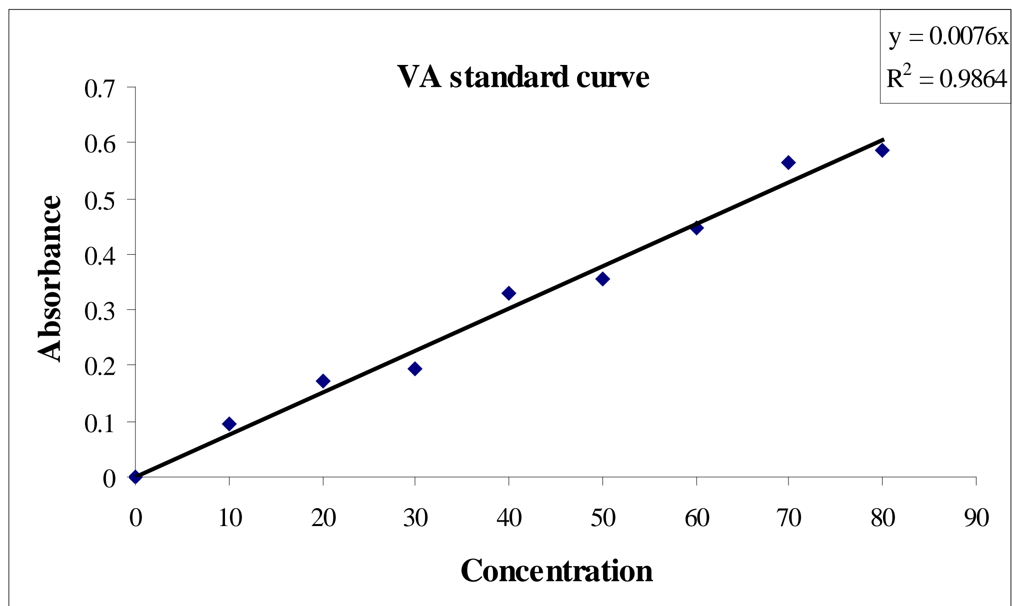

Figure B.2. VA standard curve for release test

APPENDIX C

ANALYSIS OF VARIANCE TABLES FOR MECHANICAL PROPERTIES

Table C.1. ANOVA table for mechanical properties of films incorporated with essential oils

Analysis of Variance					
Source	degrees of freedom	SS	MS	F	P
Tensile strenght	20	778.124	38.906	72.14	0
Error	84	45.3	0.539		
Total	104	823.424			
S= 0.7344	R-Sq= 94.50%	R-Sq(adj)= 93.19%			
Young' modulus	20	2616681	130834	105.44	0
Error	84	104231	1241		
Total	104	2720912			
S= 35.23	R-Sq= 96.17%	R-Sq(adj)= 95.26%			
Elongation	20	1565638	78282	65.99	0
Error	84	99643	1186		
Total	104	1665281			
S= 34.44	R-Sq= 94.02%	R-Sq(adj)= 92.59%			
Thickness	20	42037.08	2101.85	433.88	0
Error	189	915.58	4.84		
Total	209	42952.67			
S= 2.201	R-Sq= 97.87%	R-Sq(adj)= 97.64%			

Table C.2. ANOVA table for mechanical properties of films incorporated with clove extract

Analysis of Variance					
Source	degrees of freedom	SS	MS	F	P
Tensile strenght	4	81.888	20.472	38.89	0
Error	20	10.529	0.526		
Total	24	92.417			
S= 0.7256	R-Sq= 88.61%	R-Sq(adj)= 86.33%			
Young' modulus	4	576301	144075	92.19	0
Error	20	31255	1563		
Total	24	607556			
S= 39.53	R-Sq= 94.86%	R-Sq(adj)= 93.83%			
Elongation	4	274.74	68.685	445.5	0
Error	20	3.083	0.154		
Total	24	277.824			
S= 0.3927	R-Sq= 98.89%	R-Sq(adj)= 98.67%			
Thickness	4	17075.38	4268.84	1679.34	0
Error	20	114.39	2.54		
Total	24	17189.76			
S= 1.594	R-Sq= 99.33%	R-Sq(adj)= 99.28%			

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