OUTER MEMBRANE PROTEIN PROFILING OF *Escherichia coli* O157:H7 IN RESPONSE TO PHENOLIC ACID STRESS

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ABSTRACT

OUTER MEMBRANE PROTEIN PROFILING OF *Escherichia coli* O157:H7 IN RESPONSE TO PHENOLIC ACID STRESS

Escherichia coli O157:H7 is a Gram-negative foodborne and waterborne pathogenic bacterium. Low doses of *E. coli* O157:H7 are adequate for infection that results in clinical cases, namely hemorrhagic colitis, thrombotic thrombocytopenic purpura, and hemolytic uremic syndrome. These diseases can be lethal for children, elderly and immunocompromised people.

Phenolic acids are herbal secondary metabolites. They are important for the response to environmental stresses. They are significant for human diet due to their health beneficial properties.

The objective of this study was the elucidate the antimicrobial mode of action of selected phenolic acids against *E. coli* O157:H7. The antimicrobial activities of caffeic, rosmarinic, and vanillic acids on *E. coli* O157:H7 were investigated via microtiter plate assay. Minimum inhibitory concentrations were found as 6.5 mM for caffeic acid, 15 mM for rosmarinic acid and 8 mM for vanillic acid by applying a range of concentrations for each phenolic acid against the bacteria. Effect of each phenolic acid on the cell surface of *E. coli* O157:H7 was visualized by scanning electron microscopy. Microscopic examination demonstrated that while the surfaces of control group *E. coli* O157:H7 remained intact, cells treated with phenolic acids displayed disrupted cell surfaces. The bacteria were treated with each phenolic acid at a concentration which resulted in 30-50% inhibition for evaluation of outer membrane protein (OMP) profiles in response to phenolic acids stress. Following OMP isolation, SDS-PAGE and 2D-PAGE analyses were performed. By examining the OMP profiles of phenolic acid treated-bacteria, differences in expression of some proteins were observed.

ÖZET

Escherichia coli O157:H7'NİN FENOLİK ASİT STRESİNDE DIŞ MEMBRAN PROTEİN PROFİLİNİN ÇIKARILMASI

Escherichia coli O157:H7, gıda ve su kaynaklı olarak bulaşan, Gram-negatif, patojen bir bakteridir. *E. coli* O157:H7'nin düşük dozları, hemorajik kolitis, trombotik trombositopenik purpura ve hemorajik üremic sendromu gibi klinik durumlara yol açabilen enfeksiyonlar için yeterlidir. Bu hastalıklar, çocuklar, yaşlılar ve bağışıklık sistemi baskılanmış olan kişiler için ölümcül olabilir.

Fenolik asitler, bitkilerde, çevresel streslere cevap olarak üretilen sekonder metabolitlerdir. İnsan beslenmesinde, sağlığa yararlı özelliklerinden dolayı önemlidirler.

Bu çalışmanın amacı, seçilmiş olan fenolik asitlerin *E. coli* O157:H7 üzerine etki mekanizmalarını belirlemektir. Kafeik, rosmarinik ve vanilik asidin *E. coli* O157:H7 üzerine antimikrobiyal aktiviteleri, microtiter plate yöntemi ile belirlenmiştir. Her bir madde, çeşitli konsantrayonlarda hazırlanıp bakteriye uygulanmıştır ve minimum inhibisyon konsantrasyonları, kafeik asit için 6.5 mM, rosmarinik asit için 15 mM ve vanilik asit için 8 mM olarak bulunmuştur. Her bir fenolik asitin *E. coli* O157:H7 hücre yüzeyine etkisi, taramalı elektron mikroskopu ile görüntülenmiştir. Mikroskopik inceleme, kontrol grubu *E. coli* O157:H7 hücre yüzeyinin düzgün bir yapıda olduğunu gösterirken, fenolik asit ile muamele edilen hücre yüzeylerinde çeşitli bozulmalar olduğunu göstermiştir. %30-50 inhibisyon gösteren fenolik asit konsantrasyonları, fenolik asit stresine cevap olarak dış zar protein profillerinin değerlendirilmesi için bakteriye uygulanmıştır. Dış zar proteinlerinin izolasyonundan sonra, SDS-PAGE ve 2D-PAGE analizleri gerçekleştirilmiştir. Fenolik asit ile muamele edilen bakterilerin dış zar proteinlerinin profilleri incelenmesi sonucunda ekspresyon farklılıkları gözlenmiştir.

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LIST OF ABBREVIATIONS

APS	Ammonium per Sulfate
ATCC	American Type Culture Condition
a_w	Water Activity
BSA	Bovine Serum Albumin
CDC	Centers for Disease Control and Prevention
cfu	Colony Forming Unit
DAEC	Diffuse Adhering *E. coli*
DMSO	Dimethyl Sulfoxide
E. coli O157:H7	*Escherichia coli* O157:H7
EAEC	Enteroaggregative *E. coli*
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
FDA	Food and Drug Administration
g	Gram
g	Gravity
gluNAc	*N*-acetylglucosamine
h	Hour
HUS	Hemolytic Uremic Syndrome
l	Liter
LB	Lauria Bertani
LEE	Locus of Enterocyte Effacement
min	Minute
MIC	Minimum Inhibitory Concentration
ml	Mililiter
mg	Miligram
µg	Microgram
µl	Microlitre
mM	Milimolar
murNAc	*N*-acetylmuramic acid
NPN	1-*N*-phenylnaphtylamine

nm	Nanometer
2D-PAGE	Two Dimensional Polyacrylamide Gel Electrophoresis
OD	Optical Density
OM	Outer Membrane
OMP	Outer Membrane Protein
rpm	Revelotions per Minute
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
SMAC	Sorbitol MacConkey Agar
TEM	Transmission Electron Microscopy
vol/vol	Volume per Volume
w/v	Weight per Volume

CHAPTER 1

LITERATURE REVIEW

1.1. Foodborne Pathogenic Bacteria & Outbreaks

Consuming foods and water contaminated by pathogenic bacteria leads to foodborne gastrointestinal diseases. The occurrence of foodborne diseases originated from microorganisms is higher than other causes (Ray, 2005). It is stated that there are more than 250 different foodborne diseases and most of them are infectious (CDC, 2005). Combatting pathogenic bacteria is the primary aim for developed and developing countries to prevent foodborne diseases. There are some regulatory agencies in the U.S. to investigate foodborne diseases; the federal Food and Drug Administration (FDA), Centers for Disease Control and Prevention (CDC) are some of them (Ray, 2005).

A foodborne disease is defined as an outbreak when two or more people become sick due to consuming same food from the same source and when it is validated by epidemiological investigations. Some of the pathogenic bacteria which leads to infectious diseases are *Salmonella* strains, *Campylobacter enteritis*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, some *Shigella* species, *Brucella abortis* and *Vibrio parahaemolyticus.* When food or water containing pathogenic bacteria is consumed, cells able to remain alive start to proliferate in the digestive system and cause illness (Ray, 2005).

Foodborne diseases do not only result in suffering from illness but also lead to economic losses. Medical treatment, loss of business, eliminating spoilage, and investigation of outbreaks can be very expensive. It is presumed that in the USA, foodborne diseases cost more than $20 billion, annually (Ray, 2005).

According to the report of Centers for Disease Control and Prevention, between 1982 and 2002, 350 outbreaks, 8,598 cases, 1,493 hospitalizations, 354 hemolytic uremic syndrome cases and 40 deaths were observed. Transmission route for these outbreaks: 52% was foodborne, 14% was person to person, 9% was waterborne, 3% was animal contact, 0.3% was laboratory related, and 21% was unknown. In the United

States, infections related to *E. coli* O157:H7 are determined as 73,480 illness. These illnesses result in 2,168 hospitalizations and 61 deaths each year (Rangel et al., 2005).

World Health Organization (WHO) reported that in Canada, 27 people got *E. coli* O157:H7 infection from contaminated water and five of the patients died, in 2000 (WHO, 2000). In August 2006, multistate outbreak in the USA was reported by CDC resulted in 238 ilnesses, 103 hospitalizations and five deaths linked to consuming *E. coli* O157:H7-contaminated spinach (CDC, 2006). In 2009, an outbreak associated with consuming *E. coli* O157:H7 contaminated ground beef was reported by CDC in the United States. This outbreak resulted in 32 illnesses, 19 hospitalization and two deaths (CDC, 2009). Lately, it was described by CDC that consumption of *E. coli* O157:H7 contaminated romaine lettuce resulted in infections of 58 people in the USA and three of them came down with hemolytic uremic syndrome in March, 2012 (CDC, 2012).

1.2. *Escherichia coli* O157:H7

Escherichia coli (*E. coli*) is a Gram-negative, non-sporeforming, motile, mesophilic (Ray, 2005), catalase positive, oxidase negative, facultative anaerobic, rod shaped (1-4 µm) bacterium (Buchanan and Doyle, 1997; Ray, 2005). It is found in the intestines of humans, warm-blooded animals, and birds (Ray, 2005). Although most *E. coli* strains are harmless, some are pathogenic to humans. Pathogenic *E. coli* strains damage the intestines. Following infection, intestines absorb less water and this case results in diarrhea (Doyle et al., 2011).

According to their virulence properties, pathogenicity mechanisms, clinical syndromes and O:H serotypes, diarrheagenic *E. coli* strains classified into six groups: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), and diffuseadhering *E. coli* (DAEC) (Doyle et al., 2011; Fernandez, 1996; Buchanan and Doyle, 1997). EHEC members are unique for passing through animal feces, all others pass through human feces (Doyle et al., 2011). EHEC produces poisons called Shiga-toxins (also known as Verotoxins) which are encoded on a bacteriophage. Molecular weight of this protein is 70,000 Dalton and consists of five B subunits (7.7 kDa) and one A subunit (32 kDa). While B subunits assure tissue spesificity, following endocytosis, A subunit inactives ribosomal subunits. There are some other factors

contribute to pathogenicity, including the presence of a plasmid (pO157) which encodes an enterohemolysin and the locus of enterocyte effacement (LEE) that harbors genes encoding an adhesion molecule intimin (Buchanan and Doyle, 1997; Mead and Griffin, 1998; Pennington, 2010). EHEC leads to outbreaks and illnesses such as hemorrhagic colitis, hemolytic uremic syndrome and thrombotic thrombocytopenic purpura (Wu et al., 2003; Donnenberg et al., 1993). *E. coli* O157:H7 is the most known EHEC serotype, and causes more than 75,000 human infections and 17 outbreaks per year (Manning et al., 2008).

First recognization of *E. coli* O157:H7 as a foodborne pathogen happened in 1982. It caused two outbreaks of hemorrhagic colitis which is a disease having symptoms such as abdominal cramps, bloody stools and fever (Fernandez, 1996; Buchanan and Doyle, 1997; Mead and Griffin, 1998). Since it expresses the 157th somatic (O) antigen and 7th flagellar (H) antigen, it is named *E. coli* O157:H7 (Buchanan and Doyle, 1997; Mead and Griffin, 1998). For the selection of *E. coli* O157:H7, sorbitol MacConkey (SMAC) agar is used. Apart from other strains, this serotype does not have the ability to ferment sorbitol, so, after overnight incubation, *E. coli* O157:H7 strains appear as colorless colonies (Tarr et al., 2005; Wu et al., 2003).

In contrast to most foodborne pathogens, *E. coli* O157:H7 is an acid tolerant microorganism (Fernandez, 1996; Buchanan and Doyle, 1997). Optimum pH values are between 5.5 and 7.5 with minimum pH for growth is 4-4.5, nevertheless, it can survive at pH 2.5 for 2-to 7- h exposures at 37°C (Buchanan and Doyle, 1997). Being resistant to low pH is very important for survival of this foodborne pathogen in acidic foods and especially, in the gastrointestinal tract. Acid resistance that protect cells at pH 2-2.5 is provided by three mechanisms. One of these mechanisms is glucose-repressed system. This mechanism is also called oxidative system and is dependent on the sigma factor encoded by the *rpoS* gene. Second mechanism requires presence of glutamic acid to survive at pH 2. To survive at this pH, it uses glutamate decarboxylase. Third mechanism utilizes arginine and inducible arginin decarboxylase encoded by *adiA* and its antiporter *adiC* (Sainz et al., 2005; Foster, 2001).

It has resistance against most antibiotics, however, it is not resistant to heat, as it does not grow well above 44°C. The minimum growth temperature is about 8-10°C under optimal conditions (Buchanan and Doyle, 1997). Minimum water activity (a_w) for growth in foods is 0,95 (Ray, 2005).

Healthy cattle are the major reservoirs of *E. coli* O157:H7. It also resides in sheep, goats, horses, deer, birds and pests. Using manure as a fertilizer in agriculture, or irrigating or washing plants with contaminated water can give rise to fecal contamination (Doyle et al., 2011). Transmission occurs via many foods, including uncooked and undercooked meat, raw vegetables, etc. (Manning et al., 2008; Pennington et al., 2010), also by drinking fecally contaminated water, by swimming in it, or directly from person to person (Boyce et al., 1995). Transmission from cattle to people may be airborne (Tarr et al., 2005). Infections mostly occur in warmer months especially in summer (Tarr et al., 2005; Boyce et al., 1995). Ecology of the organism and the variation in the consumption of ground beef may arise this seasonal variation (Boyce et al., 1995).

The infectious dose for *E. coli* O157:H7 is determined as 2-2000 organisms (Buchanan and Doyle, 1997). Following consumption of contaminated food and ingestion of *E. coli* O157:H7, initial illness symptoms such as non-bloody diarrhea, abdominal cramps and fever occurs within 3-4 days. As the initial diarrhea increases in the next few days, hemorrhagic colitis symptoms such as bloody diarrhea, abdominal pain and dehydration increases. Hemolytic uremic syndrome symptoms, red blood cell destruction, low platelet counts, lack of urine production and renal failure occurs approximately 5-7 days after gastrointestinal symptoms. Fatality rate is 3-5% (Buchanan and Doyle, 1997).

As mentioned above, *E. coli* O157:H7 is a Gram negative bacterium, so it has different membrane structure than Gram positive ones. Cytoplasm of both Gram positive and Gram negative cells are surrounded by the cytoplasmic membrane that transfer solutes into and out of the cell by behaving selectively permeable (Sikkema et al., 1995; Alakomi, 2007; Molloy et al., 2000). External to this membrane, there is a peptidoglycan layer that provides mechanical rigidity to the cell and has role in maintaining cell shape. The peptidoglycan layer is also called murein layer is formed by repeating disaccharides. It consists of *N*-acetylglucosamine (gluNAc) and *N*-acetylmuramic acid (murNAc) molecules which are cross-linked by pentapeptides (L-alanine-D-glutamate-meso-diaminopimelic acid-D-alanine-D-alanine). This structure involves the rare enantiomers of the alanine and glutamate residues (Weiner and Li, 2008). The peptidoglycan layer is thicker and is the outermost layer of the Gram positive cells, whereas, it is thinner in Gram negative bacteria and encapsulated by an

additional outer membrane (Molloy et al., 2000; Weiner and Li, 2008; Alakomi, 2007; Epand and Epand, 2009). Outer membrane (OM) consists of phospholipids, polysaccharides, lipoproteins and membrane proteins (Molloy et al., 2000; Weiner and Li, 2008). The OM of Gram negative bacteria functions in passaging of molecules smaller than molecular mass of 600 Da with the help of the proteins termed porins, therefore, it functions as a barrier to many external agents (Epand and Epand, 2009; Sikkema et al., 1995). Structure of integral outer membrane proteins and integral cytoplasmic membrane proteins are different from each other. While cytoplasmic membrane proteins are hydrophobic and consist of α-helices 15-25 aminoacids in length, the outer membrane harbor proteins composed of β-barrel motifs with 8-22 β-barrels. The β-barrel is comprised of nonpolar and polar aminoacids localized at the lipid and protein interface, and at the interior of the barrel, respectively (Weiner and Li, 2008). Some of these bacterial outer membrane proteins including porins are highly expressed. Porins are integral outer membrane proteins that form hydrophilic channels. They allow free diffusion of small molecules through the outer membrane. Outermost layer also contains specialized porins responsible for transport of specific nutrients such as phosphate (PhoE), maltose (LamB), and nucleosides (Tsx). Some of them act as affinity receptors for ferric ion (FepA, FhuA), vitamin B (BtuB) and fatty acids (FadL). Moreover, a few OMPs serve as target receptors for bacteriophages and colicins (Molloy et al., 2000; Weiner and Li, 2008). Presence of an outer membrane is a distinctive property for Gram negative bacteria (Molloy et al., 2000). The outer membrane of Gram negative bacteria is important in being the interface between the cell and the environment (Molloy et al., 2000; Weiner and Li, 2008). The OM has roles in responding changing environmental conditions such as pH, temperature, salinity, and important for nutrition, resistance to antimicrobial compounds. Despite behaving as an efficient barrier against many external agents, weakening the OM with antimicrobial agents is possible (Alakomi, 2007).

Considering their location, OMPs can be targeted to find new strategies for protection against pathogenic bacteria (Molloy et al., 2000).

1.3. Phenolic Compounds

The phenolic term refers to any compound having a similar structure with phenol (Vaquero et al., 2007). Phenolic compounds are group of herbal secondary metabolites that naturally present nearly all plants. They are abundant in vegetables and fruit (Alakomi et al., 2007; Andjelkovic et al., 2006). These compunds have fundemental functions in plants (Einhellig, 2004). Besides having roles in lignification, they provide flavor and aroma to the plant. Also, plant phenolics serve as plant defense mechanism against invading pathogens such as microorganisms and insects, and stress (Shetty and Lin, 2006; Wells and Berry, 2005). They also protect plants from UV light and oxygen free radicals (Lacombe et al., 2010).

Phenolic compounds are synthesized via shikimate and phenylpropanoid pathways from phenylalanine (Einhellig, 2004; Shetty and Lin, 2007). Polyphenols are cyclic structures derived from benzene and they possess one or more hydroxyl groups attached to the aromatic ring (Andjelkovic et al., 2006).

Their classification is made by considering their carbon structure (Andjelkovic et al., 2006). Simple phenols, quinons, flavonoids, stilbenes, lignans and phenolic acids are subclasses of phenolic compounds. Simple phenols consist of a single substitute linked to the aromatic ring. Quinons are aromatic rings possessing two ketone substituons. They carry out the browning reaction in cut and injured vegetables and have roles in melanin synthesis in human. By making complexes with aminoacids in proteins they inactivate proteins and provide antimicrobial activity. Flavonoids are hydroxylated phenolics and synthesized in response to microbial infection. Their antimicrobial activity arise from their ability to complex with extracellular and soluable proteins (Cowan, 1999). Phenolic acids are subclassified into two classes: hydroxybenzoic acids having the chemical structure C6-C1 and hydroxycinnamic acids having C6-C3 structure (Tripoli et al., 2005). Hydroxycinnamic acids are differentiated from hydroxybenzoic acids by presence of an ethylene group. In hydroxycinnamic acids, carboxylic group is attached to the benzene ring via an ethylene group. However, in hydroxybenzoic acids the carboxylic group is directly attached to the aromatic ring. The presence of an ethylene group in hydroxycinnamic acids may affect chelation capacity and scavenging activity of the phenolic acid (Andjelkovic et al., 2006). Hydroxycinnamic acids are less polar than the corresponding hydroxybenzoic acids.

Less polarity of hydroxycinnamic acids is caused by their propenoic side chains. Having that side chain may facilitate transportation of hydroxycinnamic acids across the cell membrane and may be important for inhibitory effect (Vaquero et al., 2007).

Phenolic acids affect the microbial cells by interacting with the membrane and cytoplasm. They are weak organic acids (pKa ~ 4.2) and the concentration of the undissociated acid is directly related with the antimicrobial activity of the phenolic acid. Since they are partially lipophilic, their probable passage through cell membrane occurs by passive diffusion and they disturb membrane structure, acidify the cytoplasm and lead to protein denaturation (Campos et al., 2009).

Phenolic compounds are important part of both human and animal diets (Andjelkovic et al., 2006). They scavenge singlet oxygen and free radicals, and in this way display antioxidative property. They have potential for therapeutic applications against diseases caused by oxidation. By donating hydrogen from hydroxyl groups, they terminate the free radical oxidation of lipids and other biological molecules. This ability of phenolics makes them effective antioxidants. Prevention of such chain reaction disrupts the function of the cellular membrane and in prokaryotes leads to antimicrobial activity (Shetty and Lin, 2006). They also exhibit antimutagenic, anticarcinogenic, cardioprotective and antiinflammatory properties (Andjelkovic et al., 2006).

Hydroxylated phenols, such as catechol bearing two hydoxyl groups and pyrogallol bearing three are venomous to microorganisms (Cowan, 1999; Shetty and Lin, 2006; Sher, 2009). The position and the number of the hydroxyl groups on the phenol affect the toxicity to microorganisms. Increased number of hydroxyl groups, increases the inhibitory effect (Cowan, 1999; Tripoli et al., 2005; Sher, 2009). The types of other substituents which bound to the aromatic ring are also important (Andjelkovic et al., 2006). For cinnamic acids and benzoic acids, lipophilicity and pKa are responsible for 81% of the antimicrobial activity (Kouassi and Shelef, 1998).

Surface adhesions, cell wall polypeptides, and inhibition of the membrane bound enzymes through reaction with sulfydryl groups or through nonspesific interactions with the proteins are potential targets for inhibition in the bacterial cells (Shetty and Lin, 2006; Cowan, 1999). Also, aldehyde groups of phenolics may form Schiff's bases with membrane proteins and prevent synthesis of cell wall (Shetty and Lin, 2006).

1.3.1. Caffeic Acid

Caffeic acid is a hydroxycinnamic acid derived from phenylpropanoid pathway and commonly found in plants. In its chemical structure (Figure 1.1), the carboxyl group is bonded with benzene ring via an ethylene group. It also has two hydroxyl groups attached to the ring as substituents (Bowles and Miller, 1989; Shetty and Lin, 2007). Although caffeic acid is not approved as food additive, it is naturally taken by consuming plants as dietary intakes. Besides its antibacterial, antifungal, antioxidant properties, caffeic acid prevents the formation of carcinogens from procursors *in situ* and reduces the incidence of cancer (Bowles and Miller, 1989).

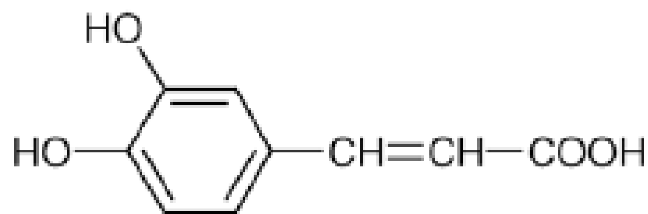


Figure 1.1. Chemical structure of caffeic acid
(Source: Peng et al., 2005)

1.3.2. Rosmarinic Acid

Rosmarinic acid is a phenolic acid which is isolated from *Rosmarinus officinalis* a plant from the family Lamiaceae (Petersen and Simmonds, 2003; Shetty and Labbe, 1998). Rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxy-phenyllactic acid (Figure 1.2). Rosmarinic acid can interact with cell membrane receptors directly and by

interacting with proteins may alter their conformation. Ability of stacking itself to membrane without penetration results in alterations in membrane fluidity (Apostolidis et al., 2008). Biologically, it exhibits antioxidative, antiinflammatory, antimutagen, antiviral and antibacterial activities (Petersen and Simmonds, 2003; Shetty and Lin, 2007). This phenolic acid also has important activities as anti-tumor and chemopreventive agent, *in vitro* (Romano et al., 2009).

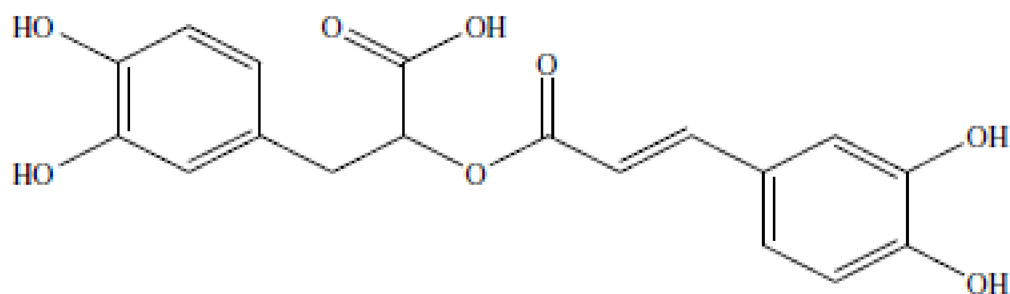


Figure 1.2. Chemical structure of rosmarinic acid
(Source: Wang, 2004)

1.3.3. Vanillic Acid

Vanillic acid is a component of solubilized lignin biomass (Chow et al., 1999). It is a hydroxybenzoic acid that its carboxyl group is directly linked to the aromatic ring. As a substituents, it has one hydroxyl group and one methoxy group (Figure 1.3).

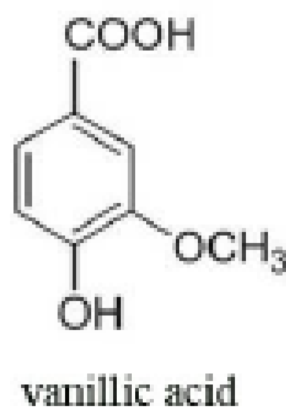


Figure 1.3. Chemical structure of vanillic acid
(Source: Boskou, 2009)

1.4. Antimicrobial Properties of Phenolic Acids

Phenol affects proper function mechanism of the membrane by influencing protein-lipid ratios in the membrane. Effects of its substitued derivatives, namely phenolics, are the object of curiosity because of the common presence of these compounds in the environment mostly in plants. Toxicity of the phenolic compound is highly correlated with its hydrophobicity (Sikkema et al., 1995).

Results of epidemiological studies have shown that phenolic acids play substantial roles for prevention of diseases (Cueva et al., 2010). Apart from their health beneficial properties, they also exert antimicrobial properties by denaturating proteins and inactivating enzymes (Karaosmanoglu et al., 2010).

Hydroxycinnamic acid derivatives such as caffeic acid, ferulic acid and *p*-coumaric acid hamper the growth of *E. coli*, *Staphylacoccus aureus*, *Bacillus cereus* (Shetty and Lin, 2007).

It had been demonstrated that the sensitivity of *E. coli* to phenolic acids was strain dependent and phenolic acid concentration altered the susceptibility of the microorganism to tested phenolic acids (Cueva et al., 2010). Benzoic acid, phenylpropionic acid and phenylacetic acid displayed the most inhibitive effect reaching 100% inhibition against *E. coli* O157:H7 at the concentration of 1000 µg/ml. At the same concentration, phenylpropionic, 4-hydroxyphenylacetic and 4-

hydroxypropionic acids showed 90% inhibition against tested two pathogenic and one non-pathogenic *E. coli* strains. Antimicrobial activities of benzoic acids, phenylacetic acids, phenylpropionic acids against six *Lactobacillus* strains were also tested in the same study and applied concentrations showed distinct inhibition values. In the case of pathogen *Staphylococcus aureus*, at 1000 µg/ml concentration of all tested phenolic acids inhibited growth. In the study, it was observed that benzoic acids were more active than phenylacetic and phenylpropionic acids relative to their different substituents (Cueva et al., 2010).

In another study, some phenolic acids including, caffeic acid, rosmarinic acid, and carnosic acid were tested against *Listeria monocytogenes* at the concentrations of 250 µg/ml, 250 µg/ml and 100 µg/ml, respectively. At 9 h exposure, all tested compounds displayed inhibitory effect and the most efficient one was found to be carnosic acid. At 72 h exposure, caffeic, rosmarinic and carnosic acids still had prohibitive impact (Del Campo, 2003).

To provide a new insight to usage of vanillin as a potential antimicrobial additive for refrigirated fresh-cut vegetables, its antimicrobial effect was tested against four pathogenic bacteria; *E. coli*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, and *Salmonella enterica* subsp. *enterica* serovar Newport. Vanillin (reduced form of vanillic acid) a phytochemical from vanilla bean widely used in foods as safe flavoring compound was prepared in different concentrations ranged between 1.5 mM and 18 mM. Prepared concentrations were applied on bacteria, and their growth was assessed by measuring optical density. Its antimicrobial effect was both dose dependent and microorganism dependent. For instance, at 6 mM vanillin concentration, growth inhibition of *E. coli* was 73%, while *P. aeruginosa*, *E. aerogenes* and *S. enterica* subsp. *enterica* serovar Newport displayed 45%, 18%, and 37% growth inhibition, respectively. Minimum inhibitory concentration (MIC) for *E. coli* was determined as 12 mM, whereas, others demonstrated MIC at 18 mM. Also, in this study it was observed that addition of 12 mM vanillin reduced the microbial growth during the 19-day post-cut storage (Rupasinghe et al., 2006).

Moreno et al. (2006) determined the antimicrobial activity of rosmarinic acid and carnosic acid against eight bacteria including, *E. coli*, *S. aureus*, *Bacillus subtilis*, *Klebsiella pneumonie* by using disk diffusion technique. Carnosic acid was effective between the concentrations of 25-200 µg/ml, whereas, rosmarinic acid did not show any

antibacterial effect at the concentration of 250 μg/ml against tested microorganisms (Moreno et al., 2006).

Percent inhibitions in the growth rates of *E. coli* O157:H7, *Salmonella* Enteritidis, *L. monocytogenes* were determined by microtiter plate method in the presence of some phenolic compounds including phenolic acids such as cinnamic acid, vanillic acid, syringic acid, 4-hydroxybenzoic acid (Karaosmanoglu et al., 2010). After the 24 h exposure, higher activity for almost all tested phenolics was observed against *L. monocytogenes*, indicating Gram positives were more susceptible to phenolic compounds than Gram negatives. While some compounds were more effective against *E. coli* O157:H7, others were effective against *Salmonella* Enteritidis. As an illustration, vanillic acid at the concentration of 0.20 mg/kg oil brought about 13.13% inhibition in the growth of *E. coli* O157:H7, whereas, it caused 6.80% inhibition in the growth of *Salmonella* Enteritidis. This study also represented that the activities of tested phenolic compounds were concentration dependent, but increased concentration did not represent increased activitiy for every compound (Karaosmanoglu et al., 2010).

Wells et al. (2005) studied the effects of *trans*-cinnamic acid, *para*-coumaric acid, and ferulic acids on the death rate of *E. coli* O157:H7 in feces of bovines fed with cracked corn. For observation of the effect of forage phenolic acids on viability of *E. coli* O157:H7, cinnamic, coumaric and ferulic acids were added to cattle feces inoculated with bacteria. This study indicated that the addition of phenolic acids into the feces, decreased the survival of pathogen even at the concentrations as low as 0.1% (Wells and Berry, 2005).

Susceptibility of *L. monocytogenes* to benzoic acid and cinnamic acid was determined by enumeration of viable cells following incubation with phenolic acids for 24 h. The concentrations were chosen between 0.1-2% (w/v) and the initial cell numbers were 10^9 cfu/ml. Cinnamic acid showed higher antilisterial activity than benzoic acid, 1% of cinnamic acid exposure did not allow the cells to recover. Moreover, it was displayed in the same study that there were changes in the extracellular glucose and ATP levels upon treatment with cinnamic acid. Increased concentrations of cinnamic acids resulted in glucose depletion and decreased ATP levels. Although pKa values of cinnamic acid (4.4) and benzoic acid (4.2) were close, there were differences between their activity. This study indicated that the lipophilicity of these phenolic acids accounted for difference in antimicrobial activity (Kouassi and Shelef, 1998).

In another study, antimicrobial activity of some plant extracts and phenolic acids were examined against 13 antibiotic resistant bacteria, including *Klebsiella pneumoniae*, *E. coli*, *S. aureus*, *B. subtilis*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Shigella* spp. which are resistant to many antibiotics such as ampicillin, amikacin, cefoxitin, chloramphenicol, erytromycin, gentamycin, kanamycin, penicillin. Antimicrobial activity caused by phytochemicals was determined via agar diffusion method. *S. aureus*, *E. aerogenes* and *E. coli* displayed susceptibility to benzoic acid and cinnamic acid at the concentration of 10%. *K. pneumonie* and *E. coli* were susceptible to benzoic acid at the same concentration with cinnamic acid displaying inhibiton zone greater than 7 mm (Nascimento et al., 2000).

Antimicrobial activities of red wine phenolic compounds were tested against *Serratia marcescens*, *Proteus mirabilis*, *E. coli*, *K. pneumoniae*, and *Flavobacterum* sp. The concentration range for the phenolics was chosen at their concentrations present in wines. Different concentrations between 5-1000 mg/l for gallic acid, vanillic acid and protocatechuic acid and 1-500 mg/l for caffeic acid were applied to bacteria. All concentrations of used phenolic acids were effective against *E. coli*, while, none of them resulted in occurance of inhibition zone in *Flavobacterium* sp. inoculated agars. Caffeic acid slightly inhibited *S. marcescens* and *P. mirabilis*, on the other hand, *K. pneumoniae* was effected from gallic and vanillic acids. This study was another example which indicated different bacterial species response the phenolic acids at different sensitivities (Vaquero et al., 2007).

The synergistic antimicrobial effect of rosmarinic acid (RA), carnosic acid (CA) and carnosol (COH) as a rosemary extract (*Rosmarinus officinalis*) was studied since rosemary was commonly used for its aroma in food and beverages, and its antioxidative properties were well known. *E. coli* and *S. aureus* were tested with rosemary extract consisting of 30% CA, 16% COH and 5% RA. Benzoic acid was also applied to tested bacteria to compare with the antimicrobial activity of rosemary extract. Minimum inhibitory concentration (MIC) of rosemary extract was 50 μg/ml to *S. aureus* and 105 μg/ml to *E. coli*, whereas, MIC of benzoic acid was 120 μg/ml for the former and 250 μg/ml for the latter bacterium (Romano et al., 2009).

To figure out the antimicrobial effect of American cranberry, its constituents were tested against *E. coli* O157:H7. Anthocyanins, organic acids and phenolics were the active components of cranberry providing various health benefits. Results of

antimicrobial activity of phenolic acids were reported in equivalents of gallic acid. For visualization of damage with transmission electron microscopy caused by phenolics, cells were treated with different concentrations of compounds. After cells had stained with negative staining, treated cells were compared with the control whether or not the cells remained intact. While the control cells kept their intactness, outer membrane disintegration, leaking of cytoplasm and irregular shape were observed in the case of phenolic acid treated cells. The outer membrane damage and irregularity in cell shape indicated spesific interactions at the cell surface (Lacombe et al., 2010).

1.5. Mode of Action of Phenolic Acids

Action mechanisms of phenolics against bacterial pathogens has not been distinctly described so far. One of the main reasons for this poor understanding is their formation process. Many antimicrobial phytochemicals, spesifically phenolics, are stress inducable. Biotic and abiotic factors affect the efficacy of plant extracts. Therefore, assurance of consistance from batch to batch and source to source can not be provided. Even so, there are possible mechanisms attemped to explain mode of action of phenolics (Shetty and Lin, 2007).

When lipophilic compounds like thymol from certain essential oils are accumulated in membranes, membrane structure and function is disrupted, as well as alterations in the enzyme activities are observed. Membrane localized hyperacidity affects membrane integrity (Sikkema et al., 1995). Hyperacidification at the plasma membrane interphase is a mechanism to explain antimicrobial activitiy of phenolic acids against pathogens. Dissociation of phenolic acids results in acidification which would change the cell membrane potential of the microorganism (Cueva et al., 2010). Since proper functioning of the membrane is very important as a selective barrier, gradients of protons and some other ions such as sodium are important. Proton motive force (PMF) which formed by chemical proton potential and electrical potential provides an energy intermediate for the cell to carry on vital processes (Sikkema et al., 1995). Because of the disruption of the PMF across the membrane as a consequence of this hyperacidification, energy depletion may occur. Cell membrane become more permeable, ATP synthesis is affected on the occasion of sodium-potassium ATPase pumps are affected (Cueva et al., 2010; Shetty and Lin, 2007).

To sum up, it can be said that action mechanisms of all antimicrobial compounds can be classed into groups: (a) antimicrobials reacting with cell membrane, (b) antimicrobials inactivating essential enzymes, and (c) antimicrobials damaging or inactivating genetic material (Fitzgerald et al., 2004).

For determination of action mechanism of vanillin against several foodborne pathogens, its antimicrobial effect was tested against *E. coli*, *Lactobacillus plantarus* and *Listeria innocua*. The antimicrobial effect of vanillin was discovered in case of its influence on respiration, membrane intergrity, potassium gradient, pH homeostasis. After 96 h incubation with different concentrations of vanillin, MICs for *E. coli*, *L. plantarus*, and *L. innocua* were found as 15, 75 and 35 mmol/l, respectively. By using oxygen electrode, oxygen consumption of cells was measured when 40 mmol/l vanillin was added to cell suspensions. 19% inhibition was achieved for *E. coli* and 52% inhibition for *L. innocua*. To investigate the membrane integrity, nucleic acid stain propidium iodide was used whether it can enter the cells or not. Within 60 min, it was observed that there was an increase in cells with disrupted membranes. Following perturbation of cell membrane, potassium levels were measured, decrease in levels of internal K^+ and increase in levels of external K^+ were determined (Fitzgerald et al., 2004).

Cranberries contain many bioactive compounds including phenolics which display antioxidant, antimutagenic, antihypercholesterolemic properties, and also have potential to prevent development of cancer. In a study which intended to use of cranberry concentrate as a natural food protector, ground beefs were supplemented with cranberry concentrate to prevent possible *E. coli* O157:H7 contamination. Cranberry concentrates displayed antimicrobial activity at the concentrations of 2.5%, 5%, and 7.5% impaired the population of *E. coli* O157:H7 by 0.4 log, 0.7 log, and 2.4 log cfu/g, respectively, at the end of five days. Appearence, flavor and taste of the burgers were not changed upon treatment with concentrate. After the antimicrobial activity had been determined, the action mechanism of this antimicrobial compound was investigated on the gene regulation level. Expression levels of some genes were changed with 30 min and 60 min cranberry concentrate application. In response to treatment, expression of *slp*, *hdeA*, *cfa*, and *ompC* were reduced when compared with control samples. It was hypothesized that since *ompC* and *slp* were outer membrane protein encoding genes, their downregulation with cranberry concentrate treatment might be associated with its

interaction with the outer membrane. Then, by entering inside the cell, it inhibited the transcription of other genes responsible for bacterial growth (Wu et al., 2009).

1.5.1. Proteomic Changes in Response to Phenolic Stress

It was known that phenol acted on cell membrane by increasing its permeability. Cytoplasmic membranes and outer membranes of phenol-treated *E. coli* cells were extracted and compared with nontreated cells. Upon phenol treatment, outer membrane protein profile demonstrated different pattern, several proteins were expressed in low levels and some were in higher levels. It was concluded that increase in expression of some proteins were related with increased membrane permeability. Since phenol disrupted the lipid composition of the membrane, increasing protein proportion was an adaptive reaction to hamper leakage of cell constituents. The proteins expressed in minor quantities were proteins induced with iron deprivation. This study indicated that there were modifications in the cell membrane of *E. coli* when cells were treated with phenol (Keweloh et al., 1990).

As being high molecular weight polyphenols, tannins displayed their antimicrobial effect particularly by interacting with bacterial proteins. It has been known that tannic acid, hydrolysable form of tannin, found in foods was able to prevent the growth of intestinal bacteria of humans, such as *E. coli*, *Bacteriodes fragilis*, and *Clostridium perfringens*. Tannins' ability to cross the cell membrane of microorganisms has been proved. In order to investigate the inhibitory concentration of tannins on *Lactobacillus hilgardii*, different concentrations of tannic acid ranged between 100-1000 mg/l were treaeted to bacteria, and 1000 mg/l was determined as MIC value. By isolating total proteins from cells treated with 100 mg/l and 1000 mg/l tannic acid and from control cells, quantification of extracted proteins was done. Identification of proteins with MS/MS demonstrated that nine proteins decreased at treated cells, and additionally 15 proteins found in 1000mg/l tannic acid treated cells were altered. Most of the downregulated proteins upon tannic acid treatment were metabolic enzymes. Owing to downregulation of metabolic enzymes, growth inhibition of *L. hilgardii* caused by tannins could be related with interaction between tannins and cell protein expression (Bossi et al., 2007).

Action mechanism of grean tea polyphenols (TP) against *E. coli* was determined with regards to protein-polyphenol interaction. Antimicrobial effect of TP was dose dependent. After 30 h treatment with 5000 μg/ml TPP or 18 h treatment with 10,000 μg/ml TP, total inhibition of *E. coli* cultures was observed. These concentrations showed bactericidal effect. To observe the damages to bacteria membrane, cells were treated with 10,000 μg/ml TP for 12 h and by examining with scanning electron microscope, wrinkled surfaces and irregularity at the rod shaped cell membrane were observed. For determination of increased and decreased proteins upon TP treatment, 3000 μg/ ml TP was applied to cells. In TP treated cells, protein expression was altered for more than 35 proteins. Within 17 proteins identified, nine were upregulated proteins. One of them was chaperone protein and eight proteins were related with cellular defense mechanism. Based on this data, it was suggested that induced proteins upon TP treatment were important in protecting cells or helping cells to survive under stress conditions (Cho et al., 2007).

In another study, proteomic changes in foodborne pathogen *Salmonella enterica* serovar Thompson upon treatment with sublethal concentrations of thymol was examined. Thymol, an essential oil, have been used as additive antimicrobial in foods. Following treatment with 0.01% thymol, total protein extraction and protein identification studies demonstrated that there were differences in protein spots displaying higher or lower expression. Cytoplasmic proteins with changed expression levels were detected as 37 spots, another seven proteins were attributed to outer membrane proteins. It was concluded that thymol acted as a global stress because many induced proteins were related with metabolism, stress response, or structure of the microorgasnism (Di Pasqua et al., 2010).

Lambert et al. (1997) demonstrated that benzoic acid induced the *E. coli* proteins. At least two-fold increase in protein expression which was decided by visual observation was determined as induction. Benzoic acid at the concentration of 20 mM was treated to cells both at an external pH of 6.5 and external pH of 8.0. These pH values were chosen because of their effect on benzoic acid accumulation where at pH 6.5 benzoic acid accumulated and at 8.0 it was taken up by the cell in the little amounts. Proteins were radiolabelled and it was observed that in untreated cells there were very little differences in protein patterns, indicating consistance of protein homeostasis between pH 6.5-8. Treatment with 20 mM benzoic acid at pH 6.5 induced 33 proteins

increasing their expression. Twelve of proteins induced by benzoic acid treatment at pH 6.5 were also induced at pH 8.0. It was concluded that proteins induced after benzoic acid treatment only at pH 6.5 displayed the modifications of cell proteins as response to internal acidification or due to accumulation of benzoic acid on the membrane (Lambert et al., 1997).

Antimicrobial effects of some phenolic compounds including *p*-coumaric acid, ferulic acid and caffeic acid against some *Lactobacillus casei* strains were elucidated. Minimum inhibitory concentrations were found for all tested phenolic acids. While *p*-coumaric acid inhibited the growth at the concentration of 25 mmol/l, ferulic and caffeic acids inhibited the growth at the concentration of 40 mmol/l. For determination of involved systems in the response to phenolic acid stress, 15 mmol/l *p*-coumaric acid was applied to cells and changes in protein patterns were observed. According to protein identification results, six proteins were upregulated and five proteins were downregulated in response to *p*-coumaric acid. Two upregulated proteins were identified as required proteins in protein turnover, and one upregulated protein detected was essential for normal protein synthesis. Two of downregulated proteins were involved in purine biosynthetic pathway. It was stated that *p*-coumaric acid damaged protein structure and affected membrane functions in *L. casei* (Rivas-Sendra et al., 2011).

Antimicrobial action mechanism of tea polyphenols (TP) on *Pseudomonas aeruginosa* was studied through damage to the cell membrane. Its antimicrobial effect was concentration dependent. When intact *P. aeruginosa* cells treated with TP for eight hours, alterations in the cell membrane were observed by transmission electron microscopy (TEM). The TEM images indicated that TP treatment disrupted integrity of the outer membrane which resulted in deformation of typical rod shape. Permeabilization of the outer membrane was also demonstrated by hydrophobic 1-*N*-phenylnaphthylamine (NPN) fluorescent probe, and permeabilization of inner membrane was determined by measuring the release of β-galactosidase activity. Moreover, membrane proteins of TP treated and untreated cells of *P. aeruginosa* were analysed by 2D-PAGE. Expression of 27 proteins were diversified in TP treated cells when compared with control samples (Yi et al., 2010).

Response of *E. coli* to phenol stress was studied by proteomic approach. Alterations in the outer membrane protein profile upon phenol treatment were observed

and changed proteins were further determined. Expression of nine proteins were different from their counterparts in control samples. Among nine differentially expressed proteins, four proteins were outer membrane proteins displaying upregulation, and one outer membrane protein was displaying downregulation (Zhang et al., 2011).

CHAPTER 2

INTRODUCTION

Consumption of foods such as raw vegetables, uncooked and undercooked meat especially ground beef and several low-pH foods is related to foodborne diseases caused by *Escherichia coli* O157:H7, *Salmonella* and *Listeria monocytogenes*. These foods have major chance to get contaminated by these pathogens (Ray, 2005). Acidic foods have been regarded as nonhazardous for consumption, however, outbreaks implicated in acidic foods are emerging associated with acid resistant pathonegic bacteria. According to outbreak records, 80% of the outbreaks are related with *Salmonella* and *E. coli* O157:H7. Adaptation to acidic environments leads to increased resistance to several environmental stress conditions such as heat and surface active agents. Resistance to irradiation and heat for acid resistant *E. coli* O157:H7 has been shown (Singh et al., 2006).

Therapeutic applications to combat with *E. coli* O157:H7 infections are limitative (Shetty and Labbe, 1998). Antibiotic therapy for treatment of *E. coli* O157:H7 infections are debated among countries (Highsmith and Favero, 1985). *In vitro* studies demonstrated that utilization of antibiotics in *E. coli* O157:H7 infections increase the Shiga-toxin production and releasing, thereby, occurance of hemolytic uremic syndrome (HUS). This concern also have been proved by clinical cases (Mead and Griffin, 1998; Acheson and Jaeger, 1999; Wong et al., 2000). *E. coli* O157:H7 also develops resistance to classic antimicrobial agents. There are many ways for bacteria to get antimicrobial resistance. Alterations in the permeability of the cell membrane, changes in the target site, inactivation of antimicrobial by enzymes, active effluxing of compounds by efflux pumps, excessive production of target enzymes are some of the mechanisms of resistance againts antibiotics (Souza et al., 2005). When bacteria gain resistance against the antibiotic, mortality rate associated with the infection increases, hospitalization and treatment process extends, expenses to overcome ilnesses and spread risks for infections increase gradually (Shetty and Labbe, 1998).

Increase in antibiotic resistance from unregulated and excess use of antibiotics reinforce the needs of new strategies which use plant based sources as antimicrobials

(Shetty and Lin, 2006). Currently, new applications as potential inhibitors of these pathogenic organisms are in interest to inhibit their presence and multiplying in food (Shetty and Labbe, 1998).

Phenolic acids obtained from plants are abundant in fruits and vegetables. Several studies have demonstrated that besides their health beneficial effects, they posses antimicrobial effects against pathogenic bacteria (Alakomi et al., 2007). Owing to their membrane active properties they disrupt membrane structure and cause leakage of cytoplasm in microorganisms (Campos et al., 2009). By means of their hydrophobic structure, phenolic acids may bind the outer membrane of Gram-negative bacteria leading to alterations in permeability and integrity of the membrane (Lacombe et al., 2010).

In this study, antimicrobial activities of phenolic acids, namely caffeic acid, rosmarinic acid and vanillic acid were investigated against foodborne pathogen *E. coli* O157:H7 by microtiter plate method. Interactions of phenolic acids with proteins were known as one of the antimicrobial action mechanisms to display their antimicrobial effects. Effect of chosen phenolic acids on *E. coli* O157:H7 cell surface was examined with scanning electron microscopy. Antimicrobial action mechanisms of these phenolic acids were studied by proteomic approach. By taking the membrane structure of *E. coli* O157:H7 into consideration, it was hypothesized that caffeic, rosmarinic, and vanillic acids affect the bacteria through their outer membrane, primarily. Therefore, outer membrane protein isolation, SDS-PAGE, 2D-PAGE analyses were performed.

CHAPTER 3

MATERIALS AND METHODS

3.1. Bacterial Strain and Culture Conditions

E. coli O157:H7 (NCTC 12900) was obtained from National Culture Type of Collection (NCTC, United Kingdom). *E. coli* O157:H7 was grown in Lauria Broth (LB), and on LB agar (Agar, Merck). LB includes 10 g of tryptone (Fluka), 5 g of yeast extract (Fluka) and 5 g of sodium chloride (Fluka) per liter.

E. coli O157:H7 was maintained in LB medium containing 20% glycerol and stored at -80°C as stock culture. It was refreshed from stock culture to soft agars in every 3 months. For the short term storage of *E. coli* O157:H7, it was sub-cultured on LB agar plate every week and kept at +4°C to maintain viability.

3.2. Antimicrobial Activities of Phenolic Acids

3.2.1. Preparation of Bacterial Culture

For microtiter plate assay, a single colony of *E. coli* O157:H7 from agar plates was inoculated into 4 ml LB medium. Incubation time was approximately 16.5 h at 37°C (Nüve) for the bacteria to reach exponential phase. After incubation period, optical density (OD) of *E. coli* O157:H7 was adjusted to desired inoculum level by Thermo Multiscan Spectra Reader (Finland) which was determined as 0.21 at 600 nm by dilution with LB medium. When optical density was adjusted to 0.21, *E. coli* O157:H7 culture was serially diluted (9:1 ratio). Bacterial culture was used from tube containing 1x10^{4} cfu/ml to determine antimicrobial activity. By using plate count method, the confirmation of proper bacterial load was done in every experiment.

3.2.2. Preparation of Phenolic Acid Solutions

Caffeic acid (Sigma, PN: C0625), rosmarinic acid (Aldrich, PN: 536954), vanillic acid (Fluka, PN: 94770) were purchased commercially.

Phenolic acid solutions were prepared in different concentration ranges as given in Table 3.1. The solutions of each phenolic acid were prepared in the highest concentration that was determinated. Ethanol content of 6 mM caffeic acid and 6 mM rosmarinic acid solutions were 0.1% (Riedel-de Haen, Germany), whereas, DMSO content was 3% for 12.5 mM vanillic acid and 17.5 mM rosmarinic acid solutions. Each of the solutions was firstly dissolved in ethanol or DMSO, and then by adding required amount of LB, the highest concentrations were diluted to intended concentrations. All solutions were prepared as two-fold of target value because in the wells the concentrations were leveled to half. Phenolic acids were prepared freshly prior to use.

Table 3.1. Concentration ranges of phenolic acids

Phenolic Compound	Concentration in tubes (mM)	Concentration in 96-well plate (mM)
Caffeic Acid	12	6
	13	6.5
	14	7
	15	7.5
	16	8
Rosmarinic Acid	2	1
	4	2
	6	3
	8	4
	10	5
	12	6
	20	10
	25	12.5
	30	15
	35	17.5
Vanillic Acid	10	5
	13	6.5
	16	8
	19	9.5
	22	11
	25	12.5

3.2.3. Determination of Antimicrobial Activity of Phenolic Acids (Microtiter Plate Assay)

Antimicrobial activity of each phenolic acid used in this study was determined by microtiter plate assay (Karaosmanoglu et al., 2010). Stock solutions of caffeic, vanillic, and rosmarinic acids were diluted to concentrations given in Table 1 with LB. 100µl of each prepared concentration was dispensed into a well of flat bottom 96-well microtiter plate (Bio-Grainer, Germany). 100µl of $1x10^4$ cfu/ml *E. coli* O157:H7 was added into each well. So, final phenolic acid concentration in each well was halved. Blanks for each concentration were prepared by adding 100µl LB and 100 µl of phenolic acid and filled into wells. As a control, 100µl *E.coli* O157:H7 and 100µl LB was loaded into wells. Optical density of plates were measured at 600 nm by Thermo Multiscan Spectra Reader (Finland) in every 3 hours for 24 h during incubation at 37°C. This experiment was performed at least four times for each phenolic acid.

3.3. Scanning Electron Microscopy (SEM) Analysis

Visualization of *E. coli* O157:H7 cells was done for both untreated and phenolic acids treated cells by examining with scanning electron microscopy (SEM).

As a control, single colony of *E. coli* O157:H7 was inoculated into 4 ml LB and incubated at 37°C for 18 hours. For treatment of bacteria with phenolic acids, 2 ml of 15 mM caffeic acid, 25 mM rosmarinic acid and 13 mM vanillic acid were mixed with 2 ml LB. Therefore, each phenolic acid concentration was halved in the tubes, namely, 7.5 mM caffeic acid, 12.5 mM rosmarinic acid and 6.5 mM vanillic acid. Single colony of *E. coli* O157:H7 was inoculated into tubes and incubated at 37°C for 18 hour. After incubation period, cells were transferred into 1.5 ml eppendorfs. Cells were harvested by centrifugation at 10,000g for 10 minutes. Obtained pellet was washed with 0.1% peptone 3 times by centrifugation at 10,000g for 10 minutes. Following washing step, cells were resuspended in 100 µl of 0.1% peptone and 10 µl of sample was spreaded onto aliminium foil. Aliminium foils containing spreaded cells were cut into small pieces, approximately 1 cm x 1 cm. For distinct visualization of samples, aliminium foil preparates were covered with gold.

Images of samples were taken at İYTE-MAM by scanning electron microscopes Phillips XL-30S FEG and FEI Quanta 250 FEG.

3.4. Protein Extraction from Outer Membranes in the Presence of Phenolic Acids

3.4.1. Preparation of Bacterial Culture

Single colony of *E. coli* O157:H7 was inoculated into 4 ml LB medium. This inoculation was done into 6 tubes because of high requirement of culture which was 200 ml. Tubes were incubated at 37°C for 16.5 h. When they reached to exponential phase, optical density was adjusted to desired inoculum level at 600 nm (Thermo Multiscan Spectra Reader) by dilution with LB medium. By spread plating from appropriately diluted cultures, the confirmation of proper bacterial load was done in every experiment.

3.4.2. Preparation of Phenolic Acid Solutions

Caffeic acid concentration which was used in outer membrane protein isolation protocol was determined as 6 mM. This concentration was chosen because of its inhibition value which retarded the growth at 18th hour. To have 6 mM final concentration in flasks, 12 mM caffeic acid solution was prepared. Ethanol (Riedel-de Haen, Germany) at the concentration of 0.2% was used as a solvent to dissolve 101 ml 12 mM caffeic acid solution.

Vanillic acid concentration that retarded the growth of *E. coli* O157:H7 was determined as 6.5 mM. 13mM vanillic acid which gave 6.5 mM in flasks was prepared at the amount of 101 ml with 0.2% ethanol. Ethanol and DMSO concentrations used in this study were at the values which do not inhibit the growth. Vanillic acid solution was mixed with 101 ml LB in the 500 ml flask.

Rosmarinic acid concentration which exhibited ~50% inhibiton was determined as 10 mM. Thus, 20 mM rosmarinic acid was prepared by dissolving the phenolic acid

in 6% DMSO firstly. Then, by addition of LB medium, the amount of 20 mM rosmarinic acid was broght up to 101 ml.

After the phenolic acid solutions were prepared, the same procedure with 96-well plate assay was performed. Solutions were transferred into 500 ml flask containing 101 ml LB (1:1 ratio) in every experiment. Therefore, caffeic acid, vanillic acid and rosmarinic acid concentrations were decreased to 6 mM, 6.5 mM and 10 mM, respectively. As a blank, 4 ml of each solution was transferred from flask into a glass tube.

3.4.3. Application of Phenolic Acids to Bacteria

For treatment of bacteria with phenolic acids, 2 ml of *E. coli* O157:H7 (0.5 ml culture from 4 tubes) of which optical density was adjusted to desired value was inoculated into 198 ml of phenolic acid solution. As a control, 2 ml of bacteria (0.5 ml culture form 4 tubes) was inoculated into 198 ml LB. Control and phenolic acid stressed bacteria were grown at 37°C for 18 h until the end of the exponential phase. For confirmation of the bacterial load, plate count method was performed and optical density measurements were taken both at the begining and at the end of incubation period in every experiment.

3.4.4. Isolation of OMPs

Outer membrane proteins were extracted by using sodium lauryl sarcosinate method (Lin et al., 2008). Following incubation period, bacterial cells were transferred from flasks to 500 ml centrifuge tubes (Beckman) and harvested at 4,000g for 20 min at 4°C by centrifugation (Sigma 6K15, Ultracentrifuge). By doing centrifugation, the *E. coli* O157:H7 pellet was obtained. The pellet was washed in 80 ml of 0,85% NaCl, twice. Then, centrifuge tubes were kept at -80°C for 90 min to weaken the cells. Pellet was resuspended in a mixture consisting of 5 ml of 100 mM Tris-HCl (pH 7.4) and 5 ml of steril ultra pure water (0.22 μm). After resuspension, cells were transferred into 50 ml centrifuge tubes (Beckman) and disrupted by intermittent sonication. Control *E. coli* O157:H7 cells were sonicated for a total of 5 minutes with 30 seconds per cycle and

intervals of 9 seconds on ice, whereas, phenolic acid treated cells were sonicated for a total of 4 minutes with the same time for each cycle and intervals. Cellular debris and unbroken cells were removed by centrifugation at 6,000g for 20 min at 4°C. Supernatant was collected and transferred into ultracentrifuge tubes. Then, supernatant was centrifuged at 100,000g for 45 min at 4°C. The obtained pellet was resuspended in 6 ml of 2% (vol/vol) sodium lauryl sarcosinate in 50 mM Tris (pH 7.4) and incubated at 25°C for 20 min. Incubation was followed by ultracentrifugation at 100,000g for 45 min at 4°C and pellet was resuspended in 250 μl of 50 mM Tris-HCl (pH 7.4). Samples were transferred into protein lo-bind tubes (Eppendorf). The samples were stored at -80°C for further usage.

3.5. SDS-PAGE Analysis

SDS-PAGE analysis was carried out in Mini-PROTEAN Tetra Cell (Bio-Rad). Concentrations of proteins were adjusted to 1.4 mg/ml; 0.70 mg/ml for caffeic acid treated OMPs, 1.0 mg/ml; 0.5 mg/ml for vanillic acid treated OMPs, and 1.5 mg/ml; 0.75 mg/ml for rosmarinic acid treated OMPs. Adjustment of desired concentration was done with diluting proteins with 50 mM Tris-HCl pH 7.4. In every experiment, 15 μl of sample was mixed with 15 μl of sample buffer and boiled at 100°C for ten minutes. 2.5 μl protein marker was also boiled at 100°C for ten minutes.

- Preparatrion of sample buffer (SDS reducing buffer): 3 ml of distilled water, 0.5 M of Tris-HCl pH 6.8, 1.6 ml of glycerol and 1.6 ml of 10% SDS were mixed. Then, 0.4 ml of β-mercaptoethanol and 0.4 ml of 0.5% (w/v) bromophenol blue were added.
- Preparation of 12% seperating gel (5 ml total volume per gel): 2 ml of 30% acrylamide-bisacrylamide solution (19T:1) was mixed with 1.675 ml of ultra pure water. Then, 1.25 ml of 1.5 M Tris pH 8.8 and 50 μl of 10% SDS were added. 25 μl of 10% APS and 2.5 μl of TEMED were added to solution, finally.

Following addition of APS and TEMED, the acrylamide solution started to soldify, so the gel was immediately poured between 2 glasses. For faster polymerization, 200 μl of tertamyl alcohol was added onto gel. After an hour, when the gel was completely solidified, the alcohol was discarded and the bottom of gel was

washed with deionize water. Then, stacking gel was prepared by adding ingredients sequentially as follows:

- Preparation of 4% stacking gel (2.5 ml total volume per gel): 0.325 ml of acrylamide-bisacrylamide solution was mixed with 1.525 ml of ultra pure water. Then, 0.625 ml of 0.5 M Tris pH 6.8 and 25 μl of 10% SDS were added into solution. 12.5 μl of 10% APS and 2.5 μl of TEMED were added into solution and it was poured onto seperating gel between the glasses. The comb was placed onto stacking gel, carefully.

After polymerization of gels, the comb was removed slowly and the wells were washed with deionize water. Required amount of 1X run buffer which was prepared by diluting 5X run buffer was added and then prepared protein samples was loaded into wells. In every experiment, 10 μl of sample was loaded into wells.

- Preparation of 5X Running Buffer (1X: 25mM Tris, 192 mM glycine, 0.1% SDS, pH8.3): 45 g of Tris base and 216 g of glycine were weighed and dissolved in 2 liter of ultra pure water. Then, 15 g of SDS was added and the volume was completed to 3 liter with ultra pure water. pH of the solution was not adjusted with acid or base. This buffer was kept at +4°C and warmed at room temperature prior to using.

When the samples were loaded, the mini SDS tank was half filled with 1X run buffer and the electrophoresis was programmed to 100V for 120 minutes. After electrophoresis had completed, the gel was taken into fixer solution and kept in fixer for overnight incubation prior to silver staining.

- Preparation of Silver Staining components:
    - Fixer (It can be used several times): 150 ml methanol, 36 ml of acetic acid and 150 μl of 37% formaldehyde were mixed and the solution was completed to 300 ml with ultra pure water.
    - 50% Ethanol: 500 ml of pure ethanol and 500 ml of ultra pure water were mixed.
    - Pretreatment Solution: 0.08 g of sodium thiosulfate was dissolved in 400 ml ultra pure water.
    - Silver Nitrate Solution: 0.8 g of silver nitrate was dissolved in 400 ml of ultra pure water and 300 μl of 37% formaldehyde was added into solution.

- Developing Solution: Into a 400 ml of ultra pure water, 9 g of potassium carbonate, 8 ml of pretreatment solution, and 300 µl of 37% formaldehyde were added.
- Stop Solution: 200 ml of methanol and 48 ml of acetic acid were mixed and the solution was completed to 400 ml with ultra pure water.

After the gel was taken from the fixer solution, washed in 50% ethanol 3 times. Time of treatment of ethanol was 20 minutes for all 3 washing steps. Gel was kept in pretreatment solution for 1 minute, and then, rinsed with ultra pure water 3 times each 20 seconds long. Gel was taken into silver nitrate solution for 20 minutes. Following silver nitrate treatment, rinsed again with ultra pure water 2 times each 20 seconds long and treated with developing solution. After waiting about a minute some water added into developing solution to slow the process. Time was determined by observation of colour development. Finally, the developing solution was discarded, and stop solution was added to stop the reaction. After waiting about 10 minutes, the image of gel was taken with VersaDoc Imaging System.

3.6. 2D-PAGE Analysis

3.6.1. Protein Solubilization with Rehydration Buffer

Protein concentration determination was done by Bradford protein assay. For Silver Blue (see page 34: very sensitive colloidal Coomassie) stained gels, the protein concentration was adjusted to 400 µg in 100 µl of sample. For Silver stained gels, the protein concentration was adjusted to 100 µg in 100 µl of sample. Protein concentrations were adjusted by dilution with 50 mM Tris-HCl (pH 7.4) or by concentrating proteins using SpeedVac (Thermo Electron Corporation). When the protein concentration was adjusted, the OMPs were cleaned with 2-D clean-up kit (GE Healthcare). Then, the cleaned samples were resuspended in 350 µl of rehydration buffer consisting of urea (Sigma), CHAPS (Sigma), DTT (Sigma), ampholytes (pH 3-10, Bio-Rad) and Bromophenol Blue.

- Preparation of rehydration solution: 4.8g Urea, 0.2g CHAPS, 0.077g DTT were weighed and dissolved in ultra pure water to a final volume of 10 ml by stirring.

The solution can be heated up to 30°C but must not be heated above. Otherwise, the heat leads to the urea breakdown. Then, 100µl of 20% 3-10 ampholytes (Bio-Rad) were added into solution. 4 µl of Bromophenol Blue was added into solution to make it possible to observe the separation of proteins during isoelectric focusing. The rehydration solution was stored at +4°C.

IEF experiments were performed in a parallel manner to provide same experimental conditions to control and stressed bacteria OMPs.

After protein samples from both control and phenolic acid treated *E. coli* O157:H7 were mixed with rehydration buffer, samples were dispensed into wells of tray. IPG strips (pH 3-10, non-linear gradient, 17 cm, Bio-Rad) were positioned into tray as their gel side down contacting the sample and rehydration buffer mixture evenly. The tray was incubated at room temperature on the bench for an hour. Afterwards, 2 ml of mineral oil (Bio-Rad) was added above the IPG strips to minimize evaporation and to prevent urea precipitation. The tray's cover was placed and the tray was placed into 20°C incubator for passive rehydration for 16.5 h.

3.6.2. First Dimension: Isoelectric Focusing

The first dimension of 2D-PAGE was carried out by using a Protean IEF Cell (Bio-Rad). The focusing tray should be clean and dry. Paper wicks were localized onto the electrodes of tray and wicks were wetted with 8 µl of ultrapure water. IPG strips were taken from incubator and their back sides were cleaned slowly with a paper towel. Then, IPG strips were put carefully into the wells of tray, especially by preventing formation of air bubles. In this step, it was important to place the strips with the positive end of the strip on the positive side of the tray. After placing IPG strips carefully, they were covered with 2 ml of mineral oil and the cover of focusing tray was localized.

IEF settings was programmed as:

- Step 1: Linear gradient, 200 V, 300 Vhours
- Step 2: Linear gradient, 500 V, 500 Vhours
- Step 3: Linear gradient, 1000 V, 1000 Vhours
- Step 4: Linear gradient, 4000 V, 4000 Vhours
- Step 5: Rapid gradient, 8000 V, 24000 Vhours

- Step 6: Rapid graident, 8000 V, 30000 Vhours

IEF program temperature was set to 20°C. After the isoelectric focusing of proteins had completed, the strips were taken from the focusing tray and equilibrated with equilibration buffers.

3.6.3. Equilibration of Strips

For solubilization of focused proteins and binding of SDS to proteins, it was important to equilibrate the IPG strips. Thus, all proteins had negative charges became unfolded state. Equilibration buffer I consisted of urea, SDS, Tris-HCl, glycerol and DTT which was required to reduce sulfhydryl groups by cleaving bonds between cysteine residues. Equilibration buffer II consisted of urea, SDS, Tris-HCl, glycerol and Iodoacetamide which was necessary for alkylation of sulfhydryl groups. Equilibration buffers were prepared as follows:

- Preparation of Equilibration Buffer I: 7.2 g urea was weighed and put into 5 ml of ultra pure water and put on a stirrer. Then, 5ml of 1.5 M Tris-HCl (pH 8.8), 4 ml of glycerol and 0.4 g of SDS were added and after completion of dissolving the volume was adjusted to 20 ml by ultra pure water. 400 mg DTT was added into equilibration buffer I, at last. The solution was kept at 4°C. For 1 strip, 6 ml of solution was used for equilibration.
- Preparation of Equilibration Buffer II: 7.2 g urea was weighed and put into 5 ml of ultra pure water and stirred on a stirrer. Then, 5ml of 1.5 M Tris-HCl (pH 8.8), 4 ml of glycerol and 0.4 g of SDS were added and after completion of dissolving, the volume was adjusted to 20 ml by ultra pure water. 500 mg/20 ml iodoacetamide was added into buffer prior to usage. 6 ml of equilibration buffer II was used per strip.

Before starting equilibration, required amount of iodoacetamide was weighed and added into 6 ml of equilibration buffer II. Strips were taken from focusing tray and placed into disposable tray as their gel side up. 6 ml of equilibration buffer I was treated with strips for 10 minutes with gentle shaking. After 10 minutes, strips were taken from the used wells and placed into clean wells of tray and treated with 6 ml of equilibration buffer II for 10 minutes with shaking.

3.6.4. Second Dimension: SDS-PAGE

Seperation of proteins in second dimension by SDS-PAGE was carried out in PROTEAN II Cell (Bio-Rad). 12% polyacrylamide gel containing 1.5 M Tris (pH 8.8), 10% SDS, 10% ammonium per sulphate (APS) and TEMED was prepared for seperation of proteins according to their molecular weight.

- Preparation of 1.5 M Tris pH 8.8: 54.45 g of Tris base was added into 150 ml of ultra pure water. pH of the solution was adjusted to 8.8 with HCl and the volume was completed to 300 ml.
- Preparation of 10% SDS: 10 g SDS was dissolved in 100 ml of ultra pure water.
- Preparation of 10% APS: 0.045 g APS was dissolved in 450 µl of ultra pure water. APS was prepared freshly in every experiment.
- Preparation of 12% Polyacrylamide Gel (for 2 gels): 32 ml of 30% acrylamide-bisacrylamide solution (19T:1) was mixed with 26.8 ml of ultra pure water. Then, 20 ml of 1.5 M Tris and 0.8 ml of 10% SDS were added. 400 µl of 10% APS and 40 µl of TEMED were added finally.

As soon as addition of APS and TEMED polymerization started, so the gel was poured between two glass plates immediately. 600 µl of tertamyl alcohol was poured onto gel for faster polymerization. The gel was put into 4°C for one day, prior to gel electrophoresis.

After equilibration step had completed, the strips were rinsed with 1X running buffer. For rinsing of strips, 100 ml of 1X running buffer was prepared by diluting 5X running buffer. 20 ml of 5X buffer was diluted with 80 ml of deionize water in a graduated cylinder. Then strips were holded with forceps and rinsed with 1X run buffer 3 times. After rinsing, strips placed onto polyacrylamide slabs and ~2 ml of low melt agarose (0.5% agarose) was poured onto the strips to seal the strip to gel.

- Preparation of low melt agarose: Low melting point agarose was prepared in 25 mM tris, 192 mM glycine, 0,1% SDS and a trace of Bromophenol Blue. For 50 ml total volume 0,15 g of tris, 0,72 g glycine, 0,05 g SDS and 8 ml Bromophenol Blue was mixed in 50 ml ultra pure water and homogenized by boiling at microwave oven.

In this step, there must be no air bubles between strip and gel, otherwise, it may prevent migration of proteins. Following the solidification of low melt agarose, the gel

was placed into a tank and half filled with 1X run buffer. Cooling of tank was provided by circulating water. Electrophoresis was started at 16 mA per one gel for the first 30 minutes, and setted to 25 mA current per one gel for the next 5 hours until the blue dye reach the bottom of the gel.

3.6.5. Staining the Gels

After electrophoresis was finished, the gels were taken into large capes for staining step. Gels which correspond to vanillic acid treated *E. coli* O157:H7 OMPs and its control OMPs were stained with very sensitive colloidal coomassie staining called 'blue silver' developed by Candiano (Candiano et al., 2004).

- Preparation of staining solution: 118 ml of 85% phosphoric acid and 82 ml of ultra pure water were mixed, then 10 g of ammonium sulphate was added. When the ammonium sulfate had dissolved, 1.2 g of Coomassie Blue G-250 was added into solution. While this solution was kept on the stirrer, 600 ml of ultra pure water and 200 ml of methanol were added. This stock dye solution was kept in a brown bottle at room temperature. The final concentrations of the components were as follows: 10% phosphoric acid, 20% methanol, 10% ammonium sulphate, and 0.12% G-250.

For staining of gels, firstly gels were washed in ultra pure water for 5 minutes, and then fixed in a mixture of 30% methanol, 10% acetic acid and ultra pure water for 1 hour. Afterwards, gels were washed in ultra pure water 4 times (20 minutes for each washing step). The water was discarded and the staining solution was added and the gels were kept in dye solution for 3-4 days. Dye solution did not create much backround on the gels, however, following staining process, the gels were washed in ultra pure water for distinct spot visualization. The images of gels were taken with VersaDoc Imaging System.

Gels which correspond to caffeic acid and rosmarinic acid treated *E. coli* O157:H7 OMPs and their control OMPs were stained with silver staining. The same procedure that applied to mini SDS-PAGE gels was applied to gels for silver staining (see page 30).

CHAPTER 4

RESULTS AND DISCUSSION

This study was comprised of two parts. In the first part of the study, antimicrobial activities of caffeic, vanillic and rosmarinic acids were investigated against *E. coli* O157:H7. Minimum inhibitory concentrations (MICs) of these phenolic acids were found by microtiter plate assay. Also, alterations in the cell membrane upon phenolic acid treatment were observed by SEM. In the second part, antimicrobial action mechanisms of caffeic, vanillic and rosmarinic acids were studied by a proteomic approach. Phenolic acid concentrations which retarded the growth were treated to bacteria to observe the proteomic changes that had occured in the outer membrane.

4.1. Antimicrobial Activities of Phenolic Acids

Activities of caffeic, vanillic and rosmarinic acids against *E. coli* O157:H7 were determined by microtiter plate assay. Considering absorbance measurements during 24 h incubation at $37^{o}C$, the growth curves in the presence of each phenolic acids were plotted as optical denstity (OD) versus time (hour). Growth curves of *E. coli* O157:H7 in the presence of caffeic, vanillic and rosmarinic acids were given in Figure 4.1, 4.2, and 4.3, respectively. Standart deviations were equal or lower than 0.01.

Some concentrations of these phenolic acids retarded the growth of bacteria, while others inhibited the growth. 6 mM caffeic acid, and 10 mM rosmarinic acid delayed the beginning of exponential phase about six hours, 12.5 mM rosmarinic acid for nine hours, whereas, 5 mM vanillic acid retarded the growth for three hours and 6.5 mM for about nine hours. Hihger concentrations treated to bacteria showed growth inhibtory effect.

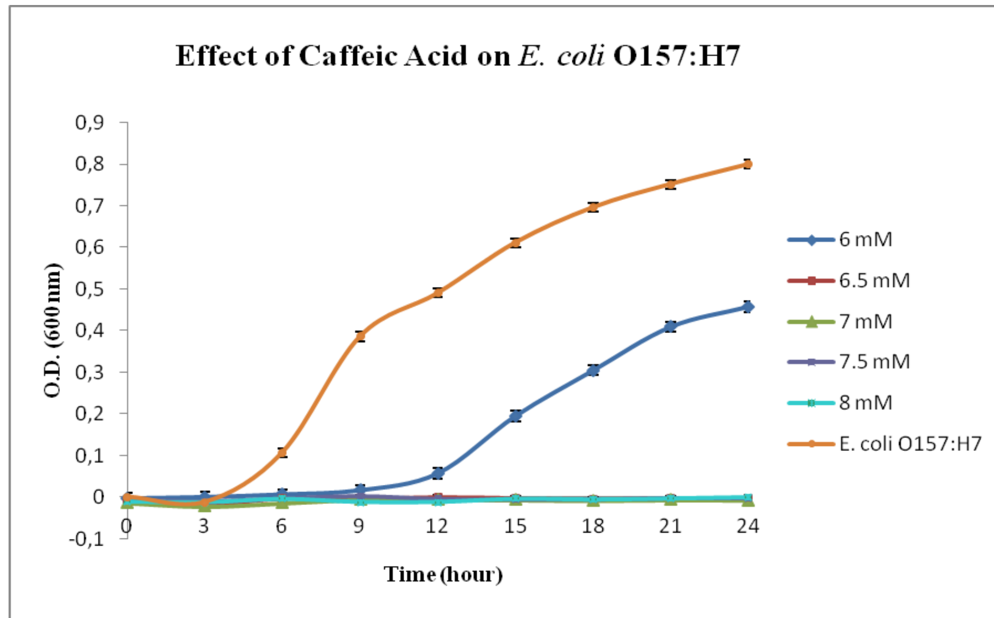


Figure 4.1. The growth curve of *E. coli* O157:H7 in the presence of caffeic acid

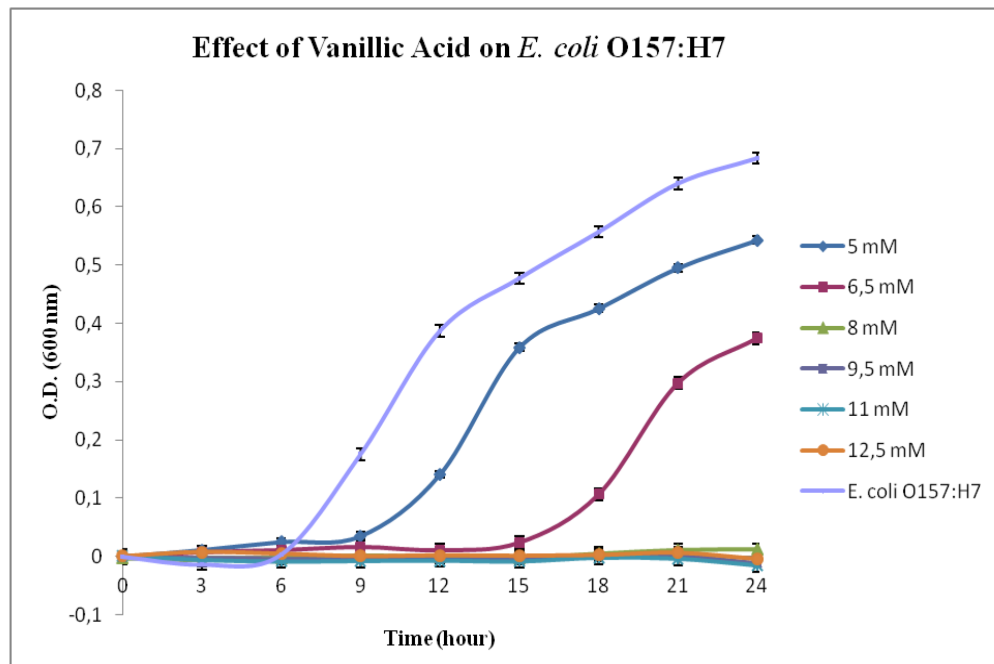


Figure 4.2. The growth curve of *E. coli* O157:H7 in the presence of vanillic acid.

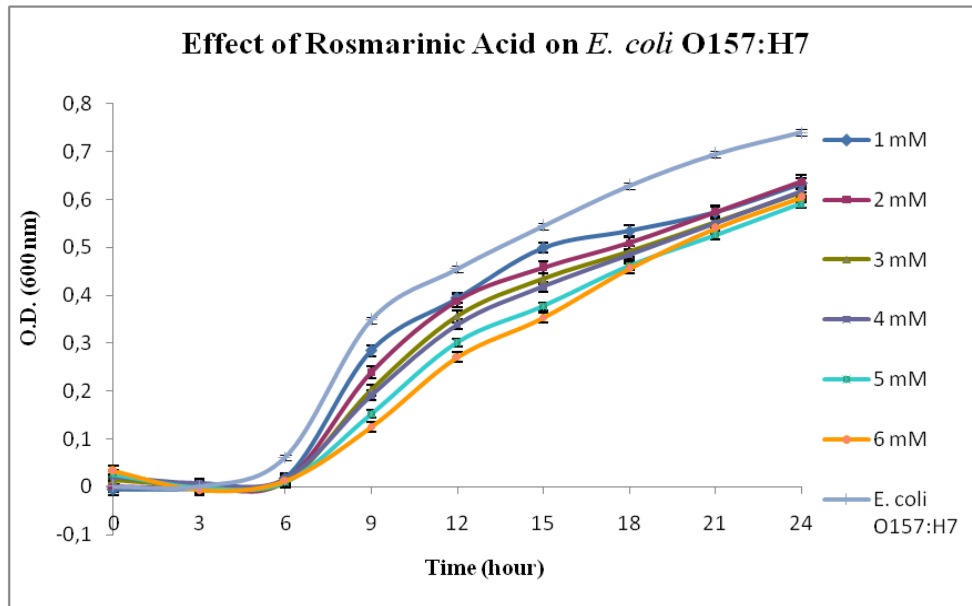


Figure 4.3. a) The growth of *E. coli* O157:H7 in the presence of rosmarinic acid (low concentrations)

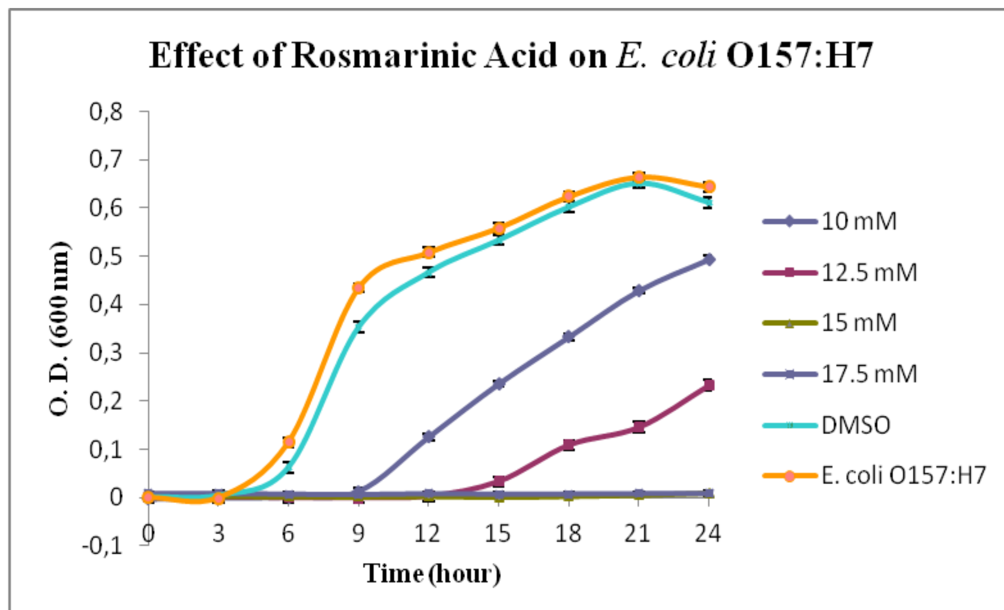


Figure 4.3. b) The growth of *E. coli* O157:H7 in the presence of rosmarinic acid (high concentrations)

Minimum inhibitory concentration (MIC) of each phenolic acid was determined as the lowest concentration that inhibited the growth of bacteria during 24 h incubation period. According to the results, caffeic acid inhibited the growth of *E. coli* O157:H7 at 6.5 mM concentration, vanillic acid at 8 mM concentration and rosmarinic acid at 15 mM concentration (Table 4.1).

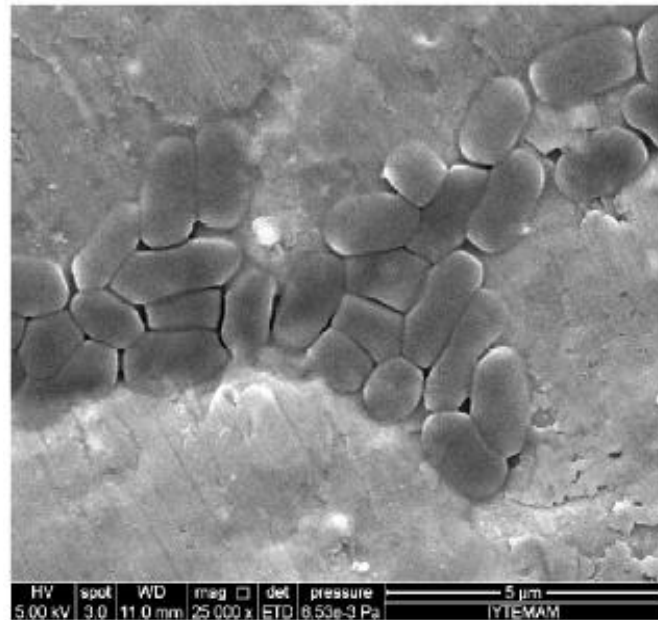
Table 4.1. Percent inhibition values of phenolic acids upon 24 h treatment

Phenolic Compound	Concentration (mM)	Percent Inhibition
	6	43
	6.5	100
Caffeic Acid	7	100
	7.5	100
	8	100
	5	20
	6.5	45
Vanillic Acid	8	100
	9.5	100
	11	100
	12.5	100
	10	23
Rosmarinic Acid	12.5	64
	15	100
	17.5	100

4.2. SEM Images

Morphological changes on *E. coli* O157:H7 cell surfaces upon treatment with certain phenolic acid concentrations were examined by SEM. The images indicated that while the control *E. coli* O157:H7 cells had intact cell surfaces (Figure 4.4), phenolic acid treated bacteria lost their intactness and displayed irregular rod shapes. Cells

treated with 7.5 mM caffeic acid (Figure 4.5), 12.5 mM rosmarinic acid (Figure 4.6) and 6.5 mM vanillic acid (Figure 4.7) were collapsed inward and wrinkled. At the cell surfaces of 12.5 mM rosmarinic acid treated cells, openings at the cell envelope were observed (Figure 4.6).



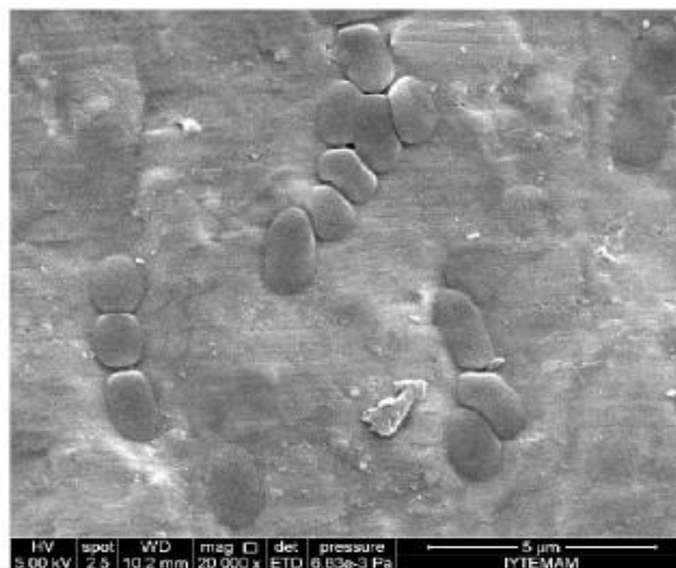
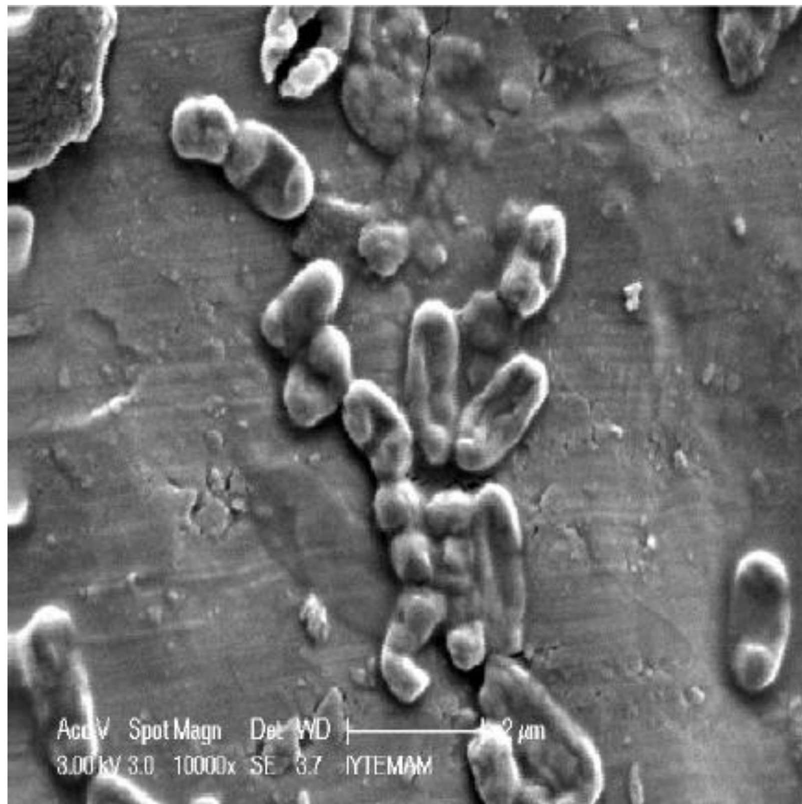


Figure 4.4. SEM images of control *E. coli* O157:H7 cells



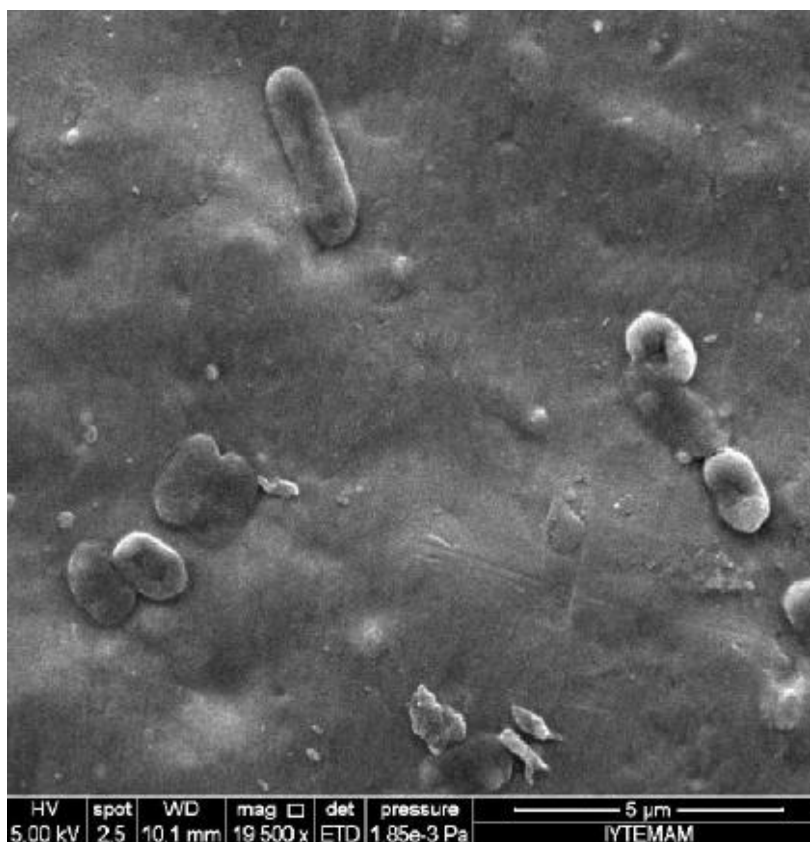
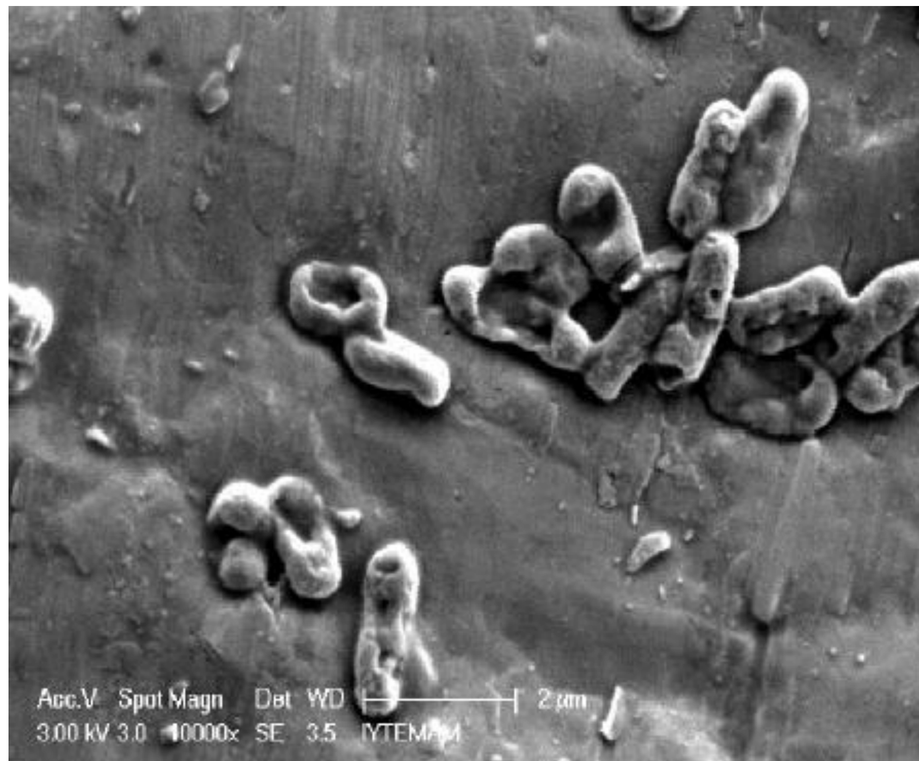


Figure 4.5. SEM images of 7.5 mM caffeic acid treated *E. coli* O157:H7 cells



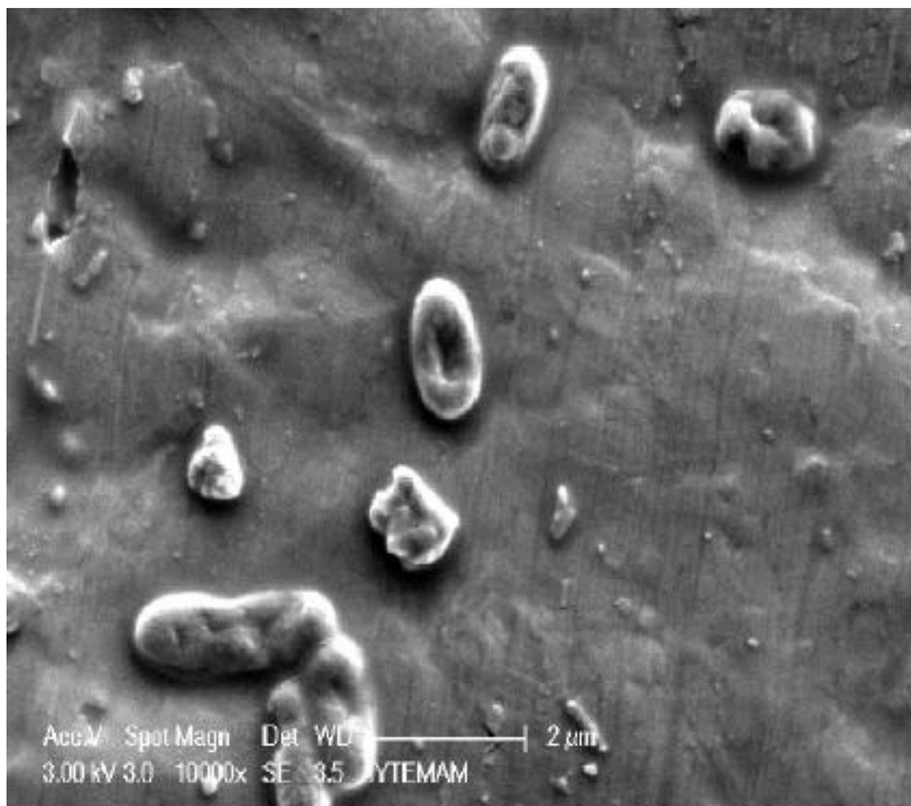


Figure 4.6. SEM images of 12.5 mM rosmarinic acid treated *E. coli* O157:H7 cells

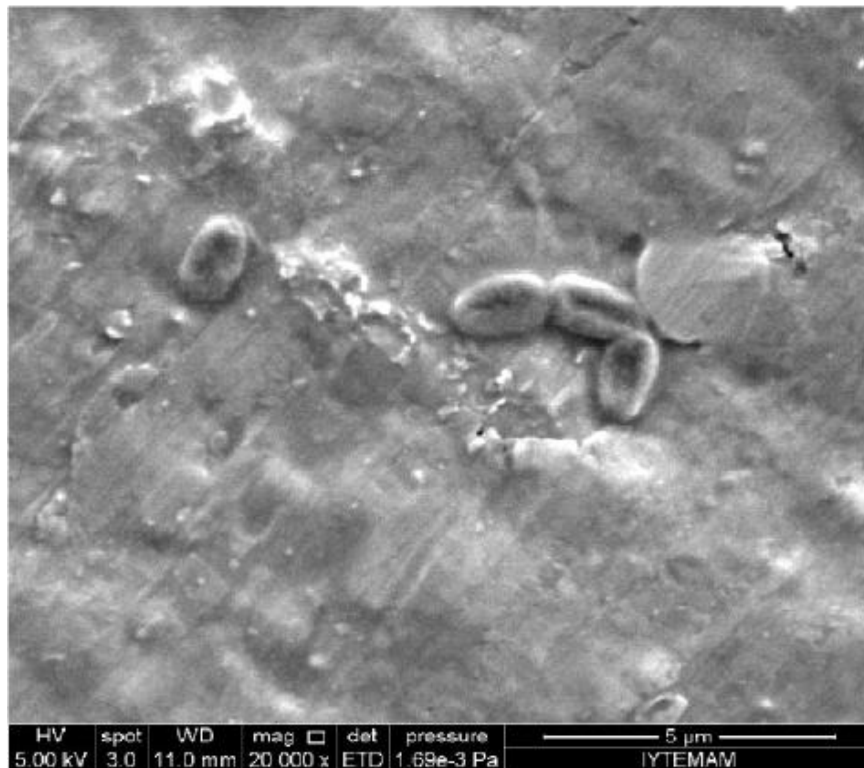


Figure 4.7. SEM images of 6.5 mM vanillic acid treated *E. coli* O157:H7 cells

4.3. Phenolic Acid Stress Responsive Proteins

In the proteomic part of the study, *E. coli* O157:H7 bacteria were grown till the end of the exponential phase of growth which took 18 h at 37°C incubation. During growth of bacterial culture for outer membrane protein (OMP) isolation, bacteria were reproduced in large scales in the presence and the absence of phenolic acids. 18th hour OD measurements gave the inhibition values of each phenolic compound by contrasting the phenolic acid treated bacteria with control.

Table 4.2. Percent inhibition values of phenolic acids upon 18 hour treatment

Phenolic Acid	Concentration	Treatment Time	% Inhibition
Caffeic Acid	6 mM	18 h	32
Vanillic Acid	6.5 mM	18 h	36
Rosmarinic Acid	10 mM	18 h	31

OMPs were isolated from both control and phenolic acids treated bacteria to see the differences in OMP patterns if their quantities were changed under phenolic acid stress or not.

4.3.1. SDS-PAGE Analysis

Following extraction of OMPs from *E. coli* O157:H7, the proteins were seperated by SDS-PAGE technique developed by Laemmli. Gel images demonstrated that phenolic acids affected the expression of some of the outer membrane protein encoding genes. The proteins were seperated on a 12% acrylamide SDS gel and the gels were stained with silver staining.

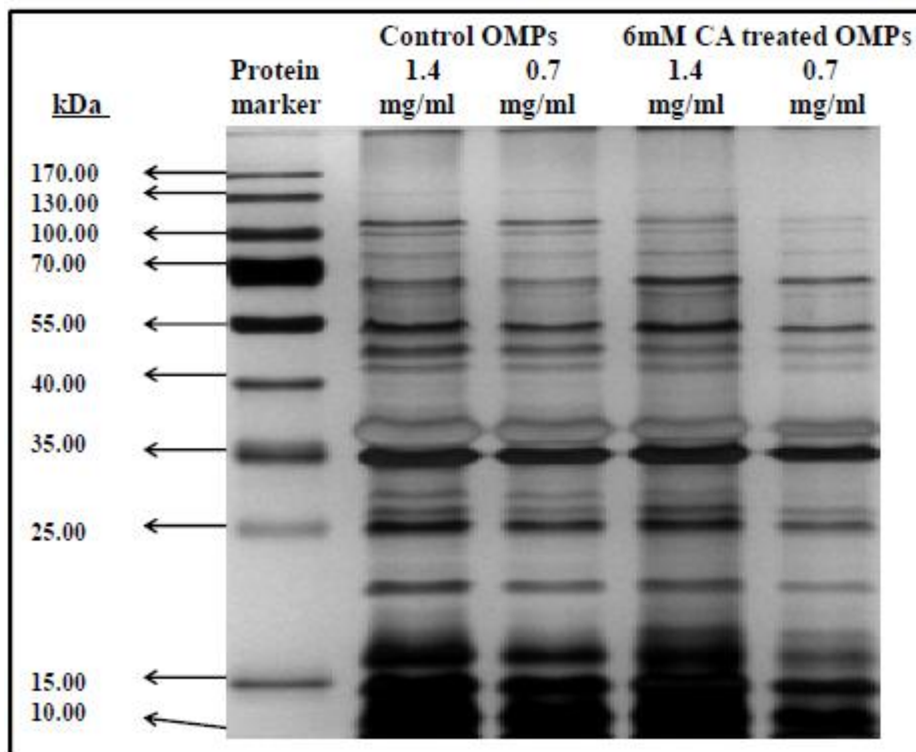


Figure 4.8. SDS-PAGE image of *E. coli* O157:H7 control and caffeic acid treated OMPs. Lane 1 corresponds to protein marker (Thermo, 26616), lane 2 and 3 correspond to the control OMPs at the concentrations of 1.4 mg/ml and 0.7 mg/ml, respectively. Lane 4 and 5 correspond to the 6 mM caffeic acid treated OMPs at the concentrations of 1.4 mg/ml and 0.7 mg/ml, respectively.

According to SDS-PAGE image, expression of 6 mM caffeic acid treated OMPs having molecular weights of ~100 kDa, ~50 kDa, 20 kDa decreased, whereas, upon treatment, expression of OMPs having molecular weights of ~70 kDa increased (Figure 4.8).

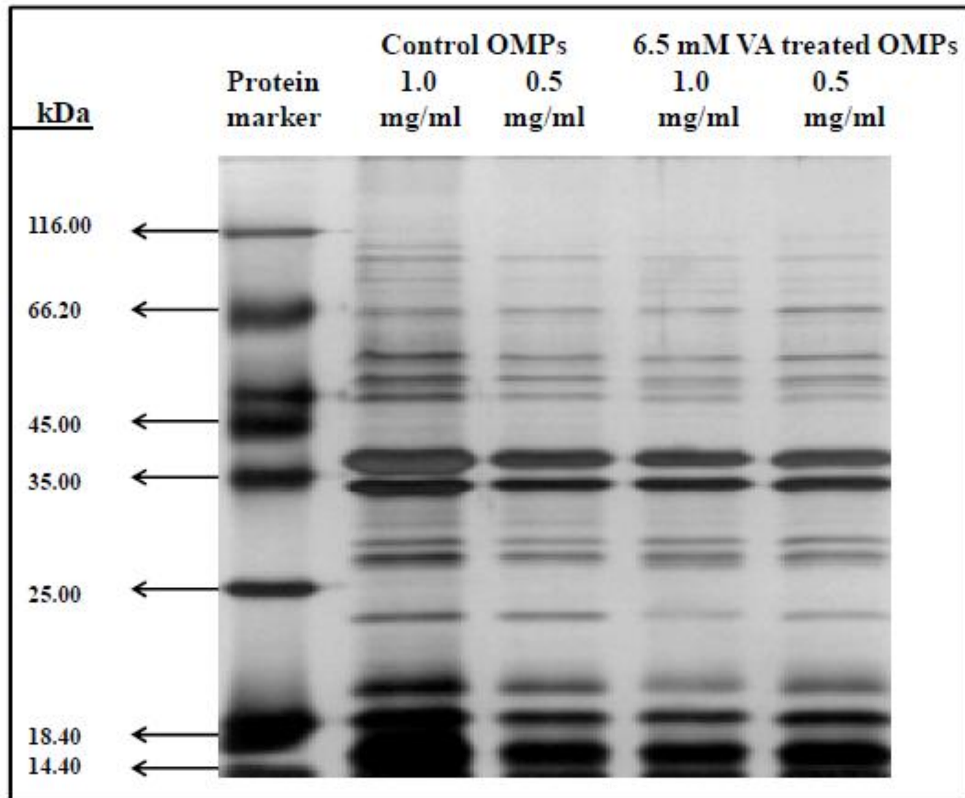


Figure 4.9. SDS-PAGE image of *E. coli* O157:H7 control and vanillic acid treated OMPs. Lane 1 corresponds to protein marker (Fermentas, SM0431), lane 2 and 3 correspond to the control OMPs at the concentrations of 1.0 mg/ml and 0.5 mg/ml, respectively. Lane 4 and 5 correspond to 6.5 mM vanillic acid treated bacteria OMPs which have the same concentrations with control.

SDS-PAGE image indicated little difference in expression of 6.5 mM vanillic acid treated OMPs at the molecular weights of ~35 kDa, ~25 kDa and ~20 kDa (Figure 4.9).

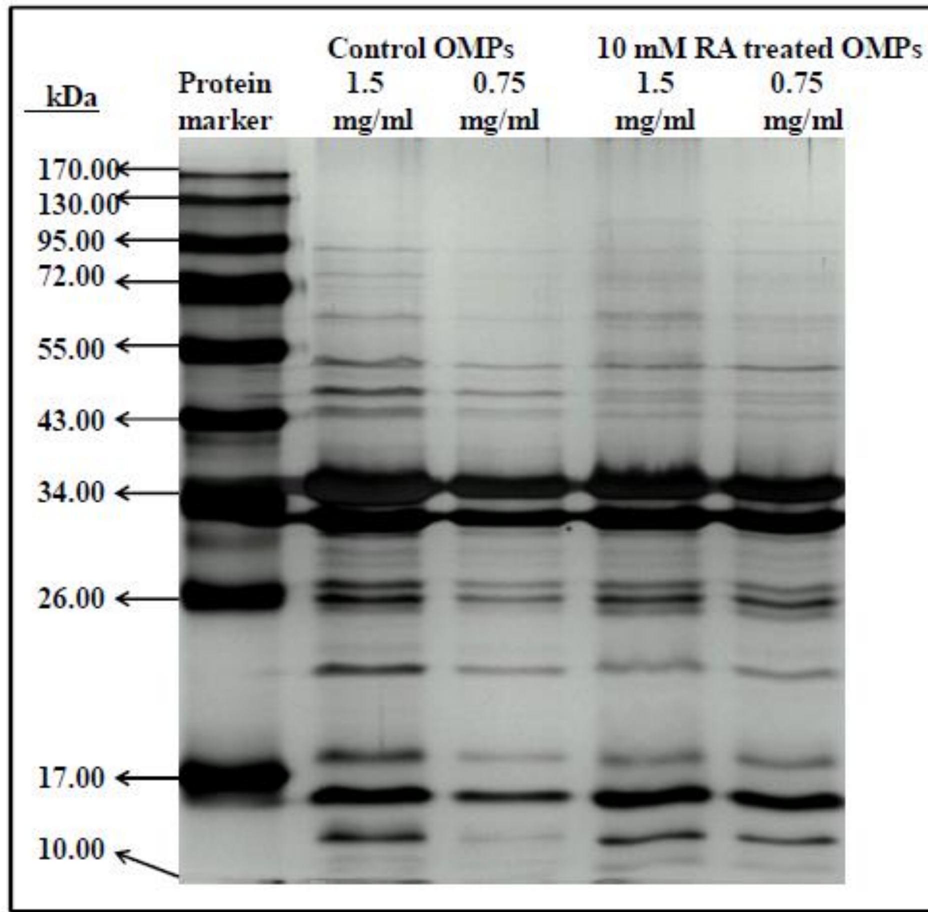


Figure 4.10. SDS-PAGE image of *E. coli* O157:H7 control and rosmarinic acid treated OMPs. Lane 1 corresponds to protein marker (Fermentas, SM0671), lane 2 and 3 correspond the control OMPs at the concentrations of 1.5 mg/ml and 0.75 mg/ml, respectively. Lane 4 and 5 correspond to 10 mM rosmarinic acid treated bacteria OMPs which have the same concentrations with control.

According to SDS-PAGE image of 10 mM rosmarinic acid treated *E. coli* O157:H7 OMPs, proteins at the molecular weights of ~52 kDa, ~35 kDa, ~22 kDa, ~17 kDa displayed decreased expression, and the proteins at the molecular weight of ~120 kDa displayed inreased expression (Figure 4.10).

Since SDS-PAGE seperated proteins according to their molecular weight, more spesific seperation was needed to identify alterations in the OMP pattern.

4.3.2. 2D-PAGE Analysis

Isolated proteins were separated according to their isoelectric points by IEF firstly, and then separated according to their molecular weights. For blue silver stained gels 400 μg of protein, and for the silver stained gels 100 μg of protein were loaded onto IPG strips.

Isoelectric focusing of proteins indicated that *E. coli* O157:H7 outer membrane proteins were mostly acidic proteins located in the acidic side of the 3-10 IPG strips.

Phenolic acid treated *E. coli* O157:H7 displayed difference in expression of some proteins (Figure 4.11, 4.12, 4.13). Differences in sizes of spots indicated difference expression levels in phenolic acid treated cells. Disappearance of some spots in OMP patterns of 6 mM caffeic acid (Figure 4.12) and 10 mM rosmarinic acid (Figure 4.13) treated cells supported the theory that phenolic acids affected the OMP profile of the bacteria. Nevertheless, in OMP patterns of caffeic and rosmarinic acid treated cells, some different spots were also observed. These spots may be related with stress response mechanism of the bacteria.

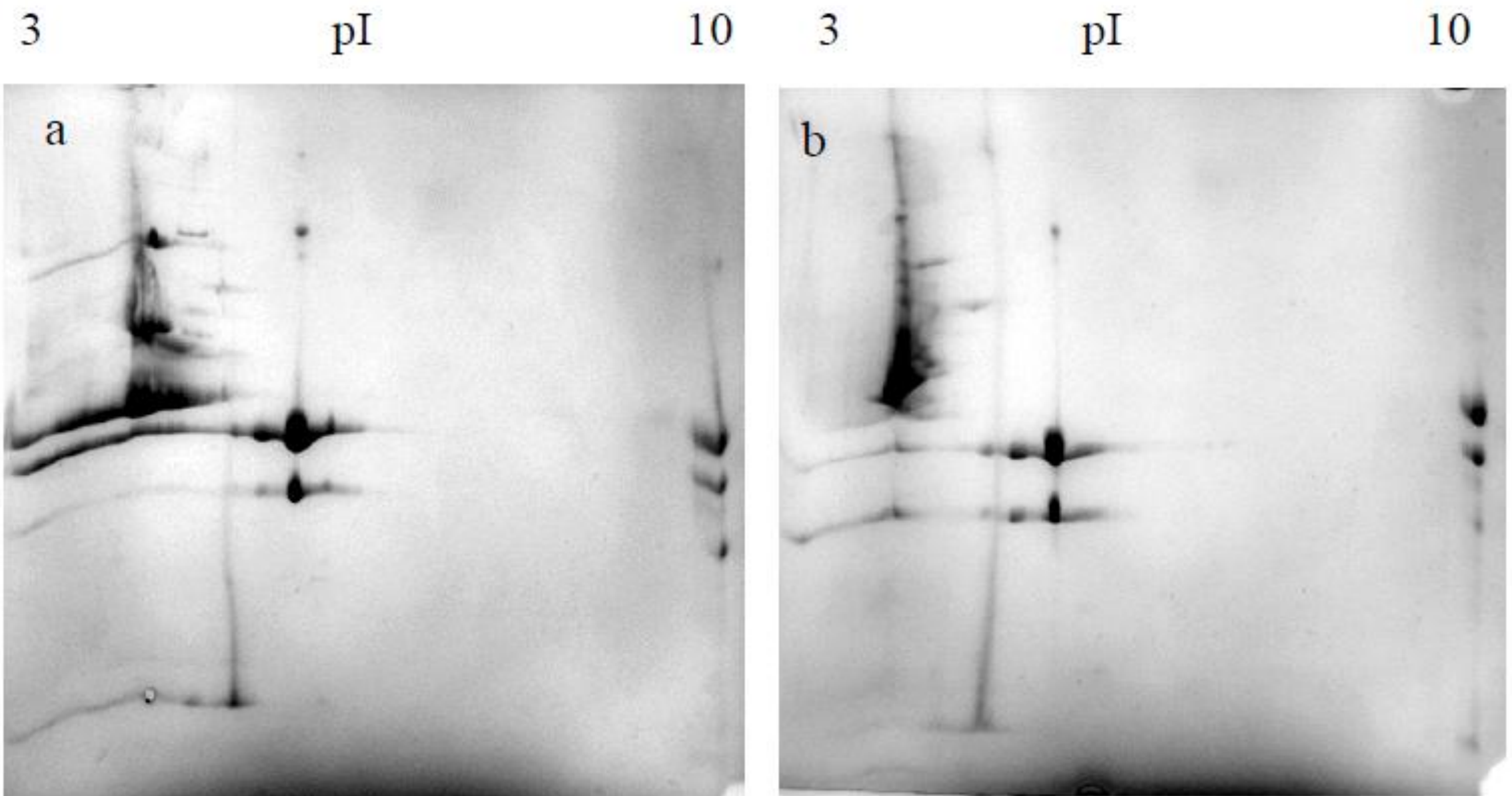


Figure 4.11. 2D-PAGE images of control (a) *E. coli* O157:H7 and 6.5 mM vanillic acid (b) treated *E. coli* O157:H7 OMPs. 400 µg protein containing gels were stained with Silver Blue staining.

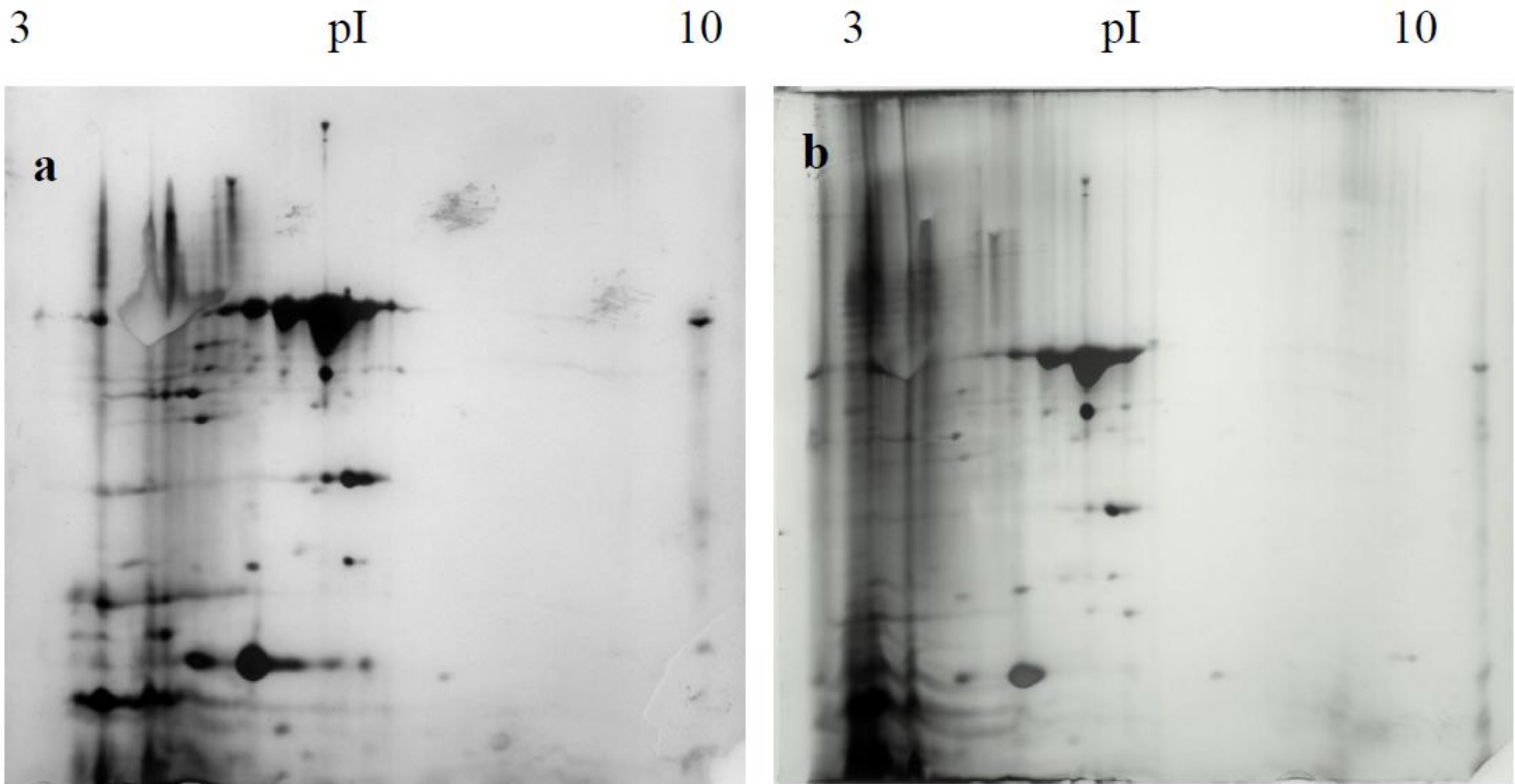


Figure 4.12. 2D-PAGE images of control (a) and 6.0 mM caffeic acid (b) treated *E. coli* O157:H7 OMPs. 100 μg protein containing gels were stained with Silver staining.

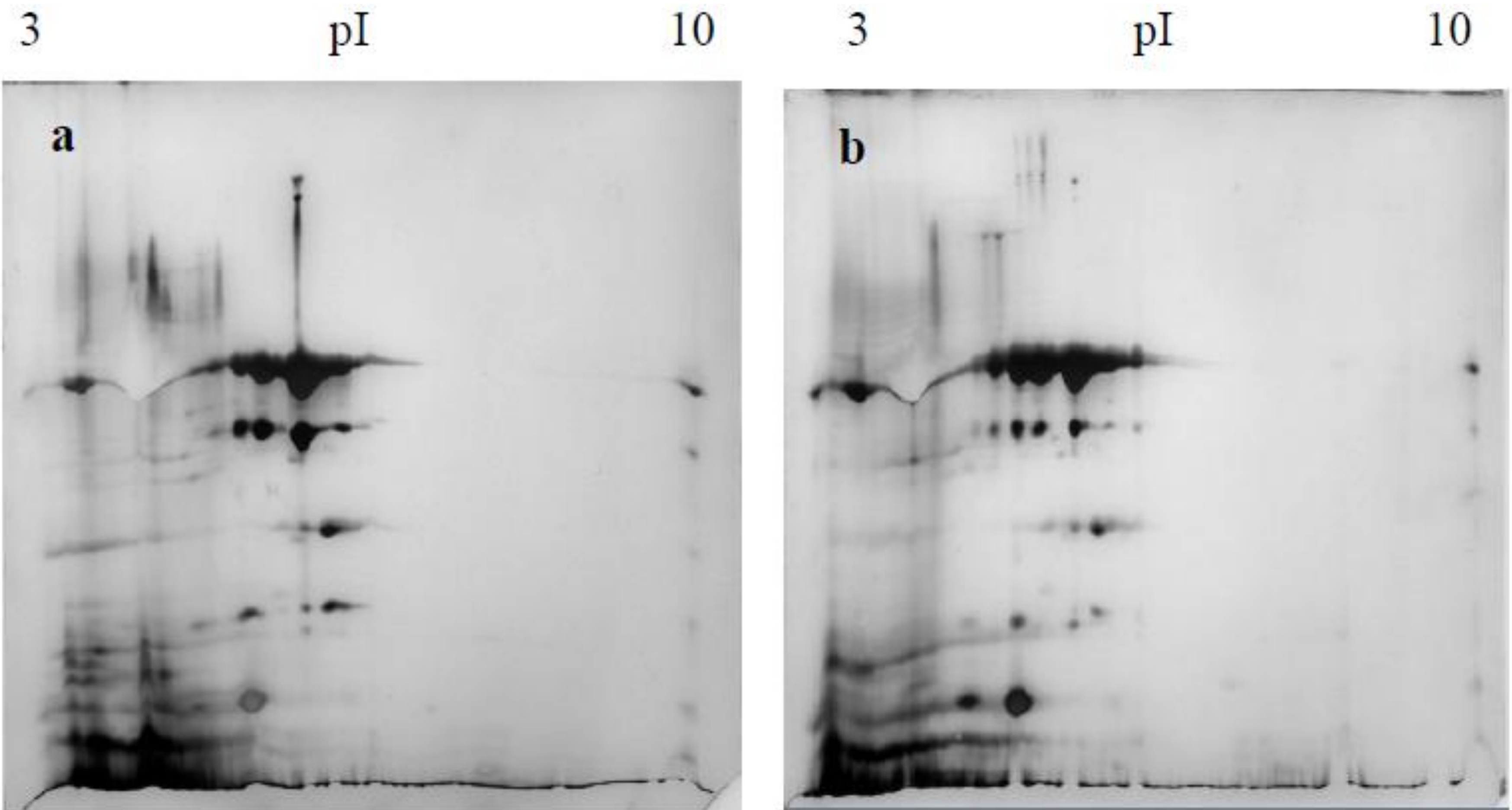


Figure 4.13. 2D-PAGE images of control (a) and 10 mM rosmarinic acid (b) treated *E. coli* O157:H7 OMPs. 100 µg protein containing gels were stained with Silver staining.

CHAPTER 5

CONCLUSION

The main goal of this study was to determine the antimicrobial mode of action of caffeic, vanillic and rosmarinic acids against *E. coli* O157:H7. Firsly, minimum inhibitory concentrations of all tested phenolic acids were found by applying a range of concentrations of each phenolic acid to the bacteria. Minimum inhibitory concentrations were found as 6.5 mM for caffeic acid, 15 mM for rosmarinic acid and 8 mM for vanillic acid. Then, alterations in the cell surfaces of *E. coli* O157:H7 in response to phenolic acid treatment were visualized by scanning electron microscopy. Irregular rod shapes were observed in phenolic acid stressed bacteria.

In the proteomic part of the study, outer membrane proteins of *E. coli* O157:H7 were isolated from phenolic acid stressed bacteria and compared with OMPs of nontreated *E. coli* O157:H7 by separating proteins via SDS-PAGE and 2D-PAGE analyses. Therefore, proteins were separated according to their isoelectric points and molecular weights. This elaborate separation revealed altered expressions of some proteins as response to phenolic acids stress.

The next step of this study is the identification of proteins whose expressions altered upon phenolic acid treatment by mass spectrometry. The further step for this research could be investigation of genes responsible for decreased or increased protein expression.

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APPENDIX A

BRADFORD PROTEIN ASSAY

For determination of protein concentration of sample, series of protein standards are needed. The standard curve for this series of protein concentrations is prepared by using a protein of known concentration. In this assay, bovine serum albumin (BSA) was used as a standard protein.

- Preparation of Coomassie Reagent: 0.1 g of Coomassie Brilliant Blue G-250 was dissolved in 50 ml absolute ethanol. 100 ml 85% phosphoric acid was added and the solution was completed to 250 ml with ultra pure water. The obtained solution was filtered through Whatman no: 1 filter paper and was stored in dark bottle.
- Preparation of BSA standards: 1 mg/ml BSA was prepared as a stock standard. 10 mg of BSA was weighed and dissolved in 10 ml of ultra pure water and aliquoted into 1.5 ml centrifuge tubes. By using the stock BSA standard, 5 μg/ml, 10 μg/ml, 25 μg/ml, 50 μg/ml, 75 μg/ml and 100 μg/ml protein concentrations were prepared by serial dilution.

Prepared protein standards and Coomassie reagent were mixed in 96-well plate in 9:1 ratio. 20 μl of each sample was mixed in 180 μl of Coomassie reagent, 20 μl of water also mixed with the 180 μl of reagent as blank. The 96-well plate containing mixture was incubated at room temperature for 10 minutes. Following incubation, absorbance of standards was measured at 595 nm by using a spectrophotometer.

The standard curve was drawn by plotting the absorbance at 595 nm versus μg of BSA standard proteins. By using the equation of $y= mx + b$ where y is the absorbance, and x is the protein concentration, the concentration of unknown protein was calculated. The standard curve for BSA was shown in Figure A.1.

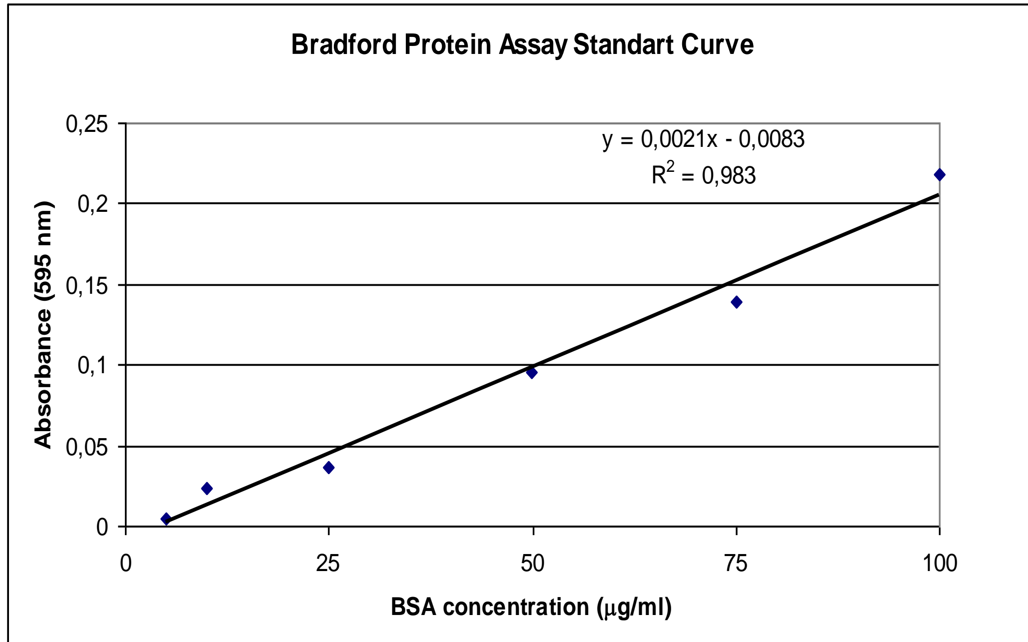

Figure A.1. Standard curve for bovine serum albumin