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ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES OF OLIVE LEAF EXTRACT AND ITS FOOD APPLICATIONS

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in Biotechnology

by
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ABSTRACT

ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES OF OLIVE LEAF EXTRACT AND ITS FOOD APPLICATIONS

In this study, aqueous/ethanol extract of olive leaves (OLE) was examined for its antimicrobial and antioxidant activities and its possible food applications. In the first part of the study, relative antioxidant capacity and total phenol content of the extract were determined and found as 966 µg ascorbic acid eq./mg and 197.42 mg GAEq/g sample, respectively.

In the second part, microdilution assays were conducted by 96 well plate for OLE to find minimum inhibition concentration (MIC) against Gram (+) and Gram (-) bacteria. The most susceptible bacteria were *Escherichia coli, Listeria innocua* and *Staphylococcus carnosus*.

In the third part OLE was applied to raw beef cubes with 1%, 2% and 3% (v/w) concentrations to examine its antimicrobial and antioxidant effects. The results clearly indicated that usage of 2% and 3% OLE had the beneficial effect in controlling the microbial load, total viable and coliform counts, of beef cubes during 9 days of storage at 4ºC. The 2% OLE treatment applied to beef cubes also delayed the oxidative deterioration compared to the other samples.

As the last part of the study, 300 ppm OLE was applied to sardine (*Sardina pilchardus*) fillets as a marinade component. Results indicated that OLE was effective in controlling microbial load of sardine fillets and also delayed the oxidative deterioration and total volatile base nitrogen formation in the fillets during marination process.

ÖZET

ZEYTİN YAPRAĞI ÖZÜTÜNÜN ANTİMİKROBİYAL VE ANTİOKSİDAN AKTİVİTELERİ VE GIDA UYGULAMALARI

Bu çalışmada, zeytin yaprağı özütü, antioksidan ve antimikrobiyal özellikleri yanısıra, muhtemel gıda uygulamaları için incelenmiştir. İlk olarak bağıl antioksidan kapasitesini ve toplam fenol içeriğini saptamak amacıyla testler uygulanmış ve sırasıyla, 966 µg askorbik asit eşd./g ve 197,42 mg GA Eşd./g örnek olarak tespit edilmiştir.

Ikinci kısımda, özütün Gram (+) ve Gram (-) bakterilere karşı minimum inhibisyon konsantrasyonlarını bulmak amacıyla 96 kuyucuk mikrodilüsyon yöntemi uygulanmış ve en duyarlı bakteriler; *Escherichia coli, Listeria innocua* ve *Staphylocuccus carnosus* olarak tespit edilmiştir.

Üçüncü kısımda zeytin yaprağı özütü, %1, %2 ve %3 lük konsantrasyonda sulu çözelti olarak antioksidan ve antimikrobiyal etkilerin belirlenmesi amacıyla kırmızı ete uygulanmıştır. Sonuçlara göre; özütün %2 ve %3'lük konsantrasyonları, 9 gün boyunca 4ºC de depolanan et örneklerindeki mikrobiyal yükü kontrol altında tutmuş ve ayrıca %2'lik konsantrasyon, diğer örneklere kıyasla oksidatif bozunmayı geciktirmiştir.

Son olarak, zeytin yaprağı özütü, 300 ppm'lik konsantrasyon halinde marinat içeriği olarak sardalyalara (*Sardina pilchardus*) uygulanmış; kırmızı ette olduğu gibi marinasyon sırasında da mikrobiyal yükü kontrol altında tutup, oksidatif bozunmayı ve toplam uçucu bazik azot oluşumunu geciktirmiştir.

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LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ACW	Antioxidant capacity of water soluble compounds
AST	Antimicrobial susceptibility testing
BHA	Butylhydroxyanisole
BHT	Butylhydroxytoluene
TBHQ	Tertiary butylhydroquinone
DMSO	Dimethyl sulfoxide
DPPH	Diphenylpicrylhydrazyl
F-C	Folin- Ciocalteu
GAE	Gallic Acid Equivalents
HAT	Hydrogen Atom Transfer
Hυ	Optical excitation
LDL	Low–Density Lipoprotein
MIC	Minimum Inhibition Concentrations
MRSA	de Man, Ragosa and Sharp Agar
OD	Optical density
PC	Psychrotrophic Bacteria Count
PCA	Plate Count Agar
PCL	Photochemiluminescence
ROS	Reactive oxygen species
SET	Single Electron Transfer
TBARS	Thiobarbituric Acid Reactive Substences
TEAC	Trolox equivalent antioxidant capacity
TMA	Trimethylamine
TVB-N	Total Volatile Base Nitrogen
TVC	Total Viable Bacteria Count
VRBA	Violer-Red Bile Agar

CHAPTER 1

INTRODUCTION

In last decade, there is an increasing interest in researches for production of biologically active compounds from natural sources. Bioactive compounds are remarkable due to prevention and/or treatment of diseases such as cardiovascular diseases and certain cancer types. These protective abilities of bioactive materials are mostly attributed to plant polyphenols and their antioxidant, antimicrobial, antiviral or anticarcinogenic effects. Furthermore, plant polyphenols are preferred as protective ingredients in pharmaceutical, food and cosmetics industries as food additives, preservatives and dietary supplements instead of synthetic chemicals.

The number of studies on use of plant polyphenols in food researches is increasing day by day. One of the most undesirable problems in food industry is deterioration of essential chemicals, such as lipids. Lipid oxidation causes formation of rancid flavors namely, off-flavors which directly affects the quality and shelf life of foodstuff. In order to eliminate this problem, synthetic antioxidants, as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary butyl hydroquinone (TBHQ), are vastly used in food industry. However, health threatening properties like carcinogenic effects of these synthetic antioxidants lead increasing affinity to natural sources of antioxidants.

In addition to chemical quality, bacterial quality of food materials also has to be taken into account. Microbiological safety and shelf life of food materials are strongly dependent. Quick bacterial spoilage of raw meats and meat products limits their shelf life even though they are stored in refrigerated conditions. Hygienic and safety problems encountered while production or storage of meat and meat products must be eliminated in corrective actions such as employing antibacterial agents.

Olive leaf is one of the potent source of plant polyphenols having antioxidant, antimicrobial, antiviral properties due to its rich phenolic content. The most abundant phenolic component of this content is oleuropein which gives the bitter taste to olive and olive oil. In order to utilize oleuropein and other bioactive components within olive

leaf effectively enough, they should be extracted from olive leaf. In this manner, solvent extraction is a highly used process in industry in order to recover plant polyphenols from their sources. Selection of solvents mostly depends on the chemical properties of polyphenols needed to be extracted, extraction yield, and the cost of extraction process and the application area of the extracts.

The main objectives of this study are; (1) to investigate the antioxidant and antimicrobial properties of olive leaf extract (OLE), (2) to monitor effects of using olive leaf extract on raw red meat quality, (3) to monitor the antioxidant and antimicrobial effects of OLE on marinated sardines.

CHAPTER 2

LITERATURE REVIEW

The chemical deterioration in food material is an important issue affecting the quality and shelf life of food products. Chain reactions encountered in these deteriorations, such as lipid oxidation limits the acceptability of food and mostly triggered by the free radicals.

2.1. Free Radicals

Oxidation metabolism is an essential process for survival of living things, drugs, and foodstuff and yet causes formation of free radicals (Antolovich at al., 2002; Pourmorad et al., 2006). Free radicals can be defined as high energy atoms with an extra unpaired electron, that's why they are defined to be highly reactive (Madhavi et al., 1996).

Although they are unwanted metabolic by-products, they are continuously released by aerobic metabolisms (Mantle et al., 2000). Free radicals can also be produced by light energy, photochemical smog, tobacco products, polyunsaturated fats (as in deep fried foods), alcohol, radiation, physical stress that leads depletion of immune system antioxidants, modification of proteins caused by gene expression changes (Pourmorad et al., 2006). Figure 2.1. summarizes the possible causes and effects of free radicals on living cells.

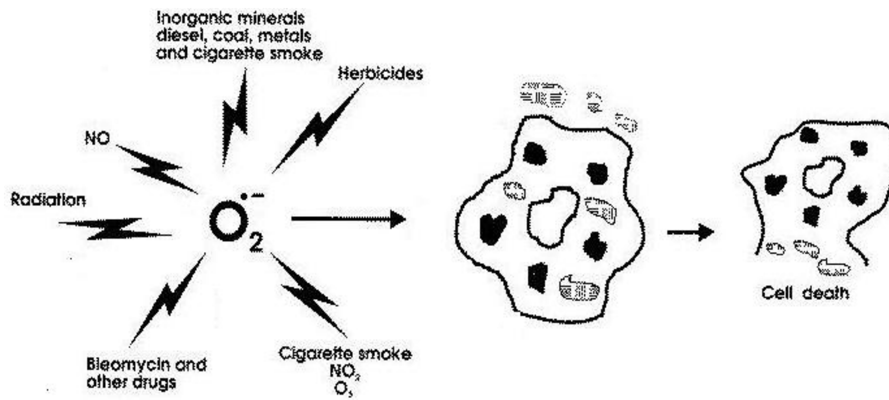


Figure 2.1. Cause and effect of free radicals
(Source: Pourmorad et al., 2006)

Free radicals, as they are unstable, have a tendency of being stabilized in a way of reducing their energy level by transferring their excess electron to nearby substances. As an example, when they are formed within body, they attack nearby tissues by oxidizing membrane lipids, cellular proteins, DNA that causes complete shutdown of cellular activities such as respiration and terminates the cell. Furthermore, the interaction of oxygen free radicals with members of lipidic portion of body leads us to formation of new radicals such as hydroperoxides, superoxide, lipoid oxides and hydroxyl radical whose type may interact with biological systems in a citotoxic manner (Benavente-Garcia et al., 2000).

2.2. Lipid Oxidation

One of the ways of lipid oxidation is non-enzymatic free-radical mediated chain reaction, which consists of four steps called; initiation, propagation, branching and termination. The initiation of process can be triggered by heat, ionizing radiation, light or chemical agents as matalloproteins and metal ions.

Initiation;

$$LH + R^{\bullet} \rightarrow L^{\bullet} + RH \quad (2.1)$$

Where; LH is the unsaturated lipid interacting with $R^{\bullet}$ as initiating oxidizing radical. This interaction forms a highly reactive allyl radical (denoted as $L^{\bullet}$) that has a tendency to react with oxygen to form lipid peroxyl radical ($LOO^{\bullet}$) as the starter of propagation step.

Propagation:

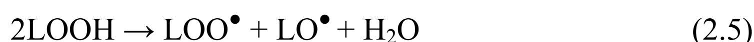
$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \quad (2.2)$$

$$LOO^{\bullet} + LH \rightarrow L^{\bullet} + LOOH \quad (2.3)$$

The peroxyl radicals are the chain carriers of the reaction that can further oxidize the lipid, producing lipid hydroperoxydes (LOOH), which then breaks into several compounds such as alcohols, ketones, alkyl formates, aldehydes and other hydrocarbons also the radicals including alkoxyl radical ($LO^{\bullet}$).

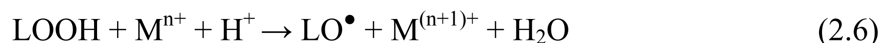
Branching:

$$LOOH \rightarrow LO^{\bullet} + HO^{\bullet} \quad (2.4)$$



$$2LOOH \rightarrow LOO^{\bullet} + LO^{\bullet} + H_2O \quad (2.5)$$

The breakdown of lipid hydroperoxides often involves transition metal ion catalysis, in reactions analogous to that with hydrogen peroxide, yielding lipid peroxyl and lipid alkoxyl radicals:

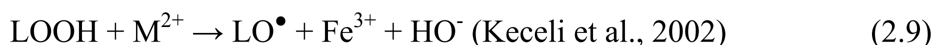


$$LOOH + M^{n+} + H^{+} \rightarrow LO^{\bullet} + M^{(n+1)+} + H_2O \quad (2.6)$$

$$LOOH + M^{(n+1)+}\ OH^{-} \rightarrow LOO^{\bullet} + M^{n+} + H_2O \quad (2.7)$$

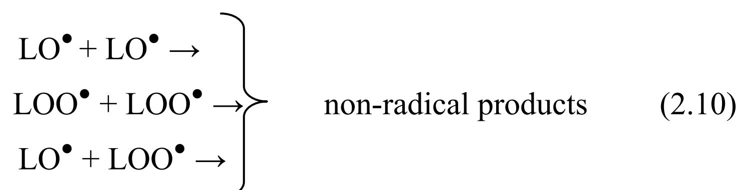
As an example of metal catalysis, the autoxidation of lipids could be attributed to the decomposition of lipid hydroperoxides by ferric and ferrous ions to give lipid peroxyl and alkoxyl radicals which initiate the free radical chain reaction, can be given:

$$LOOH + Fe^{3+} \rightarrow LOO^{\bullet} + Fe^{2+} + H^{+} \quad (2.8)$$



$$LOOH + M^{2+} \rightarrow LO^{\bullet} + Fe^{3+} + HO^{-} \text{ (Keceli et al., 2002)} \quad (2.9)$$

If the free radicals meet and form non-radical compounds, the process is called as termination (Antolovich et al., 2002):



$$\left.\begin{array}{r} LO^{\bullet} + LO^{\bullet} \rightarrow \\ LOO^{\bullet} + LOO^{\bullet} \rightarrow \\ LO^{\bullet} + LOO^{\bullet} \rightarrow \end{array}\right\} \text{non-radical products} \quad (2.10)$$

All radical groups formed during oxidation processes, are primarily responsible for certain diseases involving many organs. For example and unpredictable effect of oxidation in DNA can lead to cancers. Also following disorders are related to free radical chain reactions;

- Heart and cardiovascular diseases
- Lung diseases
- Alcohol-related diseases
- All types of cancers
- Aging
- Skin diseases
- Eye disorders
- Immune system related diseases
- Central nervous system diseases
- Radiation injury
- Kidney diseases
- Gastrointestinal diseases

The negative effects caused by free radicals can be observed not only in the body but also in food materials and drugs. Antioxidants are protective substances against these negative effects and can be found in many natural and synthetic sources (Simone, 1992).

2.3. Antioxidants

Antioxidants may be defined as substances those, when present at low concentrations compared to that of oxidizable substrates, significantly delay or inhibit oxidization of those substrates. Antioxidants, as a tradition, are divided into two groups as; primary (chain breaking) and secondary (preventing) (Antolovich et al., 2002). In a more detailed way, antioxidants can be grouped as follows;

- Inhibitors of free-radical oxidation reactions (inhibits the formation of lipid radicals)
- Inhibitors interrupting the propagation step of autoxidation
- Singlet oxygen quenchers

- Synergist antioxidants (those show no antioxidative effect when used alone but increasing the activity of primary antioxidants when used together)
- Reducing agents (transforms hydroperoxides into stable forms)
- Metal chalators (transforms metal ions into stable forms) (Pokorný, 2007)

Antioxidants can deactivate radicals by two major mechanisms, Hydrogen Atom Transfer (HAT) and Single Electron Transfer (SET). Antioxidants that work with HAT mechanism quench free radicals simply by donating hydrogen atom while those of which work with SET mechanism transfer one electron to reduce any compound such as metal ions, radicals and carbonyls as shown in equations below (Antolovich et al., 2002).

$$L^{\bullet} + AH \rightarrow LH + A^{\bullet} \tag{2.13}$$

$$LOO^{\bullet} + AH \rightarrow A^{\bullet} + LOOH \tag{2.14}$$

$$LO^{\bullet} + AH \rightarrow A^{\bullet}\ LOH \tag{2.15}$$

HAT antioxidants, when present in trace amounts, may delay or prevent the initiation step by reacting with lipid radical or directly inhibit the propagation step by reacting with peroxyl or akoxyl radicals while donating their hydrogen atom.

$$LOO^{\bullet} + A^{\bullet} \rightarrow LOOA \tag{2.16}$$

$$LO^{\bullet} + A^{\bullet} \rightarrow LOA \tag{2.17}$$

The antioxidant free radicals can interfere with chain-propagation reactions and form peroxyantioxidant groups due to their hydroxyl groups (Figure 2.2.).

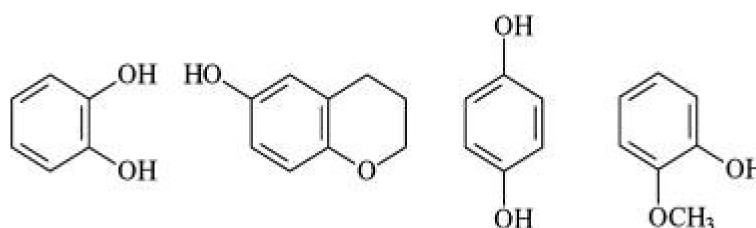


Figure 2.2. Basic chemical structure of antioxidants
(Source: Pokorny, 2007)

Antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) *tert*- butylhydroquinon (TBHQ) and propyl gallate are widely used in many foods. These compounds, for example, are added to oils and fats at low concentrations ranged from 50 to 200 ppm to prevent formation of peroxides during storage (Fki et al., 2005).

There have been some discussions about undesired effects of use of synthetic antioxidants. As an example, use of BHT in rat feed caused them to develop fatal haemorrhages in the pleural and peritoneal cavities and in organs such as epididymis testes and pancreas (Fki et al., 2005). This effect is attributed to the ability of BHT to reduce vitamin K dependent blood clotting factor (Hakkim et al., 2007). BHA, on the other hand, has become under attack due to its potential action as carcinogenesis promotion and causing lesion formation in rat forestomach (He et al., 1997; McCarthy et al., 2001a).

Although synthetic additives have been widely used in food industry, to inhibit the process of lipid oxidation, the trend is to decrease their use because of the growing concern among customers about such chemical additives (Fernandez-Lopez et al., 2005). Accordingly, there is a strong argument for the effective isolation of organic antioxidants from natural sources (McCarthy et al., 2001b), such as phytochemicals especially polyphenols and essential oils obtained from plant extracts as alternatives of synthetic antioxidants.

2.4. Plant Polyphenols

Many epidemiological studies indicate that increased consumption of fruits vegetables and grains reduce the risk of many chronic diseases. As the prevention of such diseases is more important than treatment, nutritional factors like foods and plants are gravely important for human health (Moure et al., 2000).

Polyphenols are group of organic compounds with one or more hydroxyl groups on the aromatic ring structure varying from simple molecules such as phenolic acids to highly polymerized compounds like condensed tannins (Urquiaqa et al., 2000; Lui, 2004).

Even though dietary phenolics are classified in several subgroups, major catecories of phenolic compounds are phenolic acids, flavanoids and tannins. However it is possible to name other subgroups as; lignans, coumarins, quinons and stilbenes (Pietta, 2000).

Phenolic acids:

Phenolic acids can be divided into two groups as hydroxybezoic acid and hydroxycinnamic acids both of which have single-ring structure (Manach et al., 2008).

Hydroxybenzoic acid:

Gallic acid and ellagic acid can be classified as members of this group. This acid type can be found in onions, black radish and several red fruits such as berries at very low concentrations. Teas are also available sources of gallic acid.

Hydroxycinnamic acid:

Caffeic acid, ferulic acid, p-coumaric acid and sinapic acids are examples of this group. They show heat sensitive properties. This type of acids can be obtained from kiwi, apple, blueberry, cherry and plum.

Flavanoids:

Flavanoids are the most common and most widely studied group of plant phenolics. They include several hydroxyl groups within their basic two ring carbon skeleton. Commonly they have (C_6-C_3-C_6) carbon structure consisting of two benzene rings linked by an oxygen containing heterocycle as shown in Figure 2.3 (Pietta, 1999).

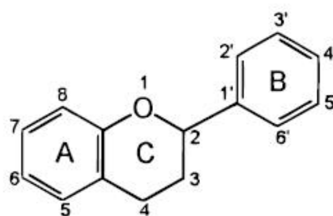


Figure 2.3. Basic flavanoid structure

(Source: Pietta, 1999)

Flavanoids can be divided into anthocyanins and anthoxanthins. Anthocyanins may have color pigments such as red, blue or purple. Anthoxanthins are rather colorless, white or yellowish (Manach et al., 2008). They can be subgrouped as flavones, flovonols, flavanols and isoflavanols as shown in Figure 2.4.

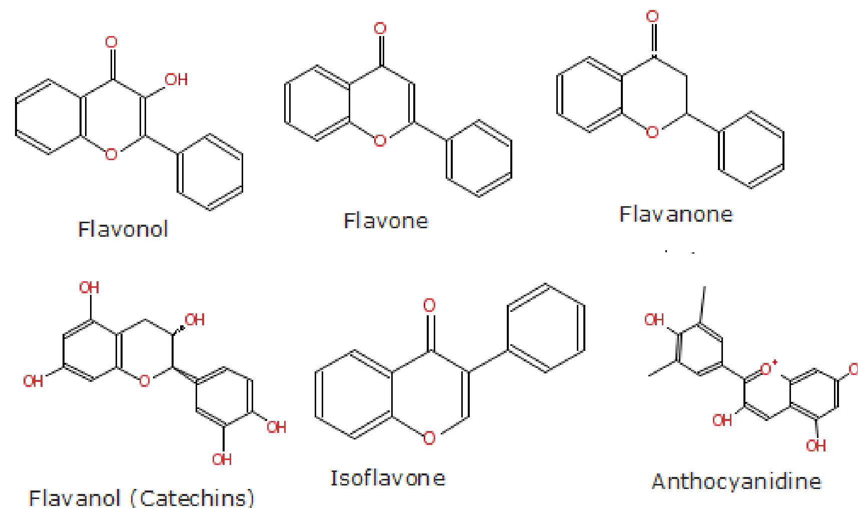


Figure 2.4. Chemical structures of flavonoids
(Source: Lakhanpal and Rai, 2007)

Flavones:

Flavones are the phenolic groups containing one carboxyl group. Flavones mainly consist of apigenin and luteolin. Olives, parsley and celery are the most important edible sources (Manach et al., 2008).

Flavonols:

Flavonols are the most common type of flavanoids. Quercetin, kaempferol and myricetin are three most widely distributed flavonols. Onion, kale, apple, red wine and tea are the most common sources of this group (King and Young, 1999; Manach et al. 2008).

Flavanols:

Flavanols can be found in both the monomer form as catechins and the polymer form as proanthociyanidins. Monomer forms can be found in apricots while polymer form can be obtained from red wine, grape seed, black tea and chocolate (Manach et al., 2008).

Isoflavones:

Isoflavones are the flavanoids showing structural similarities to estrogens although they are not steroids. This similarity causes isoflavone to bind estrogen receptors at some point (Manach et al., 2008). They are specific for legumes such as soybean. The most well-known isoflavones are genistein and daidzein (King and

Young, 1999). They are sensitive to heat and mostly hydrolyzed into glycosides during soymilk production (Manach, 2008).

Tannins:

Phenolic polymers, most commonly known as tannins, can be divided into two major classes;

Hydrolyzable Tannins:

Hydrolizable tannins contain a central core of polyhydric alcohol such as glucose and hydroxyl groups which are partially or completely esterified by gallic acid (gallotannins) or ellagic acid (ellagitannins). Acid, base or certain enxyme hydrolysis causes the break of ester bonds so that basic components are exposed again.

Condensed Tannins:

They are essentially oligomeric derivatives of flovonols (flavan-3-ols, flavan-3,4-ols or both), such as catechin and epicatechin. They are more complex than hydrolyzabl tannins. Condensed tannins can be found in fruits, vegetables, cocoa, red wine and legume family (Manach et al., 2008).

2.4.1. Extraction of Plant Polyphenols

Extraction, pharmaceutically speaking, can be defined as separation of bioactive material from plants or animal tissues by utilizing selective solvent while conducting standard extraction procedures. These bioactive constituents can then be used in pharmaceutical, food or cosmetic industry. Both extraction yield and bioactivity of extracts strongly depend on the solvent, due to different, for example, antioxidant potential of compounds with different polarity (Moure et al., 2001).

During extraction processes, the solvent is added to the sample and then removed by a proper method. This proper method either can be vacuum-drying, ultra filtration or evaporation. Method choice can be dependent on solvent cost. If the cost is high, recovery systems such as rotary evaporators can be used to remove the solvent from extract solution.

The material in which the extracts will be used is an important issue that needs to be taken into account. If the solvents will be used in extraction of food additives and biological materials they should be;

- Nontoxic

- Having high capacity
- Having high distribution coefficient
- Highly selective for the solutes
- Easily recoverable
- Stable and inert
- Environmentally safe
- Inexpensive
- Nonflammable
- Nonexplosive

Equilibrium state and mass transfer rate are two aspects that control the extraction process (Shi et al., 2005). In solvent extraction process of phenolic material from their solid hosts, there are two essential stages; dissolution of phenolic compounds in plant matrix and their diffusion to external solvent medium (Shi et al., 2005).

Initial stage:

Addition of solvent to the sample takes place. The solvent runs through the cavities and capillarities of the sample by osmotic forces and fills the plant matrix while solving phenolic compounds so that inner concentration increases by time and a concentration gradient is created. Also the polyphenols that exposed or damaged during grinding processes are washed away in this stage.

Diffusion stage:

Dissolved polyphenols diffuses from plant matrix to the solution media. Outer concentration of phenolics in external media starts to increase. This stage is similar to the initial step of delivery of a microencapsulated drug as shown in Figure 2.5.

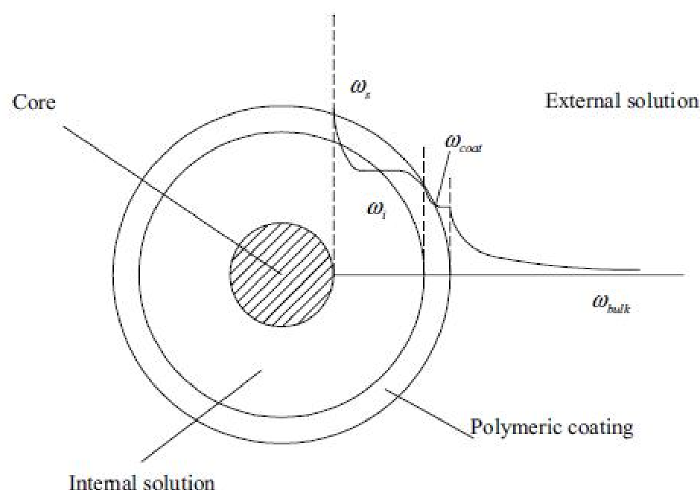


Figure 2.5. Schematic representation of dissolving phenomenon from an encapsulated material (Source: Manca and Rovaglio, 2003)

Extraction processes can be improved by selecting proper solvent type, adjusting pH, temperature, solvent to solid ratio, sample particle size, solvent viscosity. Changing these parameters to find optimum conditions can increase the efficacy of the process (Moure et al., 2001).

Solvent type:

The solvent used in extraction process should be inert against the polyphenols. It should not react with any bioactive compound of interest and should keep its stability throughout the process. Apolar solvents are the most used solvents to remove polyphenols from water (Moure et al., 2001). Ethyl alcohol and water are the most widely employed solvents due to hygienic and abundance reasons. Since ethyl alcohol is major component of alcoholic beverages, its presence in final product is also safe for consumption. Although using hot water as solvent is another health-safe method, it's not preferred for heat sensitive processes because it can cause denaturation of active compounds during the process (Moure et al., 2001; Shi et al., 2005).

Solvent viscosity:

Solvents with lower viscosities tend to increase extraction rate since they can pass through capillarity of plant cells faster than any other solvent with higher viscosities and in that way diffusion of polyphenol solution from plant matrix is altered.

pH of extraction medium:

The solubility of compounds and ions change due to pH of medium (Shi et al., 2005). That is why the pH of medium should be adjusted to suitable levels. For example

maximum solubility is reached at pH4 for polyphenols from olive rape (Moure et al., 2001).

Particle size:

Reduction in particle size either mechanical crushing or grinding and enzyme demolition boosts solvent extraction (Moure et al., 2001). Grinding shortens the path solvent has to travel to reach plant matrix also increases the surface area of particles providing more alternative routes for the solvent. Also employing pectinases and cellulases for cell breakdown enhances extraction (Shi et al., 2005; Moure et al., 2001).

Temperature:

Increasing the temperature increases the extraction rate since heat decreases viscosity of solvents while increasing solubility, diffusion coefficient of phenolic compounds being extracted and cell wall permeability by increasing the size of pores on the surface. However, increasing the temperature is limited by the nature of compounds of interest. High temperatures can cause denaturation of these compounds and all process loses its meaning that's why the optimum temperature should be lower than decomposition temperature of the phenols those need to be extracted (Shi et al., 2005; Moure et al., 2001).

Solvent-solid ratio:

If the solvent to solid ratio is high, polyphenol concentration in the bulk solution will be low so that a greater concentration gradient will be formed between inner and outer surface of plant material (Shi et al., 2005).

Although solvent extraction is widely employed, other methods such as percolation, digestion, decoction, maceration, infusion, hot continuous extraction (soxhlet), microwave assisted extraction, ultrasound extraction (sonication), supercritical fluid extraction (SFE), aqueous-alcoholic extraction by fermentation, counted current extraction (CCE), phytonic extraction (by hydro-fluoro-carbon solvents) are also used. Furthermore, for aromatic plants there are also methods such as head space trapping technique (HSTT), solid phase micro extraction (SPME), protoplast extraction technique (PET), micro distillation, thermo-micro-distillation, and molecular distillation techniques present (Ics-Unido, 2006).

2.4.2. Determination of Antioxidant Activity of Plant Polyphenols

The methods employed to determine antioxidant capacity (AOC) of plant extracts can be classified in two groups: methods utilizing HAT reaction mechanisms and SET reaction mechanisms.

Methods Utilizing HAT Reaction Mechanisms

Oxygen Radical Absorbance Capacity (ORAC) Method:

Measures the antioxidant inhibition of peroxyl radical induced oxidations so that it reflects radical chain breaking antioxidant activity by transferring hydrogen atom. In the basic assay, the peroxyl radical reacts with a fluorescent probe to form a nonfluorescent product which can be quantified by fluorescence.

Total Radical-Trapping Antioxidant Parameter (TRAP) Method:

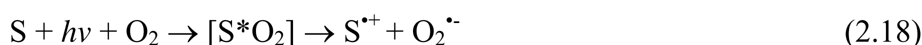
Monitors the ability of antioxidant compounds to interfere with reaction between peroxyl radicals generated by AAPH or ABAP [2.2'- azobis(2-amidinopropane) dihydrochloride] and a target probe.

Chemiluminescence (CL) Method:

The chemistry is based on the reaction of radical antioxidants with marker compounds to produce excited state species that emit chemically induced light. Any compound that reacts with the initiating radicals inhibits the light production. The most commonly used marker is luminal which is acceptable while measuring single oxidants.

Photochemiluminescence (PCL) Assays:

The assay involves the photochemical generation of superoxide ($O_2^{\bullet -}$)free radicals combined with CL detection. The assay is initiated by optical extraction of photosensitizer (S), resulting in the generation of the superoxide radical anion.



$$S + h\nu + O_2 \rightarrow [S^*O_2] \rightarrow S^{\bullet +} + O_2^{\bullet -} \qquad (2.18)$$

Low-Density Lipoprotein (LDL) Oxidation:

LDL is isolated fresh from blood samples, oxidation is initiated by Cu(II) or AAPH and peroxidation of lipid components is followed at 234nm for conjugated dienes or by peroxide values for lipid hydroperoxides (Prior et al., 2005).

Methods Utilizing SET Reaction Mechanisms

Ferric Reducing Antioxidant Power (FRAP):

The reaction measures reduction of ferric to a colored product. The reaction detects compounds with redox potentials of <0.7 V, so FRAP is a reasonable screen for the ability to maintain redox status in cells or tissues. Reducing power appears to be related to the degree of hydroxylation.

Methods Utilizing SET and HAT Reaction Mechanisms

TEAC or Other ABTS Assays:

This method is based on the scavenging ability of antioxidants to the long-life radical anion $ABTS^{\cdot +}$. In this assay, ABTS is oxidized by peroxyl radicals or other oxidants to its radical cation, $ABTS^{\cdot +}$, which is intensely colored, and antioxidant capacity is measured as the ability of test compounds to decrease the color reacting directly with the $ABTS^{\cdot +}$ radical .Results of the compounds are expressed relative to Trolox.

DPPH Assay**:**

DPPH radical is commercially available and does not have to be generated before assay like $ABTS^{\cdot +}$. This assay is based on the measurement of the reducing ability of antioxidants toward DPPH radical by measuring the decrease of its absorbance (Prior et al., 2005).

2.5. Natural Antioxidants

Numerous naturally occurring phenolic antioxidants (see Table 2.1.) that readily deal with oxidation and have potential to minimize the effects of autoxidation, have been identified in many plant sources (Weel et al., 1999).

Table 2.1. The most frequently encountered natural antioxidants in plants (Source: Pokorny, 2007)

Antioxidant class	Examples of substances
Phenolic acids	
Hydroxybenzoic acid series	Vanillic acid
Hydroxycinnamic acid series	Ferulic acid, chlorogenic acid
Flavonoids	Quercetin, catechin, rutin
Anthocyanins	Delphinidin
Tannins	Procyanidin, ellagic acid, tannic acid
Lignans	Sesamol
Stilbenes	Resveratrol
Coumarines	*ortho*-Coumarine
Essential oils	*S*-Carvone

Phenolic content of plants may be different in different growing stages, as an example, *Aloe vera* shows equal or higher antioxidant and radical scavenging activity compared to BHT and α-tocopherol, as the extracts of *Aloe vera* of different growth periods vary in phenolic content (Hu et al., 2003).

In Vitro Studies of Plant Phenolics and Essential Oils as Natural Antioxidants

The antioxidant activity of methanol extracts of holy basil (*Ocimum sanctum* L.) leaves, stems and inflorescence callus, for example, was found to have strong reducing power, effective Fe^{2+} chelating activity and high scavenging activity against superoxide anion radicals, hydroxyl radicals and hydrogen peroxide. These effects were contributed to the abundant presence of phenolics having one or more hydroxyl groups (Hakkim et al., 2007). Other methanolic extracts obtained from areca nut and *Piper betle* inflorescence contained high concentrations of antioxidant polyphenols those able to protect LDL from Ca^{2+} -catalyzed oxidation (Owen et al., 2007).

Summer savory (*Satureja hortensis* L.) is a well-known aromatic and medicinal plant, widely distributed Anatolian region of Turkey. Studies showed that antioxidant activity of extracts of this plant indicates that they have a protective effect against ROS and can therefore be used as a natural preservative ingredient in food or pharmaceutical industry (Güllüce et al., 2003). Crude extract obtained by maceration of Summer savory by acidified methanol and its ethyl acetate, n-buthanol and hexane fractions were screened to characterize their antioxidant properties by Fe(III) reduction assay and $DPPH^{\cdot}$, $ABTS^{\cdot+}$ and hydroxyl free radical scavenging assays. The crude extract and

EtOAc fraction were the most effective while other fractions were considerably weaker (Dorman and Hiltunen, 2004).

Water and ethanol extracts of basil which is a commonly used kitchen herb and an ornamental garden plant were found to be effective on ferrous in chelating, lipid oxidation, total reduction, ferrous ion chelating, hydrogen peroxide scavenging and radical scavenging. They showed significant difference compared to control but also showed lower capacities compared to BHA, BHT and α-tocopherol in all analyses except ferrous ion chelating (Gülçin et al., 2007).

Yıldırım et al., (2000) suggested that, although reducing power of a substance may indicate its potential antioxidant activity, there may not always be a linear correlation between these two properties. In their study, they compared water extracts of sage, tilia (linden flowers) and various black teas for their potential antioxidant activity and reducing power. Results indicated that, even though sage extract had the lowest antioxidant activity, it was the most effective extract in reducing power.

Jirovetz et al., (2006) reported that the antioxidant activity of 0.005% clove oil was identical to that of standard BHT at a concentration of 0.01%. It shows considerable chelating activity against Fe(III), that results in the prevention of formation of hydroxyl radicals. That means clove oil presents high antioxidant activity (Gülçin et al., 2004).

Food Application of Plant Phenolics and Essential Oils as Natural Antioxidants

In food industry, especially meat industry is in search of natural solutions for minimization of oxidative rancidity and extension of the shelf-life of meat products as alternatives of synthetic antioxidants such as BHT, BHA, propyl gallate and TBQH (Naveena et al., 2008). Therefore, there is an increase in comparative researches employing natural extracts and essential oils. For instance, water extracts of pomegranate rind powder and pomegranate juice have substantial amounts of phenolic compounds. Application of rind powder extract (10mg equivalent phenolics/100g meat) to chicken patties resulted in significant inhibition of lipid oxidation in cooked patties more sufficiently than pomegranate juice and BHT (Naveena et al., 2008). McCarthy et al., (2001a) reported that, tea catechins were the most effective ingredient among aloe vera, fenugreek, ginseng, mustard, rosemary, sage, and soy protein in comparative study with BHA/BHT and α-tocopherol in raw and cooked pork patties. In addition, tea

catechins exhibited similar or better antioxidative activity than that of BHA, BHT and TBHQ in mackerel meat system. Commonly used antioxidants such as BHT and α-tocopherol did not control the oxidative deteriorations effectively in fish meat (He and Shahidi, 1997). Antioxidant effectiveness of caffeic acid was reported to be better than that of BHA in hydrophobic systems (Medina et al., 2007). Hydroxycinnamic acids such as caffeic acid, ferulic acid and chlorogenic acid represented to be more effective than catechins when equal concentrations such as 10ppm were employed. Effect of catechins was developed with increasing concentration. This difference was attributed to the structural difference between these phenolic families (Medina et al., 2007).

Lipid oxidation and development of warmed-over flavor (WOF) of precooked meat products during their storage has an increasing interest as a matter of quality of ready-to-eat meat products (Nielsen et al., 1997). Pre-cooked meat products have relatively short shelf-life due to microbiological, oxidative and other quality concerns such as color changes. In cooked beef, supplementation of oleoresin rosemary extract (Herbalox®) Grape seed extract (ActiVin™) pine bark extract (Pycnogenol® and BHA/BHT retarded lipid oxidation, measured by TBARS, during 9 days storage time and immediately after cooking (Ahn et al., 2007). The use of rosemary extracts has been proved to be effective in control of development of rancidity and off-flavors in Swedish-style meatballs. Addition of citrus extracts such as lemon and orange also increased the acceptability of the product (Fernandez-Lopez et al., 2005).

Storage conditions of raw material affects the oxidative stability of pork patties and natural antioxidant effect of plant extracts (McCarthy et al., 2001b). The test ingredients were more effective in reducing lipid oxidation in patties manufactured from previously frozen pork rather than the ones from fresh pork. It may be contributed to free radicals those are tend to be more stable in lower temperatures (McCarthy et al., 2001b).

Phenolic compounds within plant essential oils can inhibit protein degradation and ROS during cooking or storage of muscle foods. These compounds may also retard protein oxidation by inhibiting lipid oxidation by binding to the proteins to form complexes. Essential oils from oregano, rosemary, thyme, sage, basil, turmeric, ginger, garlic, nutmeg, clove, mace, savory, and fennel have been researched to improve the sensory characteristics and shelf-life of food (Du and Li, 2007).

Employing BHT, sage and rosemary essential oils (SEO and REO respectively) retarded protein oxidation in porcine liver pâtés compared to control samples. Effect of

BHT on protein oxidation has been reported to be better than that of REO and SEO. Results in protein oxidation and lipid oxidation showed positive correlation during storage of pâtés. This correlation suggested that oxidative deterioration of proteins such as myoglobin could promote lipid oxidation by degradation of heme group and release of iron into the media (Estévez et al., 2006).

Although the use of plants and essential oils on fatty and meat products is common in industry, it can cause different results depending on meat characteristics and the concentration of additives. While deciding addition amount of such additives, systematical experiments should be carried out if the interactions between essential oils and meat is undefined (Estévez and Cava, 2005).

2.6. Antimicrobial Agents

Due to their disease treatment and microorganism elimination features, antimicrobials are very important chemicals. There are a great variety of antimicrobial agents currently available. Before choosing a particular antimicrobial agent to employ against a disease or a particular microorganism, its selective toxicity must be taken into account, doe to the fact that it is more important to eliminate the bacteria without harming the host organism.

Antibiotics are important biochemicals produced by microorganisms and widely employed in current medical use for a long time in semi-synthetic forms. Unfortunately, uncontrolled use of antibiotics, caused from either patients or prescriptions made without cell cultures analyses, increased resistance of bacteria. Increment in resistance and some other problems caused an increasing interest in antimicrobial plant extracts (Freidman, 2007).

Each and every class of antimicrobial agents represents a unique mode of action against a particular microorganism. These actions are mostly dependent on the type of microorganism, which can be related to the cell structure. As an example membrane structures of gram negative and gram positive have essential differences which totally affect their antimicrobial resistance mechanisms (Holley and Patel, 2005).

There is an increasing concern about safety and quality of foods, especially in meat products, which led numerous developments in meat preservation. Although, synthetic preservatives have usually been employed for this purpose but their use is

limited due to their side effects. That is why, in order to overcome the microbial contamination in meat, the use of bioactive phytochemicals as natural preservatives are more preferred by both customers and food industry (Ahn et al., 2004).

2.6.1. Mode of Antimicrobial Action

Antimicrobial agents use different antimicrobial activities in which they may interfere with cell wall synthesis, inhibit protein synthesis, inhibit nucleic acid synthesis or block metabolic pathways to inhibit growth of microorganisms or eliminate them.

Interference with cell wall synthesis:

Antimicrobial agents can prevent cell wall synthesis, simply by blocking the synthesis of peptidoglycan layer which covers the outer surface of the cytoplasmic membrane.

Interference with protein synthesis:

A number of antibacterial agents act by inhibiting ribosome function. Bacterial ribosomes contain two subunits, the 50S and 30S subunits, binding to these sites cause protein chain termination and inhibit protein synthesis.

Interference with cytoplasmic membrane:

This type of antimicrobial agents play role in distruption and destabilization of the cytoplasmic membrane.

Interference with DNA synthesis:

A large number of agents interfere with purine and pyrimidine synthesis or with the interconversion or utilization of nucleotides. Other agents act as nucleotide analogs that are incorporated into polynucleotides. Antimicrobial agents may also bind to the enzyme gyrase to block DNA replication.

2.7. Plant Derivatives as Antimicrobials

The most common plant derivatives those have antimicrobial activities can be grouped as follows;

Phenolic acids and simple phenols

Members of this group are the bioactive phytochemicals with the simplest molecular structures. They generally include a single phenolic ring and one or more

hydroxyl groups. These hydroxyl groups are considered to be the source of antimicrobial activity, namely the toxicity against microorganisms. Some authors contributes the toxicity level of phenolics is directly proportional to the hydroxylation level. Furthermore, highly oxidized phenols show more inhibitory effects.

Their main mechanism as antimicrobials may be related to cause enzyme inhibition through reactions between oxidized compounds and sulphydryl groups or through other interactions of proteins and oxidized compounds (Cowan, 1999).

Quinones:

Besides being source of stable free radicals, quinines are known to be phenols form irreversible complexes with nucleophilic amino acids in protein that most of the time leads protein inactivation and loss of function. That is why; quinines have a vast range of effects as antimicrobials. Possible targets of quinones in a microbial cell could be the surface-exposed adhesions, cell wall polypeptides and membrane-bound enzymes.

Flavones, flavanoids and flavonols:

They are known to be synthesized by plants as a response to microbial attacks. They have been found in vitro to be effective against a wide range of microorganisms. Their activity is probably due to their ability to form complexes with bacterial cell walls and soluble proteins. With this specialty, they are similar to quinones.

Tannins:

Tannins may be formed by condensations of flavan derivatives those have been transported to wood tissue of plants or polymerization of quinone units. Tannins can be toxic to bacteria, filamentous fungi and yeasts. As antimicrobial action, condensed tannins mostly interact with cell walls, preventing growth and protease activity within cells (Cowan, 1999).

Alkaloids

Alkaloids are heterocyclic nitrogen compounds. The most famous member of this group is morphine which was discovered in 1805 from opium poppy *Papaver somniferum*. Berberine is an important member of the alkaloid group. It is potentially effective on plasmodia and trypanosomes. The mechanism of action of highly aromatic planar quaternary alkaloids such as berberine and harmane is attributed to their ability of inclusion within DNA (Cowan, 1999; Vilinski, 2003).

2.7.1. Determination of Antimicrobial Properties of Plant Polyphenols Using Antimicrobial Susceptibility Tests (AST)

Bacterial species are not always predictably susceptible to drugs of choice. That's why susceptibility testing is important and useful. In clinical microbiology, the goal of susceptibility testing is to predict the possible outcome of treating a patient's infection with a particular antimicrobial agent (Jorgensen and Ferraro, 1998).

One of the earliest methods in AST is macrobroth or tube-dilution method, which involves preparation of two-fold dilutions of antibiotics in a liquid bacterial growth medium. Generally, eight or more dilutions are inoculated with standardized bacterial suspensions and incubated overnight at required temperature. Tubes are then examined for microscopically visible evidence of bacterial growth in a form of turbidity. The lowest concentration of antimicrobial agent that prevents visible growth presents the minimum inhibition concentration (MIC) of the agent (Jorgensen and Ferraro, 1998).

Antimicrobial susceptibility testing can be done using various methods which can be classified in three groups, including dilution, diffusion and bioautographic methods. Currently there is no standard reproducible method for determining antimicrobial activity of plant extracts and their active components, although its need is stressed (King et al., 2008).

In time this method was modified and miniaturized by using 96 well micro trays instead of tubes, which allows performing eight different two-fold dilutions up to 12 dilutions in a row. That's why the method is called microdilution test (Jorgensen and Ferraro, 1998).

Diffusion methods hold a number of advantages over quantitative methods used to determine MIC. They require less work force and smaller amounts of test agents. These methods provide information that reports whether the substance of interest shows an antimicrobial activity or not, before an MIC determining method is used (MacKenzie et al., 2008).

There has been much research employing agar-based testing of disc-diffusion method due to its simplicity. Antimicrobial susceptibility discs are absorbent, mostly paper, that allows impregnation of a specific antimicrobial agent. Method includes placing discs impregnated with an antimicrobial, on the surface of agar plate which is

inoculated with the organism to be tested then overnight incubation of the plates (Scorzoni et al., 2005). For agar well diffusion technique, instead of using paper discs, approximately 6mm diameter wells are made on the surface of the agar plate and wells are filled with approximately 10µl of antimicrobial agent and incubated overnight (King et al., 2008). In both methods, sizes of inhibition zones are determined as a measure of antimicrobial activity.

In Vitro Studies of Plant Phenolics and Essential Oils as Natural Antimicrobials

Aromatic and medicinal plants and their extract have been recognized for their antiseptic qualities since antiquity, yet the studies to characterize these properties in laboratory just date back to early 1900's (Dorman and Deans, 2000). The interest that began in first quarter of 20th century, decreased with the discovery of antibiotics in 50's, but after the increasing resistance to available antibiotics have been noticed, plant derivatives managed to re-attract attention of researchers (Cowan, 1999).

Cistus species are traditionally used for various purposes such as antiulcer, sterility treatment in women, rheumatism, antidiabetic and cancer treatment in Anatolia. Water, buthanol, chloroform, ethyl acetate and methanol extracts of these species showed activity against *S. aureus* 29213. Compounds that are soluble in polar solvents such as water and buthanol were found to be more effective than the compounds soluble in nonpolar solvents (Güvenç et al., 2005).

Seabuckthorn (SBT) which is a native Eurasia plant, domesticated in many countries at 2500-4300m altitudes. Its berries are known to be rich in vitamin C, flavanoids, oils and oil soluble compounds. Methanolic extract of SBT seeds was found to be the most effective compared to its acetone and chloroform extracts. It was found that extracts obtained from SBT were more effective on Gram-positive than Gram-negative bacteria probably due to membrane surface differences (Negi et al., 2005).

Cardoon, as known as, wild artichoke is grown in Mediterranean regions. Since the flowers of this species contain high amounts of proteases, their water extracts are used for manufacturing cheese in Iberian Peninsula. Ethyl acetate extract of leaves, rich in luteolin showed the highest activity against test bacteria including *S. aureus*, *B. subtilis* and *S. typhi*. (Kukic et al., 2008). Another Mediterranean species, also fractions of bergamot extract were tested against strains of Gram-positive and Gram-negative

bacteria. Although they were strongly active against Gram-positive bacteria, they were not active against any of Gram-negative bacteria (Mandalari et al., 2007).

Volatile oils which contain the essential aromatic and flavoring components of herbs and spices would retard the bacterial contamination even added in small amounts. Their addition would not cause loss of organoleptic properties (Dorman and Deans, 2000). Antimicrobial activity of essential oils is related to activity of their components. It is often to observe synergistic effects of essential oils (Kalemba and Kunicka, 2003). In their research, Oussalah et al., (2006) found that many essential oils have in vitro antibacterial activity against *Pseudomonas putida*. Out of twenty eight oils including, oregano, savory, Chinese cinnamon and thyme showed the strongest antimicrobial activity (Oussalah et al., 2006).

Essential oils are mostly effective against Gram-positive bacteria rather than Gram-negative. However, cinnamon and clove oil were in vitro found to be the most effective essential oils among other volatile oils against *Helicobacter pylori* which are able to colonize in human stomach and cause chronic gastritis and gastric cancers in long term infections (Kalemba and Kunicka, 2003). The ethanol extracts of local herbs grown in Greece and used as herbal medicines, showed inhibitory effect at much higher concentrations compared to claritromycin, which is used to treat *H.pylori* infections, (Stamatis et al., 2003).

The antimicrobial effects of spices and herbs from eighteen plant species those are used in global cuisines were screened on foodborne pathogen *Vibrio parahaemolyticus*. It was found that basil, clove, garlic, horseradish, marjoram, oregano, rosemary and thyme showed antibacterial effects at 30ºC. Furthermore, horseradish was also effective at 5ºC, which allows its use in refrigerated storage of foods (Yano et al., 2006).

Eugenol is the major component of clove oil. Sub-lethal concentrations of eugenol was found to inhibit the amylase and protease synthesis by *B. cereus*, also caused high frequency of cell lysis in this species (Burt, 2004).

Food Application of Plant Phenolics and Essential Oils as Natural Antimicrobials

Spoilage of foods due to bacterial infections has been a major problem for many years and still a great amount of foodstuff become unusable doe to these infections worldwide (Negi et al., 2005). Some plant extracts have been known as antimicrobials

as well as antioxidants in food systems. These naturally occurring food preservatives can extend the shelf-life of fresh and cooked meat products (Ahn et al., 2007).

Application of plant extracts or essential oils on foodstuff may not always reflect the results obtained from preliminary in vitro studies of the same compounds as foods are complex, multicomponent systems consisting of different and connected microenvironments (Tiwari et al., 2009). For example, water and oil soluble extracts of rosemary showed activity against lactic acid bacteria and *Listeria* in agar diffusion test but when applied in meatballs, they only slightly reduced the lactic acid bacteria counts (Fernandez-Lopez et al., 2005).

Garlic is one of the most commonly used ingredient and flavor enhancer for sausage. Employing garlic in formulation of chicken sausages kept the aerobic plate counts below 7 $\log_{10}$CFU/g, while BHA formulated sausages exceeded the maximum permissible limit after 21 days refrigerated storage at 3 ºC (Sallam et al., 2004).

Hao et al., (1998) studied the efficacy of a range of plant extracts to inhibit *A. hydrophila* and *L. monocytog*enes in refrigerated storage of poultry products and found that eugenol reduced the pathogen counts 4 $\log_{10}$ CFU/g over a 14 day storage.

Escherichia coli O157:H7 has a low infectious dose and can easily cause severe diseases. With this characteristic, it is a problem for food preventive strategies (Albright et al., 2003). Ahn et al., (2007) reported that, grape seed, pine bark extracts rapidly reduced the levels of *E.coli* O157:H7 in cooked beef, in first three days of its refrigerated storage. At the end of nine days refrigerated storage, it was seen that rosemary extract significantly inhibited growth of *E.coli*. On the other hand, psychrotrophic *L. monocytogenes* counts increased directly proportional to storage time (Ahn et al., 2007).

Storage temperature may enhance the antimicrobial activity of essential oils. For instance, treatment of minced meat with thyme oil caused an inhibitory effect against E.coli O157:H7 at 10ºC but did not show the same activity at 4 ºC (Solomakos et al., 2007).

2.8. Antioxidative Properties of Olive Leaf

The olive is the fruit of an evergreen olive tree that grows in the temperate climate of the Mediterranean region (Soni et al., 2006). Olive tree is one of the most

important fruit trees in these regional countries, where they cover eight million hectare, counted as 98% of the world crop (Pereira et al., 2007). Historical records indicated that the olive tree was first cultivated in ancient Crete as early as 3500 BC. It has been used as a representation of abundance, peace and glory. Its leafy branches were used to crown rulers of vast lands and champions of either friendly games or bloody wars (Soni et al., 2006).

Besides its decorative properties, as a folk remedy, olive leaf has been used for combating fevers and malaria; also some reports indicated that its extract had a capacity to lower blood pressure in animals, and increase blood flow in the coronary arteries, relieved arrhythmia and prevented intestinal muscle spasms, (Benavente-Garcia et al., 2000; Pereira et al., 2007) diarrhea, to treat respiratory and urinary tract infections (Khan et al., 2007); also olives, olive oil and olive leaf extracts are some of these foodstuffs with recognized medicinal benefits and food preservation properties dating back to the Egyptian empire (Medina et al., 2007).

There is an uprising interest in antioxidants and bioactive substances from natural sources. The protective effect of diets rich in fruits and vegetables against cardiovascular diseases (Benavente-Garcia et al., 2000; McDonald et al., 2000) and it is also supported by large body epidemiological studies that certain cancers such as breast and colon cancers were the lowest in the Mediterranean Basin where the diets are rich in olives and olive products (Al-Azzawie et al., 2006). These effects have been attributed, in part, to the presence in the Mediterranean diet of antioxidant vitamins, flavanoids and polyphenols that play an important role in disease prevention (Briante et al., 2002; Benavente-Garcia et al., 2000). In other words, flavonoids and phenolic compounds obtained from olive leaf are known to have diverse biological activities and may also be responsible for the pharmacological actions of olive leaf or, at least synergistically reinforcing those actions (Abaza et al., 2007)

Even though, there are several groups, remarkable for determination of antioxidant capacity of flavonoids, it is mainly the o-dihydroxy (cathechol) structure which bestows the antioxidant properties to the olive leaf extracts (Benavente-Garcia et al., 2000). However, this is not the only factor that determined the antioxidant capacity of a compound; the stability of formed aroxyl group is another factor that needs to be taken into account. The aroxyl radical species are famous for their extensive electron delocation ability, which is a prerequisite for radical stabilization and generating multiple mesomeric structures. The decay constant of flavonoid aroxyl radicals those

formed during interaction with other radicals show that all most stable aroxyl species contained 3’-4’-catechol B-ring substitution pattern. All other polyphenols form far less stable aroxyl radicals (Benavente-Garcia et al., 2000). The phenolic groups in olive leaf extract, their examples and relative amounts contained within OLE are presented in Table 2.2. and molecule structure of most commonly encountered ones are given in Figure 2.6.

Table 2.2. The phenolic groups in OLE, their examples and relative amounts in OLE (Source: Benavente-Garcia et al., 2000).

Group Name	Example Compound	% Amount in OLE
Oleuropeosides	Oleuropein	24.54
	Verbascoside	1.11
Flavones	Luteolin-7-glucoside	1.38
	Apigenin-7-glucoside	1.37
	Diosmetin-7-glucoside	0.54
	Luteolin	0.21
	Diosmetin	0.05
Flavonols	Rutin	0.05
Flavan-3-ols	Catechin	0.04
Substitued Phenols	Tyrosol	0.71
	Hydroxytyrosol	1.46
	Vanilin	0.05
	Vannilic acid	0.63
	Caffeic acid	0.34

Garcia et al. (2000) reported the sequence of the antioxidant capacity of the flavanoids in olive leaf extract as; rutin > catechin ≈ luteolin > OL ≈ hydroxytyrosol > diosmetin > caffeic acid > verbascoside > oleuropein > luteolin-7-glucoside ≈ vanillic acid ≈ diosmetin-7-glucoside > apigenin-7-glucoside > tyrosol > vanillin.

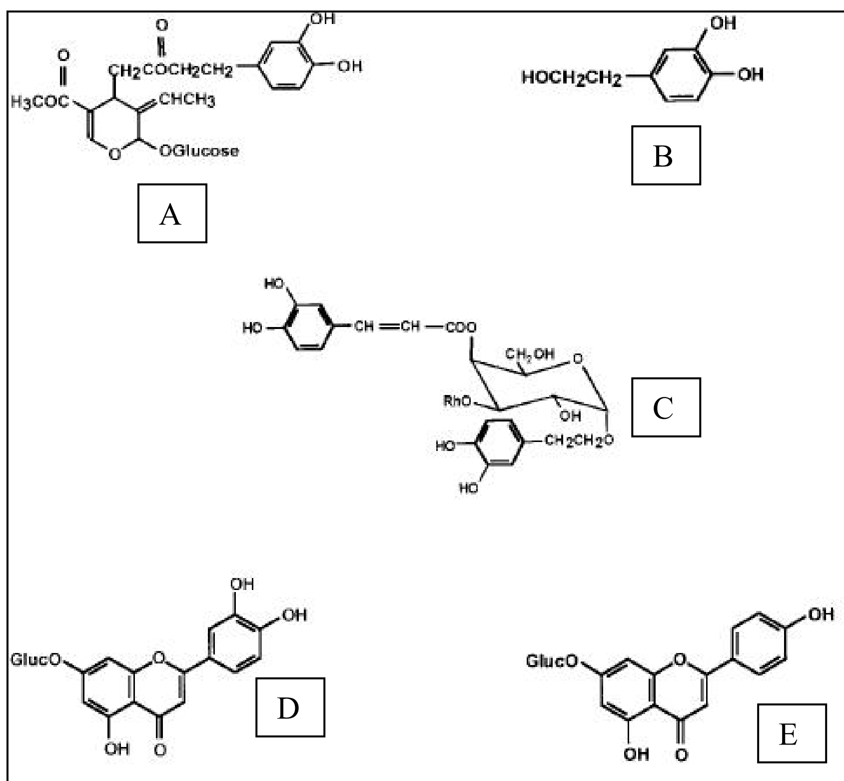


Figure 2.6. Molecular Structure of Phenolics Richly Obtained from OLE A: Oleuropein, B: Hydroxytyrosol, C: Verbascoside, D: Luteolin-7-glucoside and E: Apigenin-7-glucoside (Source: Benavente-Garcia et al., 2000).

Low cost phenolic extracts could be obtained from commercially available olive mill waste water (OMWW) to be used as alternatives to synthetic antioxidants as BHA and BHT. Furthermore, hydroxytyrosol derived from OMWW can be used to stabilize edible oils (Fki et al., 2005).

Hayes et al., (2010), reported that, OLE at a concentration of 100 and 200µg/g muscle had consistently lower levels of lipid oxidation compared to control in both aerobic and modified atmosphere pack conditions.

Caffeic acid has been identified as one of the most active antioxidants in different in vitro assays in which it has been compared to synthetic antioxidants such as BHT, BHA, α-tocopherol or trolox. Medina et al., (2007) found that supplementation of low amounts of caffeic acid in minced horse mackerel muscle showed a high inhibition of rancidity.

Pazos et al., (2007) also reported that, supplementing hydroxytyrosol or grape procyanidins via spraying and glazing significantly decreased the high susceptibility of horse mackerel fillets for lipid oxidations. However, spraying method was more effective probably due to better penetration and accordingly better absorption of

polyphenols by fillets. Hydroxytyrosol concentrations ranging from 10 to 100 ppm managed to increase the oxidative stability in bulk fish oil, oil-in-water emulsions and frozen minced fish muscle, however 50 ppm hydroxytyrosol concentration was found to be the most advantageous in delaying lipid oxidation in fish muscle (Pazos et al., 2008).

2.9. Antimicrobial Properties of Olive Leaf

In addition to its antioxidant properties, phenolic compounds within olive leaf extract have shown antimicrobial activities against several microorganisms including; E. *coli, Staphylococcus aureus, Klebsiella pneumoniae, Bacillus cereus, Salmonella typhi* and *Vibrio parahaemolyticu* (Markin et al., 2003). Furthermore, OLE affects macrophage function and modulates inflammatory response; those may contribute to activity against infectious agents (Lee-Huang et al., 2003).

Although the individual phenolic compounds in olive leaf extract may show strong in vitro activities, the antioxidant and antimicrobial activities of combined phenolics showed similar or better effects than the individual phenolics (Lee at al. 2010). It had also been previously supported by Pereira et al., (2007) that, extracts may be more beneficial than isolated constituents since a bioactive component can change its properties in the presence of other compounds present in the extract. They also reported the antimicrobial capacity order for several concentrations of OLE as follows; *B. cereus ~C. albicans > E. coli> S. aureus> C. neoformans~ K. pneumoniae~ P. aeruginosa> B. subtilis.*

Markin et al., (2003) also reported that water extract of olive leaf with a concentration of 0.6% (w/v) killed *E.coli*, *Ps. aeruginosa*, *S. aureus* and *K. pneumonia* in 3h exposure. *B. subtilis* on the other hand was inhibited only when the concentration was increased to 20% (w/v) possibly due to spore forming ability of this species.

Sudjana et al., (2009), studied antibacterial activity of olive leaf extract with large variety of bacteria. Results indicated that OLE did not present broad-spectrum antibacterial activity, but had appreciable activity on *H. pylori* and *C. jejuni*.

2.10. Oleuropein

Oleuropein is the principal active phenolic compound of olive leaf extract, as presented in Table x.x., and also that of each and every part of olive tree (*Olea auropaea* L.). Oleuropein was discovered in 1908 by Bourquelot and Vintilesco (Benavente-Garcia et al., 2000). Oleuropein is a bitter, secoiridoid glycoside that can be found in fruit, bark and leaves of olive tree (Soni et al., 2005; Markin et al., 2003; Benavente-Garcia et al., 2000). Oleuropein is an ester that consists of elenoic acid and 3,4- Dihydroxyphenylethanol, known as, hydroxytyrosol and elenolic acid, shown in Figure 2.9.

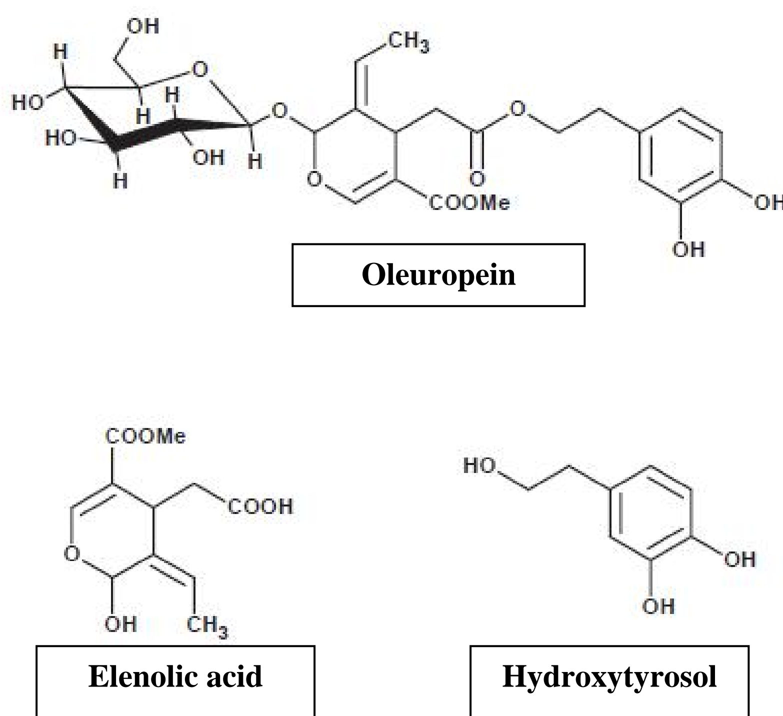


Figure 2.7. Structure of oleuropein and its components

(Source: Al-Azzawie et al., 2006)

Oleuropein has various pharmacological and health promoting properties including, antiarrythmic, spasmolytic, immıne-stimulant, cardioprotective (by inhibiting low-density lipoprotein oxidation), hypotensive and anti-inflamatory (responsible for inhibition of 5-lipoxygenase enxyme), hypoglycemic, antiviral (even against HIV), cytostatic (against McCoy cells), molluscicidali endocrinal and an enzyme modulator effects due to its antioxidative properties (Lee-Huang et al., 2003; Al-Azzawie et al.,

2006; Ranalli et al., 2006). Even earlier, Fleming et al., (1973), theorized that, green olives had an enzymatic system, activated on brining, that allows hydrolysis of oleuropein into its aglycone which is an antibacterial compound. Oleuropein can easily be transformed into glucose and oleuropein aglycon in presence of β-glucosidase enzyme (Ronalli et al., 2006).

Oleuropein prevents formation of free radicals by its ability to chelating metals such as copper and iron, which catalyze free radical generation reactions such as lipid oxidation. As a protective action oleuropein may also directly neutralize radicals by providing hydroxyl groups (Galli and Visioli, 1999).

Oleuropein and its metabolite hydroxytyrosol both have a catechol group which is required for optimum antioxidant and/or scavenging activity. Both oleuropein and hydroxytyrosol have been reported to be scavengers of superoxide anions and inhibitors of the respiratory burst of neutrophils and hypoclorus acid-derived radicals (Al-Azzawie et al., 2006). They have also been proven to inhibit or delay th growth rate of several human intestinal or respiratory track pathogens such as *Heamophilus influenza*, *Moraxella catarrhalis*, *Salmonella* Typhimurium, *Vibrio parahaemplyticus*, *Staphylococcus aureus*, *Vibrio cholera* and *Vibrio alginolyticus* mainly due to their protective action provided by their phenolic structures (Pereira et al., 2007).

CHAPTER 3

EXPERIMENTAL

3.1. Materials

3.1.1. Chemicals

For the assays, Dimethyl sulfoxide (DMSO) (99.5%) was obtained from Amersco and gallic acid was purchased from Merck. Food grade ethanol and Folin-ciocalteu reagent were obtained from Sigma, sodium carbonate anhydrus (99.5%) was obtained from Fluka and for the photochem analysis ACW and ACL kits were purchased from Analytik Jena AG. The bacterial strains used in this study, *Listeria innocua* (NRRL B-33314), *Escherichia coli* (NRRL B-3008) and Staphylococcus *carnosus* (NRRL B-14760) were supplied from the United States Department of Agriculture, Microbial Genomics and Bioprocessing Research Unit, Peoria, Illinois. In addition to these strains, *Escherichia coli* O157:H7 (ATCC 700728, Dr. Ali Aydın, Department of Food Hygiene and Technology, Faculty of Veterinary, İstanbul University, Turkey), *Salmonella* Typhimurium (CCM 5445), Dr. A. Handan Baysal, Deparment of Food Engineering, İzmir Institute of Technology, Turkey, and *Staphylococcus aureus* (RSKK 95047), Dr. Gülsün Evrendilek, Department of Food Engineering, Abant Izzet Baysal University, Turkey) were used in this study. The frozen stock cultures were maintained in nutrient broth containing 15% glycerol at –80°C prior to analyses.

3.2. Methods

3.2.1. Preparation of Olive Leaf Extract

Olive leaves used in this study were collected from Zeytincilik Araştırma Enstitüsü in İzmir/Turkey. They were collected in winter (January) and properly prepared for drying process in the day they were collected. Leaves were washed to remove impurities such as dust and then dried in an air oven for 3 days at 38^0C. A standardized solvent extraction protocol was used for the plant material. The air dried plant materials were ground in a blender with a particular size to ensure the plant powders in identical size. 10 g of each plant powder was extracted for 2 hrs with 200 ml of 70% (v/v) aqueous ethanol at 38 ºC by a thermo-shaker which is fixed to 180 rpm. Then the samples were centrifuged at 5000 rpmfor 15 minutes and the supernated parts of the samples were carried to a rotary evaporator to remove ethanol under reduced pressure at 38 ºC, 120 rpm. The remaining aqueous solutions were lyophilized at -50 ºC, 0.028 mbar and the percent (w/w) extraction yields of plant materials were calculated. The crude extracts were kept in refrigerator in glass bottles until the further experiments.

3.2.2. Analyses of Olive Leaf Extract

3.2.2.1. Determination of Phenolic Compounds in Olive Leaf Extract

The HPLC analysis was used for the determination of phenolic compounds and especially for the quantification of oleuropein. The HPLC equipment used was a Hewlett-Packard Series HP 1100 equipped with a diode array detector. The stationary phase was a C18 LiChrospher 100 analytical column (250 mm×4 mm i.d.) with a particle size of 5 mm thermostated at 30 ºC. The flow rate was 1 mL/min and the absorbance changes were monitored at 280 nm. The mobile phases for chromatographic analysis were: (A) acetic acid/water (2.5:97.5) and (B) acetonitrile. A linear gradient was run from 95% (A) and 5% (B) to 75% (A) and 25% (B) during 20 min; it changed to 50% (A) and (B) in 20 min (40 min, total time); in 10 min it changed to20% (A) and

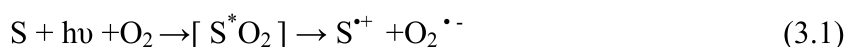
80% (B) (50 min, total time), after reequilibration in 10 min (60 min, total time) to initial composition. Oleuropein in OLE was identified by comparing its retention times with the corresponding standards. Coumarin was used as an internal standard for the quantification of oleuropein and rutin. Other standards were used only for identification of these compounds in OLE.

3.2.2.2. Determination of Antioxidant Activity of Olive Leaf Extract

PCL (Photochemiluminescence) assay, determined by Popov et al. (1999), was commercialized by Analytic Jena AG (Jena Germany), and is sold as a complete system under the name PHOTOCHEM. PCL method was used to determine the relative antioxidant activities of plant extracts. Antioxidant capacity of crude OLE was detected by ACW method for which it used to detect antioxidant capacity of water soluble compounds. Besides it's also possible to detect antioxidant activity of lipid soluble compounds within the same Photochem by a different method called ACL.

3.2.2.2.1. Mechanism of PCL

In the PCL assay the photochemical generation of free radicals is combined with a sensitive detection method using chemiluminescence (Vertuani et al., 2004). The reaction is induced by optical excitation (hυ) of the photosensitizer S which results in the generation of the superoxide radical $O^{\bullet}_2{}^{-}$ which is one of the most dangerous ROS, also occurs in the human body (Prior et al. 2005).



$$S + h\upsilon + O_2 \rightarrow [\,S^{*}O_2\,] \rightarrow S^{\bullet +} + O_2^{\bullet -} \quad (3.1)$$

There are two basic kinds of radicals present in the PCL measuring system; $O_2^{\bullet -}$ and luminol radicals (Prior et al. 2005). Luminol acts as photosensitiser as well as oxygen radical detection reagent (Vertuani et al., 2004). The antioxidants can be quantified from the changes of the measurement signal caused by them. The antioxidant capacity thus determined is referenced to equivalent concentration units of the standards used (e.g., ascorbic acid, and trolox as a tocopherol equivalent) (Margrit et al.,2006).

Standard kits provided by the manufacturer are used to measure hydrophilic and lipophilic antioxidant activity (ACW and ACL kits respectively). Calibrations and measurements for ACW (antioxidant capacity of water soluble compounds) are based on the difference in the lag time between sample and the blank. Measurements for the ACL (antioxidant capacity of lipid soluble compounds) are based on the inhibition of the area under curve (AUC) of the blank by sample (Harrison et al., 2007).

Measuring Kits Principles

The ACW and ACL kits provided by the manufacturer are used to measure hydrophilic and lipophilic antioxidant capacity, respectively, of biological samples (Prior et al., 2005). Calibration and measurements for ACW are based on the difference in lag time between sample and blank. The ACL calibration and measurements were based on inhibition (Harrison et al., 2007). Ascorbic acid and trolox are typically used as calibration reagents for hydrophilic and lipophilic antioxidant capacity, respectively (Prior et al., 2005).

The hydrophilic antioxidant capacity is assayed by means of lag phase (L) in seconds.

$$L = Lo - L_1 \qquad (3.2)$$

Where Lo and L_1 are the respective parameters of the blank and sample.

The lipophilic antioxidant capacity is assayed by the degree of PCL inhibition (I), according to the calculation.

$$I = 1 - S/So \qquad (3.3)$$

Where So is the integral under the blank curve and S is the integral under the sample curve.

3.2.2.2.2. Sample Preparation for PCL

In water soluble fraction antioxidants such as flavonoids, ascorbic acid and aminoacids are detected while in the lipid soluble fraction tacopherols, tocotrienols, carotenoids, etc. These antioxidant capacities of fractions depend on their polarities and

together give the integral antioxidant capacity as the sum of the separated values for ACW and ACL (Vertuani et al., 2002).

3.2.2.2.2.1. Sample Preparation for ACW

Crude plant extracts were firstly dissolved in DMSO as the presolution of 1g extract in 20 mL DMSO. Samples were diluted with deionized water for ACW measurements to keep the concentrations in the calibration range. In the study, measurements were conducted with the standard ACW kit (Analytik Jena): 1.5 mL of reagent 1 (solvent), 1 ml reagent 2 (water buffer solution pH: 10.5), 25 µL reagent 3 (photosensitizer-luminol) and 1-4 nmol standard (ascorbic acid) solutions.

3.2.2.3. Determination of Total Phenol Content

Total phenol content of OLE was determined by using Folin- ciocalteu method with a modification of Lako (Lako et al., 2007). Crude extract were dissolved in DMSO in a ratio of 1 g extract in 20 mL DMSO. 500 µL OLE or standard (gallic acid) solutions were mixed with 2.5 mL Folin- ciocalteu reagent (1:10 dilution with deionized water) and left to stand 2.5 min at room temperature and then 2 mL of sodium carbonate solution (7.5 % in deionized water) was added. After incubating 1 hr at room temperature in a dark place, the absorbances were measured at 725 nm by UV spectrophotometer (Perkin Elmer). Results were expressed as milligrams of gallic acid equivalents (GAE) per fresh weight.

3.2.2.3.1. Folin- Ciocalteu Method

The Folin-Ciocalteu assay has been used as a measure of total phenolics in natural products, for many years, and the mechanism is an oxidation – reduction reaction. This method was developed in 1927 originated chemical reagents used for tyrosine analysis in which oxidation of phenols by a molybdotungstate reagent yields a colored product with λ_{max} at 745 – 750 nm. The method is simple, sensitive, and precise. However, the reaction is slow at acid pH, and it lacks specificity. Results are expressed

as the standard based equivalents such as, gallic acid, catechin, tannic acid, caffeic acid equivalents. On the contrary, this method has some disadvantages for instance, suffers from a number of interfering substances particularly sugars, aromatic amines, sulfur dioxide, ascorbic acid, and some other organic acids and also Fe (II) that react with F-C reagent (Prior et al. 2007).

3.2.2.4. Determination of the Relative Antimicrobial Activities of Olive Leaf Extracts

3.2.2.4.1. Determination of the Microbial Load in Assays

It is essential to ensure that the same microbial load is used each time for antimicrobial susceptibility tests (AST) conducted in order to obtain reliable results and comparisons. Incubation periods before the inoculation of the microorganisms depend on each and every strain, but it is advised to inoculate them when microorganisms are in their logarithmic phases. In this study, adjustment of the microbial load, the incubation and inoculation procedures were kept the same and microbial loads were confirmed each time by fixing the McFarland values of cultures inoculated into 96 well plates that correspond to a certain numbers of bacteria (CFU/ml) that were determined by colony counting method.

3.2.2.4.2. Minimum Inhibition Concentrations (MIC) of Olive Leaf Extract by Micro-dilution Assay

Minimum inhibition concentration (MIC) is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism. Minimum inhibition concentrations are important to monitor the activity of new antimicrobials (Andrews, 2001).

In order to determine the minimum inhibition concentrations (MIC) of the OLE, *E. coli*, *E. coli* H157:O7, *Staphylococcus aureus*, *Staphulococcus carnosus*, *Salmonella* Typhimurium and *Listeria innocua* were used. For the study, a serial 2-fold micro-broth dilution method (Kuete et al., 2007) was performed to determine the MICs of plant

extracts. For the MIC of any tested extract, a standard protocol was followed for each test. First, the test samples were prepared by dissolving crude extract in sterile deionized water as a stock solution with a concentration of 1000 mg/mL in daily basis for each test. Two fold serial dilutions of each extract were carried out, with a final concentration of 50 mg/mL by using sterile deionized water. Then 100 µL of each extract concentration and 95 µL nutrient broth were added in each well of 96 well microplate and each well inoculated with 5 µL of 6 hr incubated bacterial suspensions after standardization by adjusting their optical densities at 420 nm by UV spectrophotometer (Perkin Elmer) to obtain 0.8- 1.2 absorbance that corresponds to approximately 10^7 CFU/mL. Negative and positive controls were also carried out for each strain. Negative controls were performed by serial dilutions of OLE in sterile distilled water (50%) and the other negative control well consisted of 195 µL of NB (Nutrient broth) and 5 µL of the standard inoculum. Positive control wells consisted of serial dilutions of penicillin (400 IU) antibiotic. The assay plates were incubated at appropriate temperature required by each bacterium for 24 hr and the growth kinetic assays for each strain were performed by duplicate growth curves and observed as turbidity determined by a microplate reader (Varioskan) at 620 nm. MIC results for extracts were reported as mg/mL. These spectrophotometric measurements of MICs were carried out with a standardized protocol of Varioskan multiplate reader (see Table 3.1). After the 24 hr incubation, INT was added into each test well in order to ensure visible indication of MICs. INT reacts with the metabolites produced by the microorganisms and the wells with the microorganism turn to pink color.

Table 3.1. Parameters of Varioskan

Shaking Parameters	
On time	23:59:59.0
Off time	00:00:00.0
Speed (spm)	120
Diameter (mm)	3
Shaking mode	Background
Kineticloop parameters	
Reading number	48
Interval (min)	30
Photometric Assay Parameters	
Wave length	620 nm
Measurement time	100 ms

3.2.3. Food Applications of Olive Leaf Extract

3.2.3.1. Application of Olive Leaf Extract on Beef Cubes

3.2.3.1.1. Preparation of Meat Samples

The vacuum packed beef loaf was obtained from Pınar Meat Co. (İzmir/Turkey). The outer surface of meat was removed to avoid possible contamination before cutting into approximately 1.5x1.5 cubes. The beef cubes were separated into four batches as control and treated samples. Control samples were immersed into distilled water and treated samples were immersed into solutions containing 1%, 2% and 3% OLE (w/v) in 1:1 ratio (beef:distilled water w/v) for 20 hr at 4 ºC. After treatment, samples were drained and divided into portions. The portions were then placed into Ziploc® bags and stored at 4 ºC for 9 days.

3.2.3.1.2. Determination of Moisture Content of Beef Cubes

Moisture content of control and treated samples was analyzed in triplicate by AOAC method at days 1, 3, 6, and 9 (AOAC 1999). Petri dishes were constant weighed after being kept at 105ºC for 1 hour then cooled in desiccators for 30 minutes and initial weight of each dish was recorded. Beef samples approximately 2 g were placed into dishes; dishes were reweighed and kept at 105ºC for 3-4 hrs. Petri dishes then final weighed and moisture contents of samples were calculated as moisture percent/g meat based on difference between initial and final weights of dishes.

3.2.3.1.3. pH Determination of Beef Cubes

Beef cubes were weighed approximately 2-2.5 g and mixed with distilled water (1:10 w/v) and homogenized at 12000 rpm for 2 min. The pH of samples was measured in duplicate by a pH meter (Hanna Instruments, Portugal).

3.2.3.1.4. Antimicrobial Activity of Olive Leaf Extract on Beef Cubes

Samples weighing approximately 10 g were aseptically mixed with 90 mL of 0.1% sterile peptone water and homogenized with stomacher (Bagmixer® 400, Interscience, France) for 60 seconds at room temperature. Mixtures were serially diluted (1:10) in 0.1% peptone water. Sample dilutions were plated and incubated to determine the microbial counts of total viable bacteria on plate count agar (PCA) at 30 ºC/48 hrs and coliforms on violet red bile agar at 37 ºC/24 hrs (VRBA). Lactic acid bacteria were enumerated using DeMan, Rogosa and Sharp agar (MRS) and Petri plates were incubated at 37 ºC/48 hrs in a CO_2 incubator (5% CO_2 and 50% humidity). Experiments were performed in triplicate. Microbial counts were expressed as $\log_{10}$ CFU/g of sample.

3.2.3.1.5. Determination of Oxidative Stability of Beef Cubes

For the determination of oxidative stability of refrigerated stored beef cubes at days 0, 1, 3, 5, 7 and 9 thiobarbituric acid-reactive substances (TBARS) assay was performed with a slightly modified method of Bekhit et al., (2003). For analysis, 2.5 g sample was placed into a beaker containing 25 mL of 0.38 % TBA and 15 % TCA prepared in 0.25 N HCl solution. The sample was homogenized at 10000 rpm for 3 min and three 5 mL aliquots obtained from homogenate was heated for for 10 min in a boiling water bath to develop a pink color and then cooled in tap water. The boiled samples were then clarified by centrifugation at 5000 rpm for 10 min and their absorbance was measured at 532 nm by using a Shimadzu (Model 2450, Japan) spectrophotometer. Average of three absorbance values was used to determine the oxidative stability of stored samples.

3.2.3.1.6. Color Analysis of Beef Cubes

Surface color attributes of control and treated beef cubes were determined by color machine vision system (ECS, Inc., USA) at days 0, 1, 3, 6, and 9. Three samples from each treatment were placed in a light box and a 24-bit image was taken with a charged couple device (CCD) video camera, located inside the light box. The image was analyzed to generate a discrete spectrum of the colors present in the sample, and the average L*, a*, b* values of all the pixels representing the fresh beef sample. The L* value was reported as lightness value, whereas a*/b* value was reported as redness index.The average L*, a*, b* values of all the pixels representing the sample were calculated. Chroma $[C^* = (a^{*2}+b^{*2})^{0.5}]$ and hue angle $[h^* = \arctan (b^*/a^*)]$ were also calculated (Little, 1975).

3.2.3.2. Application of OLE on Sardine Fillets

3.2.3.2.1. Preparation of Sardine Fillets

Fresh fish were obtained from a local fisherman in Dalyanköy-Çeşme/İzmir immediately after returning to port. Fish were cleaned with tap water and placed into 10% (w/v) salty water (1:1, fish:water) for 1 hr. Fish were then divided into six portions, three of which formed the control group and the other three were the experimental group. Both groups were placed into marinade solutions consisting 10% NaCl, 2% Acetic acid and 0.5% Citric acid in common while experimental group also had 300 ppm OLE. Fish fillets were kept in marinade solutions in a ratio of 1.5:1 (fish:marinade) for 22 days till muscle structure was completely modified.

3.2.3.2.2. Determination of Moisture Content

Moisture analyses of fish samples were carried out as follows; Petri dishes were constant weighed after being kept at 105ºC for 1 hour then cooled in desiccators for 30 minutes and initial weight of each Petri dish was recorded. Fish samples (5g) were placed into dishes and kept at 105 ºC for 3-4 hrs. Petri dishes then reweighed and moisture contents of samples were calculated as % moisture/g meat based on difference between initial and final weights of dishes.

3.2.3.2.3. Crude Protein Analysis

Protein contents of fish samples were determined according to (AOAC, 981.10, 1984). Samples with 1 g portions were placed into Kjeldahl tubes. After, 20 mL of H_2SO_4 (96%), 10 mL of 35% (w/v) H_2O_2, 1 tablet of Hg/Se catalyst and a few drops of silicone antifoam agent were added, samples were then wet-burned at 250 ºC for 15 min. and at 410 ºC for 50 min. Following that stage, samples were then placed into Kjeldahl automatic distillation unit and treated with 90 mL of distilled water, 80 mL of 40% (w/v) NaOH and 75 mL of H_3BO_3. After distillation, distillates were titrated with

0.1N HCl solution. The crude protein content was measured in duplicate at days 0, 1, and 22.

3.2.3.2.4. Crude Fat Content Analysis

Sample (20g) was homogenized in 100 mL of methanol: chloroform (1:2) mixture by using an ultratorrax homogenizer and filtered into volumetric flasks containing 20 mL of 4% (w/v) $CaCl_2$. Lids were closed and flasks were kept in dark room for 24 hrs. Mixture was then transferred into a separatory funnel and lower phase was transferred into constant-weighed containers to evaporate solvents. After evaporation, containers were reweighed and crude fat content was calculated as g crude fat/g fish meat. The crude fat content was measured in duplicate at days 0, 1, and 22.

3.2.3.2.5. Ash Analysis

Ash analyses of fish samples were carried out according to (A.O.A.C., 935.47, 1984) method. Ceramic pots were heated at 550 ºC for 1 hr, then cooled to room temperature in desiccators and tared. Samples (2 g) were placed into pots and heated in an ash oven for 3-4 hrs till colors of the samples turned into grey. Pots were then cooled and reweighed. Ash content of samples was calculated as ash percent/g fish meat. The ash content was measured in duplicate at days 0, 1, and 22.

3.2.3.2.6. Acidity in Marinade

For determination of the acidity of marinade; 2 g of marinade sample was placed into an erlenmeyer flask and diluted with 100 mL of distilled water. Flask was closed with a watch glass and the content was heated till boiling starts. After cooling, approximately for 10 min a few drops of phenolphthalein were added sample was titrated with 0.1N NaOH. The acidity in marinate was measured in duplicate at days 0, 1, 4, 7, 14, and 22.

For acetic acid;

$$Acidity\ (\%) = \frac{0.1\text{N NaOH Consumption} \times 0.6}{2} \tag{3.4}$$

For citric acid;

$$Acidity\ (\%) = \frac{0.1\text{N NaOH Consumption} \times 0.7}{2} \tag{3.5}$$

3.2.3.2.7. Salt in Marinade

To determine saltiness of marinade; 5g of marinade sample was placed into a volumetric flask and diluted with a ratio of 1:20, marinade and distilled water, respectively and filtrated. 10 mL (F=25) of dilution was taken and 5 drops of phenolphthalein were added and titrated with 0.1N NaOH. Then, into that titrated solution, 5 drops of K_2CrO_4 (5% aqueous solution) added and mixtue was neutralized with 0.1N $AgNO_3$.

$$Salt\ (\%) = \frac{AgNO_3\ \text{Consumption} \times 0.585 \times F}{\text{Mass of Sample}} \tag{3.6}$$

If salt percentage is predictable, amount of filtrate taken can be adjusted as follows; 0-3% salt content, 50 mL (F=5), 3-10% salt content, 25 mL (F=10), 10-20% salt content 10 mL, (F=25). The salt in marinate was measured in duplicate at days 0, 1, 4, 7, 14, and 22.

3.2.3.2.8. Acidity and Saltiness in Fish Fillets

Fish sample (20g) was homogenized with 100 mL of heated water for 2 min. Mixture was then diluted to 250 mL with distilled water and filtrated. The filtrate (20

mL)was taken and titrated with 0.1N NaOH in the presence of phenolphthalein. After the filtrate was neutralized with NaOH, a few drops of K_2CrO_4 (10% w/v) was added to the solution as an indicator and solution was titrated with $AgNO_3$. NaOH and $AgNO_3$ consumptions were recorded and used for further calculations. The acidity and saltiness was measured in duplicate at days 0, 1, 4, 7, 14, and 22.

3.2.3.2.9. Trimethylamine (TMA-N) Determination

Minced sample (50g) was homogenized with 100 mL of 7.5% TCA (w/v) solution for 2 min and centrifuged at 3000 rpm for 20 min. Liquid portion (4 mL) was then filtrated and placed into test tubes containing 1 mL of 20% (v/v) formaldehyde, 10 mL anhydrous toluene and 3 mL 50% (w/v) K_2CO_3 solution and shaken vigorously and left steady for a few minutes to obtain a phase separation. Toluene phase (8 mL) from each tube were transferred into a new set of tubes containing 0.1g Na_2SO_4 anhydrous and tubes were shaken again to remove remaining water molecules from solution. After precipitation, 5 mL of liquid phase were transferred into a third set of tubes, and 5 mL of 0.02% (v/v) picric acid solution. After 5 minutes of reaction, spectrophotometric readings of solutions were taken at 410 nm. The TMA-N was measured in triplicate at days 0, 1, 4, 7, 14, and 22.

3.2.3.2.10. Total Volatile Base Nitrogen (TVB-N) Determination of Fish Fillets

Fish flesh (100g) was homogenized with 300 mL 7.5% TCA solution for 2 minutes and filtrated through a Whatman no3 filter paper. Distillation was carried out by a Kjeldahl-type distillator. Filtrate (30 mL) was taken into Kjeldahl tubes and diluted with 150 mL distilled water. Then, 10 mL of 20% (w/v) NaOH, a few drops of silicone antifoam agent and boiling stones were added into tube as well. Five mL of 2% (w/v) boric acid solution and 0.04 mL of methylred and bromcresol green were added into the Erlenmeyer flask at the outlet of distillation bridge and tube content was distilled with 100% steam flow. Distillate was then neutralized with 0.05N HCl and TVB-N amounts

were calculated based on consumption of HCl. The TVB-N was measured in triplicate at days 0, 1, 4, 7, 14, and 22.

3.2.3.2.11. Determination of Oxidative Stability of Fish Fillets

TBA analyses were carried out according to the method proposed by Talgadris et al. (1960). Ten g of fish sample was homogenized with 97.5 mL of distilled water and 2.5 mL of 4N HCl solution for 2 minutes. Small amount of antifoaming agent and a few boiling stones were placed into homogenate and blend was distilled until 50 mL of distillate was obtained. Five mL of distillate and 0.02M TBA in 90% acetic acid were mixed in a test tube. The tubes were then placed in a boiling water bath for 35 min. After cooling under running tap water, the absorbances were measured at 538 nm.

3.2.3.2.12. Antimicrobial Activity of Olive Leaf Extract on Fish Fillets

Approximately 10 g of sardine sample was mixed with 90 mL 0.1% sterile peptone water in a stomacher for 1 min at room temperature. Decimal dilutions were performed for plating. In addition to total viable and lactic acid bacteria counts, for fish samples, psychrotrophic bacteria and yeast and mold counts were also monitored during storage. For psychrotrophic bacteria count, sample dilutions were pour plated in plate count agar (PCA) and incubated at 7ºC for 10 days; for yeast and mold counts, potato dextrose agar (PDA) was acidified to a pH value of 3.5 by tartaric acid 0.1 mL of sample dilutions were spread on PDA and incubated at 30 ºC for 5 days.

3.2.3.2.13. Color Analysis of Fish Fillets

Surface color attributes of sardine fillets were determined by CR 400 chromometer (Konica Minolta, Japan) at days 0, 1, 4, 7, 14, and 22. The instrument was using illuminant D65 and it was calibrated using a standard white tile. The colors of two samples from each treatment were measured. CIE L* (lightness), a* (redness/greenness) and b* (yellowness/blueness) of the fish fillets were measured. Total of five readings were averaged for each sample.

3.2.4. Data Analysis

The data was analyzed using one-way ANOVA and means were compared using Duncan multiple range test.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Olive Leaf Extract Analyses

In order to obtain OLE, powdered olive leaves were mixed with 70% aqueous ethanol solution in 1:20 solid/liquid ratio at 38ºC for 2 hrs. The obtained crude extract was then ready to be used in further analyses.

4.1.1. Phenolic Compounds in Olive Leaf Extract

Oleuropein is the major phenolic compound that contributes to total antioxidant capacity of OLE. Efficiency of the extraction process depends on the amount of oleuropein obtained from the process, since it increases the bioactivity of olive leaf extract. The qualitative and quantitative determination of oleuropein can be achieved by performing HPLC analysis. HPLC chromatogram of crude OLE is given in Figure 4.1. Major compounds and their retention times are given in Table 4.1.

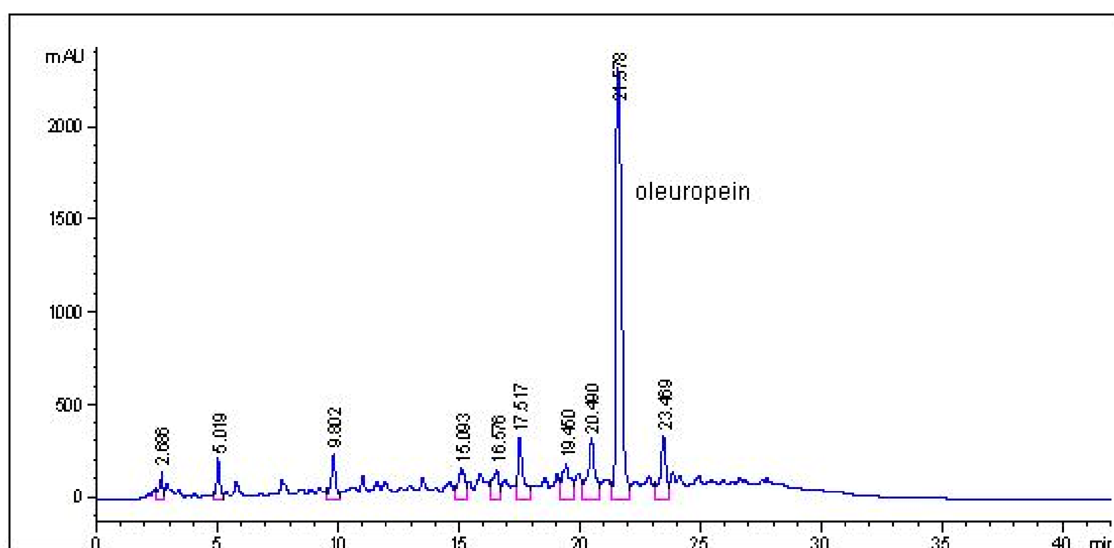


Figure 4.1. HPLC chromatogram of crude OLE

Table 4.1. Retention times of main phenolic compounds in crude OLE

Phenolic compounds	Retention time (min)	Phenolic compounds	Retention time (min)
Hydroxytyrosol	5.02	Rutin	17.52
Catechin	9.80	Luteolin-7-glucoside	19.45
Vannilic acid	15.10	Verbascoside	20.50
Vannilin	16.58	Oleuropein	21.58

Qualification of oleuropein and other phenolic constituents were first qualified by comparing the retention times with corresponding references and then quantified by using external and internal calibration curves given in Appendix A Figures A.1.-A.2.

Chromatogram shows that the retention time of oleuropein was 21.58 min. Reverse phase HPLC analysis is combination of non-polar column and highly polar mobile phase, that is why compounds having higher polarity, leaves the column faster than less polar compounds. In this manner, it could be concluded that oleuropein was less polar compared to other main phenolic compounds in crude OLE as seen in Table 4.1.

Identification of oleuropein in OLE was carried out by calculating the quantity of oleuropein in unit amount of OLE. A detailed calculation is given in Appendix A. According to these calculations, the average amount of oleuropein obtained from four successive extraction processes was 121.83 mg/g OLE. Savournin et al., (2001) investigated the oleuropein amounts obtained from 14 different olive cultivar, results showed that amount of oleuropein was ranged from 9.04% to 14.32%. In this study, oleuropein amount found in Gemlik olives was 12.18% which lies within literal range.

4.1.2. Antioxidant Capacity of Olive Leaf Extract

Antioxidant capacity determination of crude OLE was carried out by performing PCL assay. As the crude extract was used in aqueous solutions in the following experiments, ACW protocol was applied for the detection of the antioxidant capacity (AOC) of OLE. Ascorbic acid calibration curves, blank and sample curves of crude OLE are given in Figure 4.2.

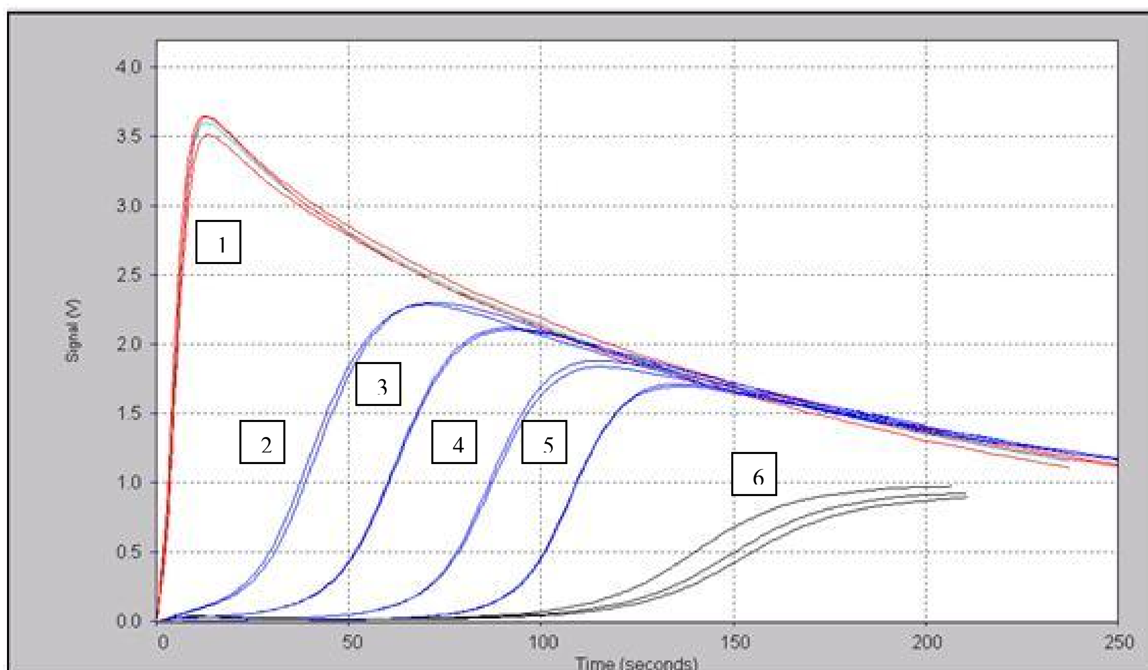


Figure 4.2.Ascorbic acid calibration measurements on ACW, 1: Blank curves; 2-5: Curves obtained from different concentrations of ascorbic acid, ranged 1-4 nmoles. 6: Sample curves of OLE.

Antioxidant capacity of OLE, in terms of ascorbic acid equivalent concentration, was 966 µg/mg OLE. Briante et al., (2001) studied the relative antioxidant efficiencies of phenolics derived from oleuropein by hydrolysis. According to this study, by using (DMDP)*N*-(3,4-dimethoxyphenethyl)-*N*-methyl-2-(2-napthyl)-*m*-dithane-2-propylamine method, the relative antioxidant efficiency (RAE) of oleuropein was found to be 33% of ascorbic acid. In addition, Hayes et al., (2009) reported that 0.035 mg/mL of olive leaf extract was enough to decrease the concentration of DPPH radical by 50%, which was also attributed to the synergistics among polyphenols present in OLEs. The same phenomena had also been previously reported by Benavente-Garcia et al., (2000). They proposed that synergistic of poplyphenols may provide better action against radicals when mixed than that of the individual phenolics used alone.

4.1.3. Total Phenol Content of Olive Leaf Extract

Total phenol content of OLE was determined by Folin-Ciocalteu assay and results were expressed as mg gallic acid equivalent per g of extract (mg GAEq./g). Total phenol content calculations of OLE were carried out by the gallic acid calibration curve

shown in Figure A.3. Total phenol content of OLE was calculated as 197.42±2.97 mg GAEq/g extract.

4.1.4. Minimum Inhibition Concentration Assays

In order to obtain MIC values of OLE against several pathogenic and spoilage microorganisms, two-fold broth microdilution assay was conducted by using 96 well microtiter plates (Thermo) and readings of plates were performed by Varioskan microplate reader (Thermo, Varioskan Flash, U.S.A.). Broth microdilution assays have benefits to overcome the difficulties such as laborious procedures used in determination of MICs by macrodilution assays in tubes (Burrows et al., 1993). MIC values of OLE and penicillin for *E. coli*, *E. coli* O157:H7, *S. aureus*, *S. carnosus*, *L. innocua* and *S.* Typhimurium are shown in Figures 4.3 - 4.13.

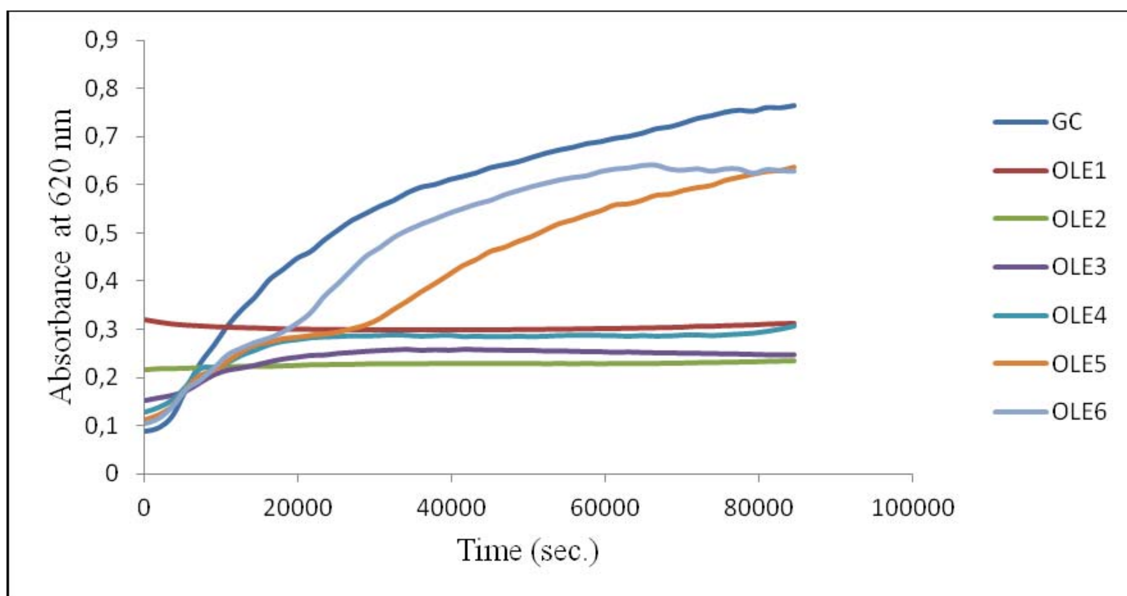


Figure 4.3. MIC of OLE for *E.coli*; GC: Negative control having 195 µL nutrient broth and 5 µL inoculums, OLE1: 250 mg/mL, OLE2:125 mg/mL, OLE3: 62.5 mg/mL, OLE4: 31.25 mg/mL, OLE5:15.6 mg/mL, OLE6: 7.8 mg/mL.

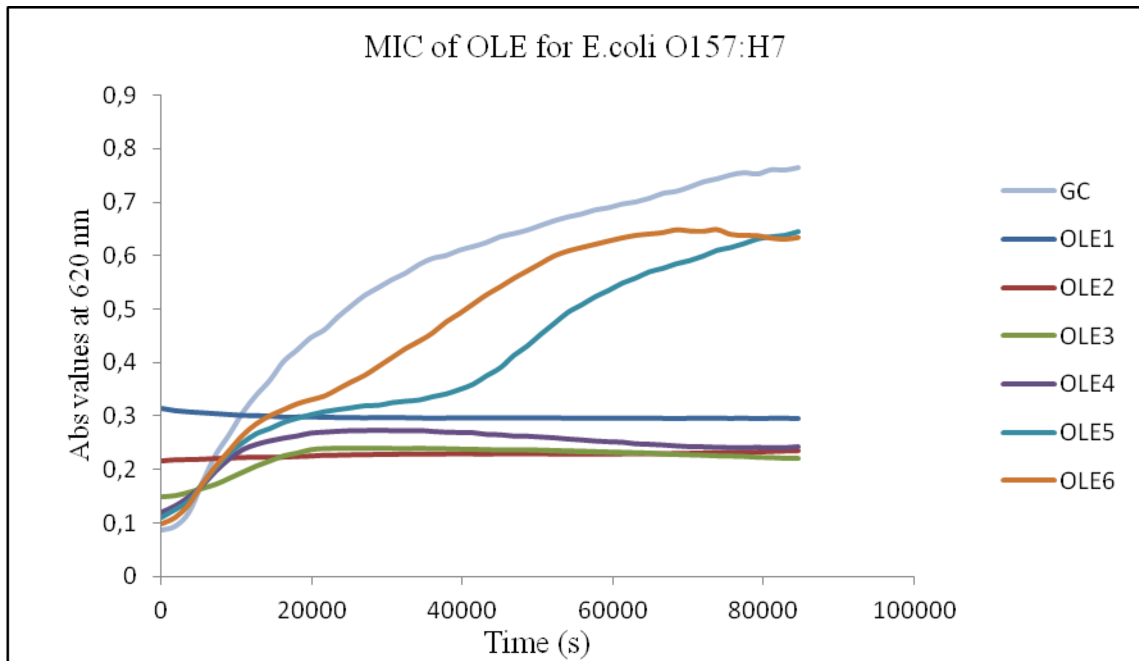


Figure 4.4. MIC of OLE for *E.coli* O157:H7, GC: Negative control having 195 µL nutrient broth and 5 µL inoculum. OLE1 : 250 mg/mL, OLE2: 125 mg/mL, OLE3: 62.5 mg/mL, OLE4: 31.25 mg/mL, OLE5: 15.6 mg/mL, OLE6: 7.8 mg/mL.

Absorbences read from OLE1 and OLE2 are higher than initial absorbences of other dilutions and negative control due to dark color of the extract. According to the graph, MIC value of OLE for both strains of *E. coli* can be said to be greater than 62.50mg/mL. However, it is not certain that the concentration is even higher than 125mg/mL or 250mg/mL due to color attributes of the extract. Sudjana et al., (2009) reported that MIC of OLE for *E. coli* was between 25 and 50% (v/v) OLE extract used in their study was liquid.

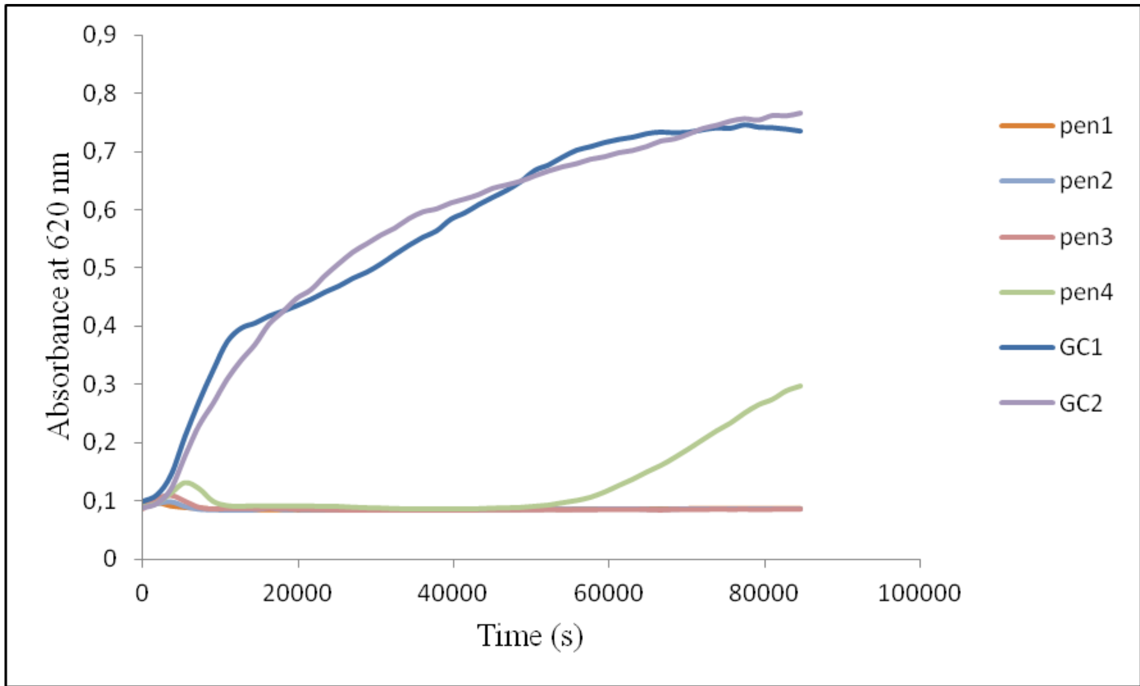


Figure 4.5. MIC of Penicillin for *E. coli* GC1: Negative control for *E.coli* O157:H7, GC2: Negative control for *E.coli*, pen1: 31.25 IU, pen2: 15.6 IU, pen3: 7.8 IU, pen4: 3.9 IU

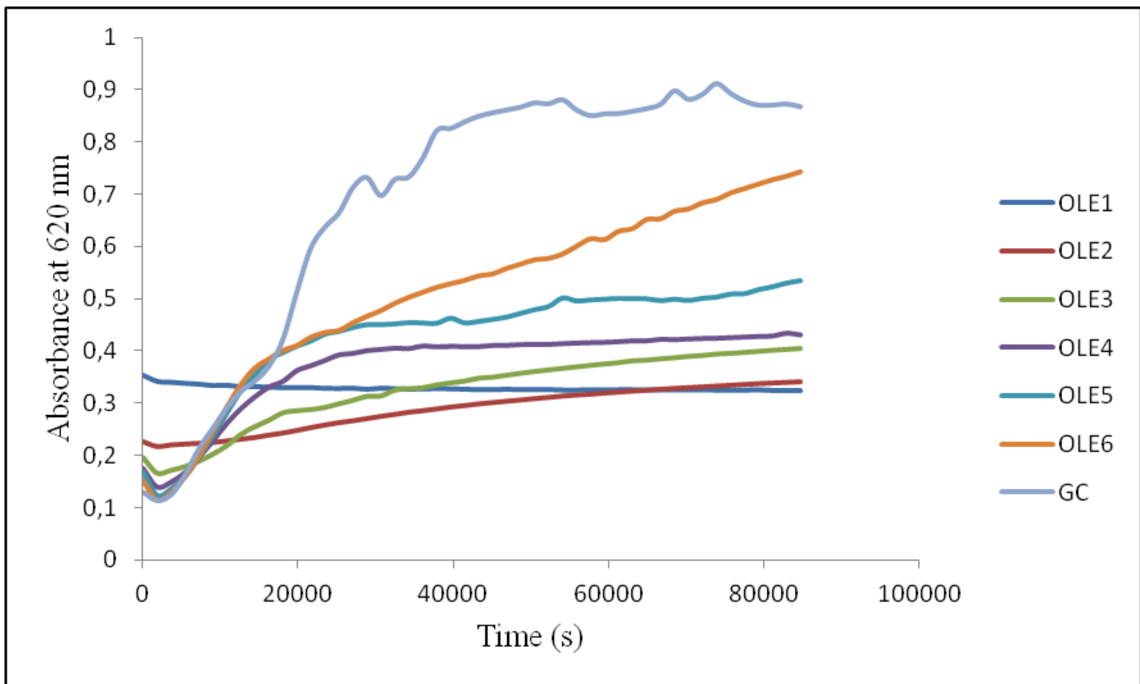


Figure 4.6. MIC of OLE for *S. aureus* GC: Negative control having 195 µL nutrient broth and 5 µL inoculum. OLE1: 250 mg/mL, OLE2:125 mg/mL, OLE3:62,5 mg/mL, OLE4:31.25 mg/mL, OLE5:15.6 mg/mL, OLE6:7.8 mg/mL.

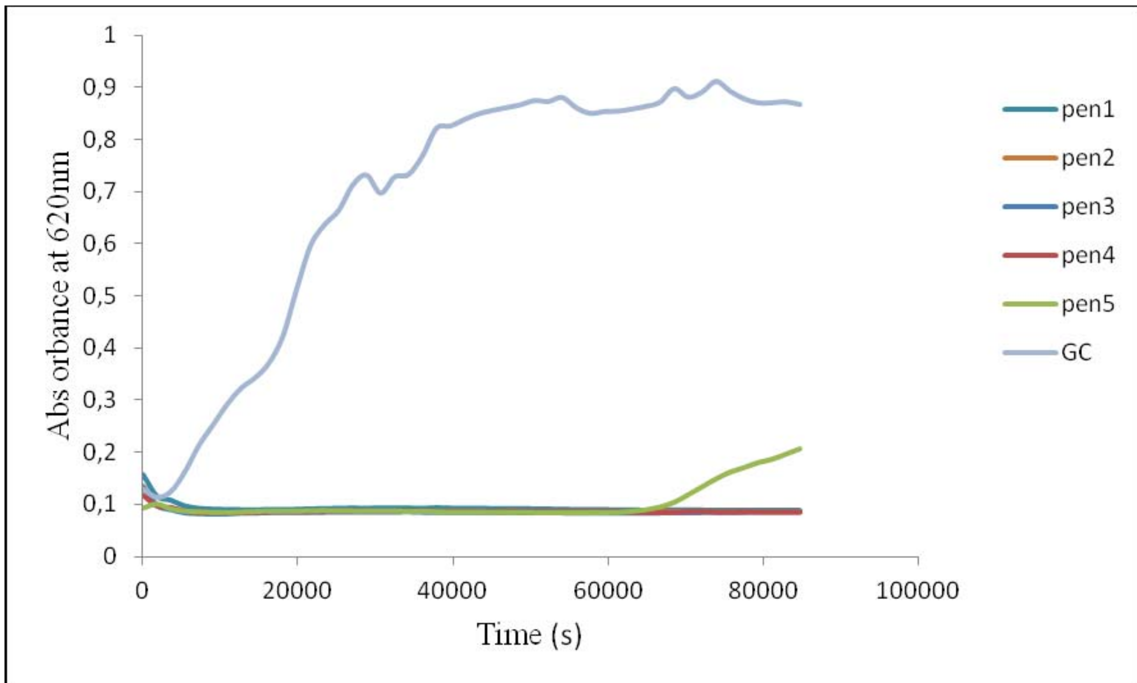


Figure 4.7. MIC of penicillin for *S. aureus*, GC: Negative control having 195 µL nutrient broth and 5 µL inoculum, pen1: 250 IU, pen2: 125 IU, pen3: 62.5 IU, pen4: 31.25 IU, and pen5: 15.6 IU.

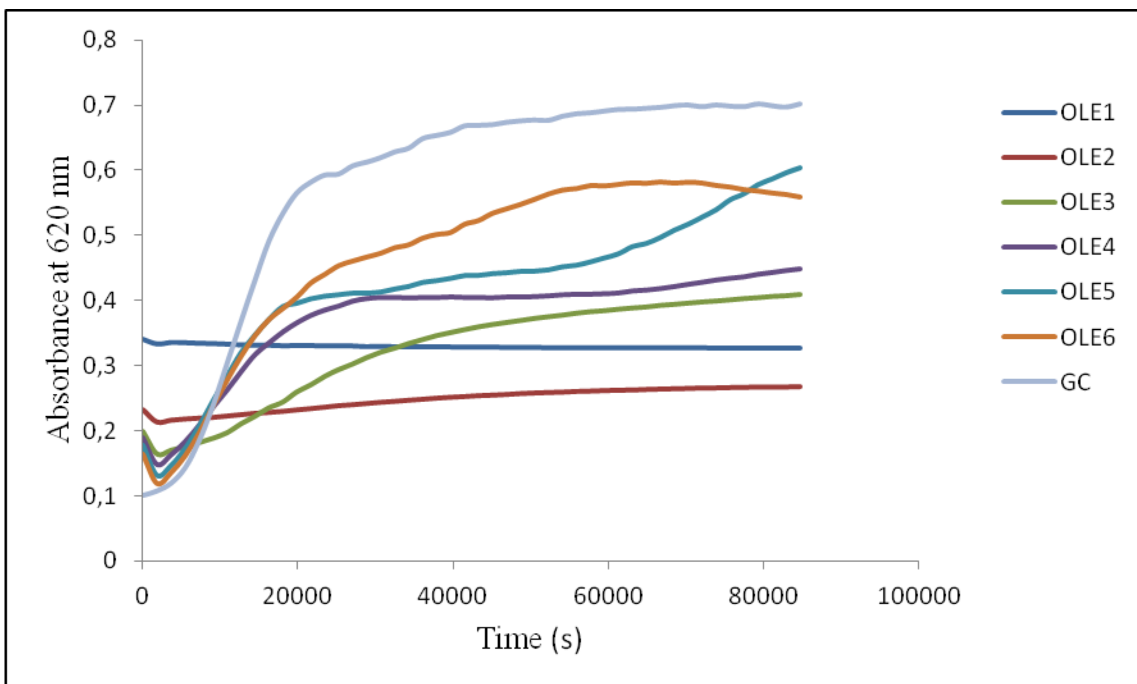


Figure 4.8. MIC of OLE for *S. carnosus*, GC: Negative control having 195 µL nutrient broth and 5 µL inoculum, OLE1: 250 mg/mL, OLE2: 125 mg/mL, OLE3: 62.5 mg/mL, OLE4: 31.25 mg/mL, OLE5: 15.6 mg/mL, and OLE6: 7.8 mg/mL.

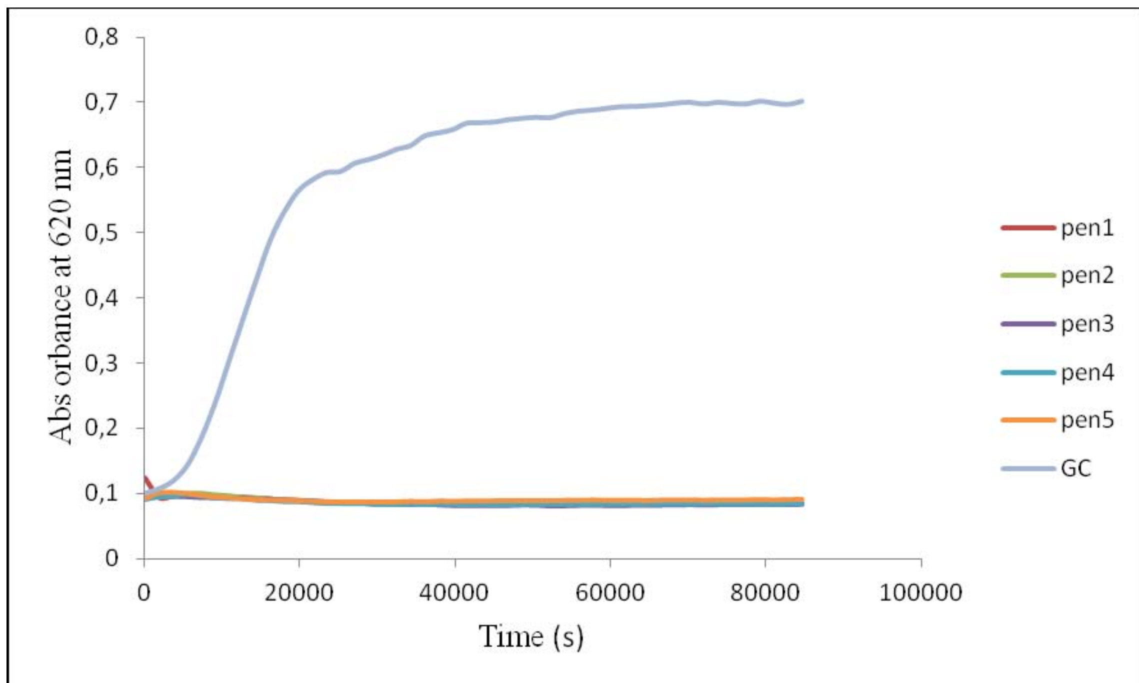


Figure 4.9. MIC of penicillin for *S. carnosus*, GC: Negative control having 195 µL nutrient broth and 5 µL inoculum, pen1: 31.25 IU, pen2: 15.6 IU, pen3: 7.8 IU, pen4: 3.9 IU, and pen5: 1.95 IU.

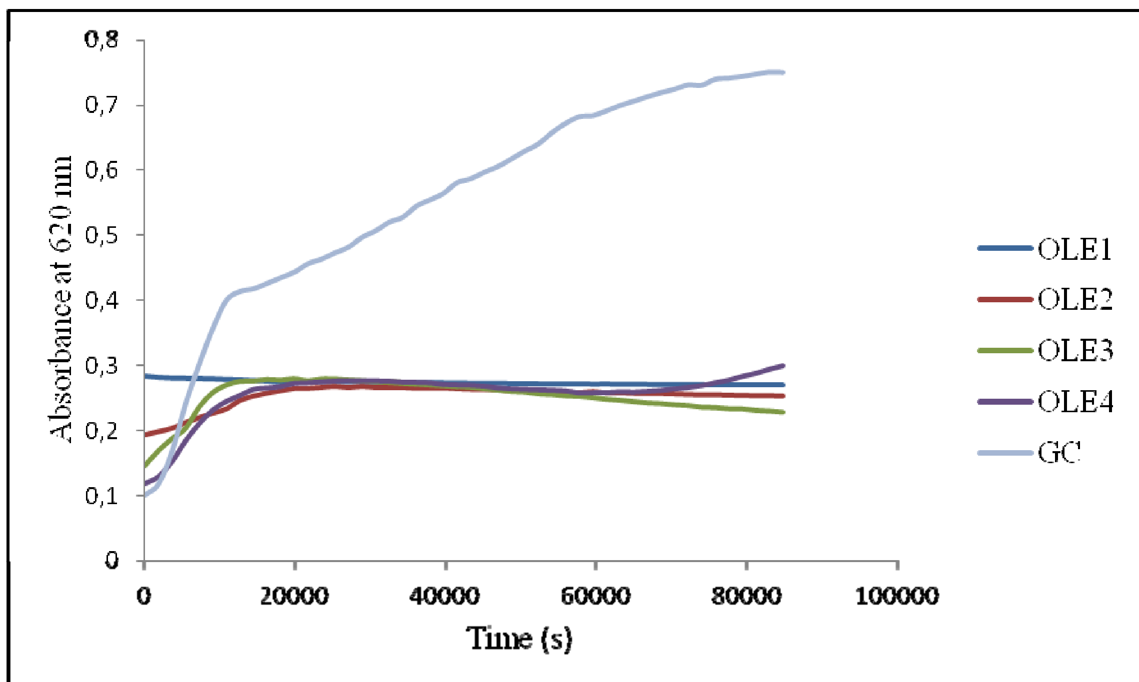


Figure 4.10. MIC of OLE for *S.* Typhimurium, GC: Negative control having 195 µL nutrient broth and 5 µL inoculum, OLE1: 250 mg/mL, OLE2: 125 mg/mL, OLE3: 62.5 mg/mL, and OLE4: 31.25 mg/mL.

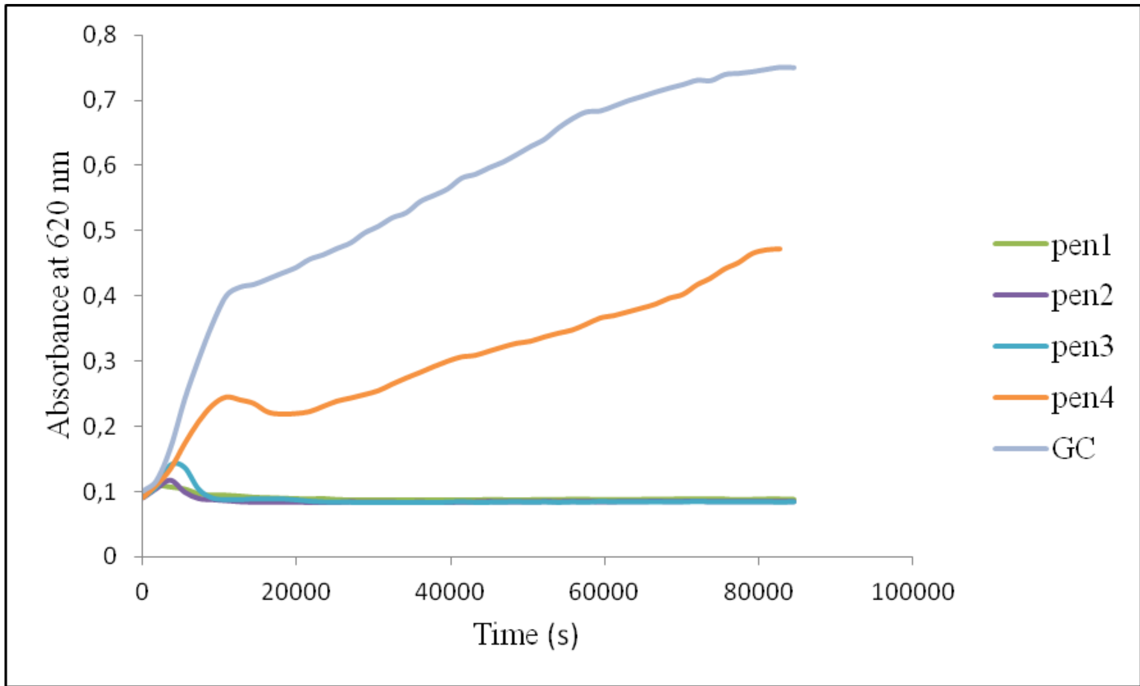


Figure 4.11. MIC of penicillin for *S.* Typhimurium, GC: Negative control having 195 µL nutrient broth and 5 µL inoculum, pen1: 15.6 IU, pen2: 7.8 IU, pen3: 3.9 IU, and pen4: 1.95 IU.

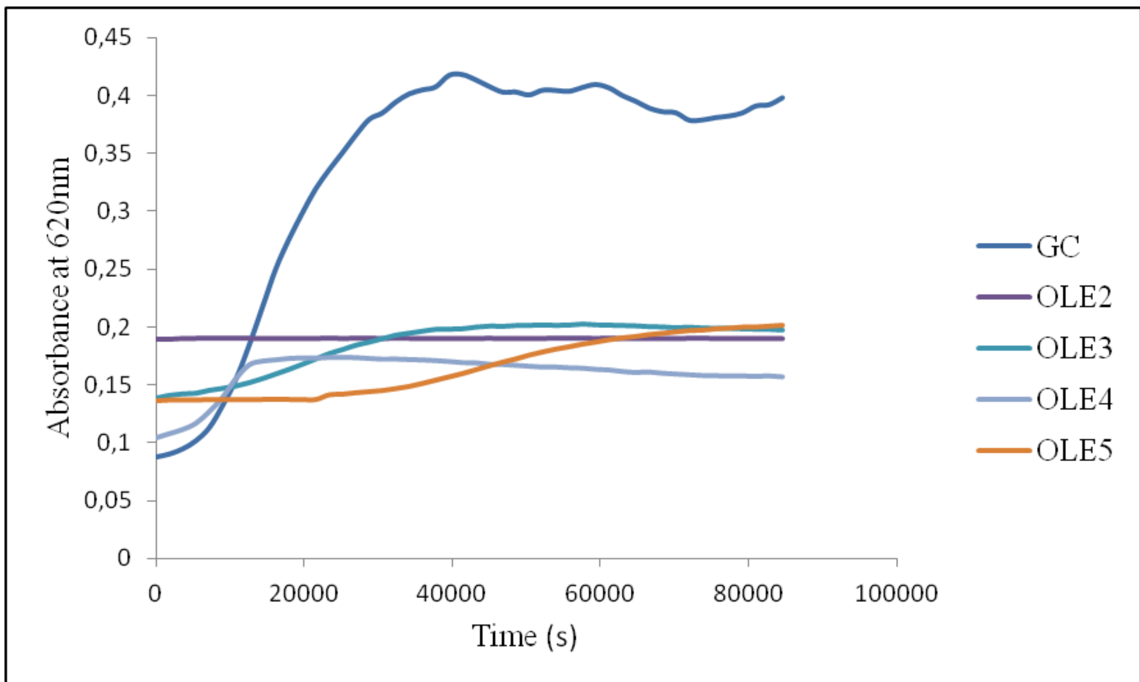


Figure 4.12. MIC of OLE for *L. innocua*, GC: Negative control having 195 µL nutrient broth and 5µL inoculum, OLE2: 125 mg/mL, OLE3: 62.5 mg/mL, OLE4: 31.25 mg/mL, and OLE5: 15.6 mg/mL.

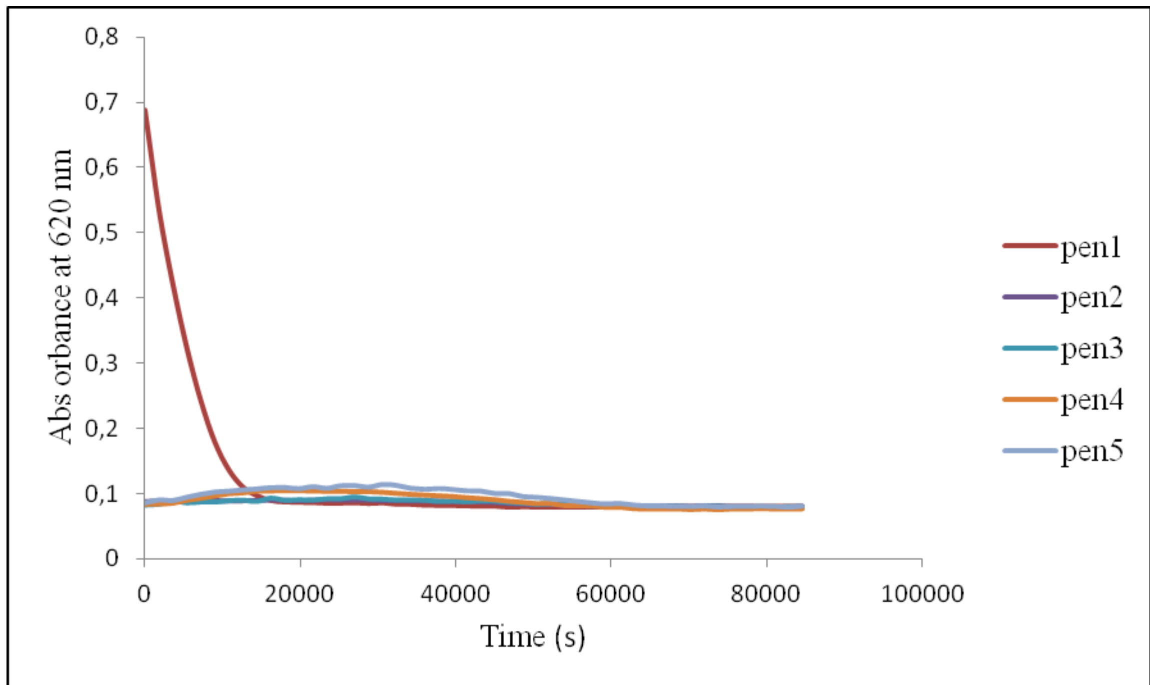


Figure 4.13. MIC of penicillin for *L. innocua*, GC: Negative control having 195 µL nutrient broth and 5 µL inoculum, pen1: 500 IU, pen2: 125 IU, pen3: 62.5 IU, pen4: 31.25 IU, and pen5: 15.6 IU.

According to the results shown in Table 4.2., the most susceptible bacteria were determined as *L. innocua* followed by *E. coli*, *E. coli O157:H7*and *S. carnosus.*

Table 4.2. Minimum inhibition concentrations of OLE and Penicillin for some bacteria

Bacteria	OLE (mg/mL)	Penicillin (IU)
E.coli (NRRL B 3008)	MIC ≥62.50	3.9< MIC<7.8
E.coli O157:H7	MIC ≥62.50	3.9< MIC<7.8
S.aureus	MIC ≥125	MIC=15.6
S.carnosus	MIC ≥62.50	MIC <1.95
*S.*Typhimurium	MIC ≥125	MIC <1.95
L.innocua	MIC ≥31.25	MIC <31.25

4.2. Food Applications Using Olive Leaf Extract

4.2.1. Antimicrobial and Antioxidant Activities of Olive Leaf Extract on Beef Cubes

The vacuum packed beef loaf was cut into approximately 1.5x1.5 cm cubes and separated into 4 batches. One of the batches were immersed into distilled water (control) and the rest of the batches were treated with 1%, 2%, and 3% OLE (w/v) in 1:1 ratio (beef:distilled water with OLE) for 20 h at 4 °C. After treatment samples were drained, placed in Ziploc® bags in portions, and stored at 4 °C for 9 days. The moisture content and pH of the beef cubes were determined during storage. The change in moisture content of beef cubes was ranged between 66% and 78%. No significant differences were detected among control and treated samples during storage. The initial pH of the raw meat was 6.48 and the pH values of samples varied from 6.11±0.02 to 6.83±0.03 during storage (Table 4.3).

Table 4.3. The pH values of beef cubes treated with OLE during storage

Treatment	pH of Beef Cubes			
	Storage Time (Days)			
	1	3	6	9
Control	6.49±0.07^{a,w}	6.39±0.05^{a,wy}	6.33±0.03^{a,y}	6.83±0.03^{a,z}
1% OLE	6.55±0.10^{a,x}	6.59±0.02^{b,xy}	6.11±0.02^{b,w}	6.71±0.04^{b,y}
2% OLE	6.80±0.05^{b,y}	6.83±0.03^{c,y}	6.43±0.03^{c,x}	6.23±0.04^{c,w}
3%OLE	6.58±0.05^{a,w}	6.51±0.03^{d,wy}	6.48±0.04^{c,y}	6.67±0.02^{b,z}

$^{a-d}$: Means having different letters within each storage time differs significantly ($p<0.05$).
$^{w-z}$: Means having different letters within each treatment differs significantly ($p<0.05$).
Data are mean values ± S.D. (n=3)

The pH values of all samples decreased up to the 6th day of storage. However, at the 9th day pH values increased except for the sample treated with 2% OLE. Control samples had the lowest pH values compared to the samples treated with OLE up to the 6th day, but at the 9th day control samples had the highest pH values. No particular trend was observed among the pH values of control and treated samples.

4.2.1.1. Effect of Olive Leaf Extract on Total Viable Count of Beef Cubes

The effect of treatment with 1%, 2%, and 3% OLE on total viable counts (TVCs) of refrigerated stored beef cubes was determined (Figure 4.14 and Table B.1). The initial microbial load of beef cubes was 5.08 log CFU/g.

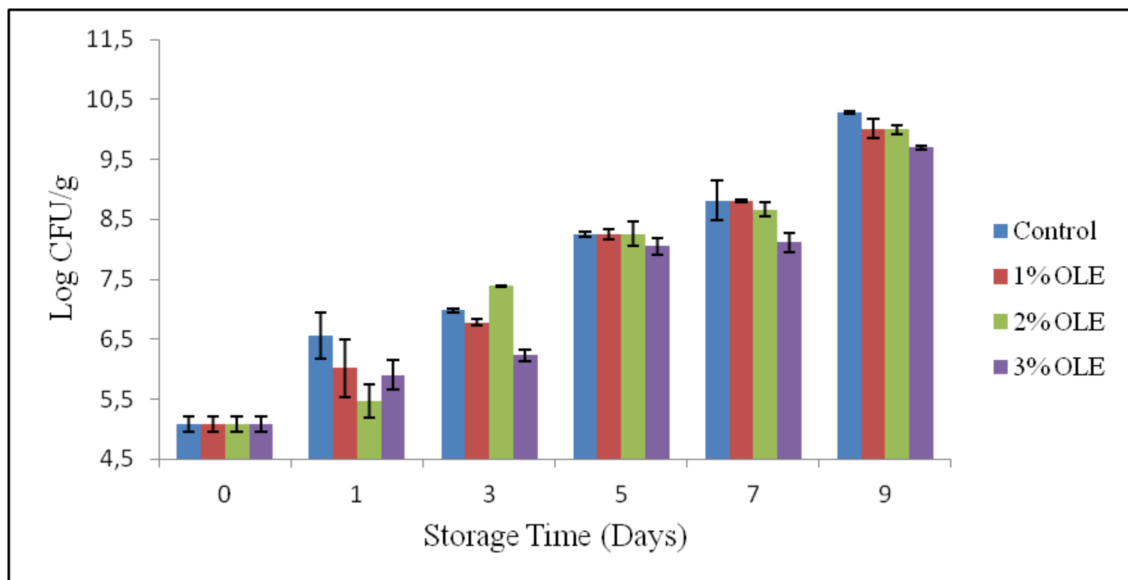


Figure 4.14. Effect of OLE on TVCs of beef cubes cold stored at 4ºC for 9 days

The TVCs of control and OLE treated samples increased dramatically during 9 days of storage at 4 ºC. Although at day 1, only TVC of the samples treated with 2% OLE were significantly lower than the control, 1% and 3% OLE treated samples, microbial counts of all treated samples were lower than that of the control samples. Control samples and 1% OLE treated samples exceeded 6 log CFU/g after 1 day of storage while other treated samples had lower microbial loads. At the end of 5 days of storage, microbial load of all samples exceeded 8 log CFU/g and there was no significant difference between treated and control samples. At 9 days of storage, although there was not a great difference among samples, the differences were statistically significant between control and treated samples. The lowest microbial load at the end of 9 days of storage was observed on the samples treated with 3% OLE.

Treatment with OLE did not reduce the microbial count of beef cubes, but provided a slight inhibition on growth after 9 days of cold storage.

4.2.1.2. Effect of Olive Leaf Extract on Total Coliform Count of Beef Cubes

The effect of treatment with 1%, 2%, and 3% OLE on total coliform counts (TCCs) of refrigerated stored beef cubes was determined (Figure 4.15 and Table B.2). The initial TCC of fresh beef cubes was 2.81 log CFU/g.

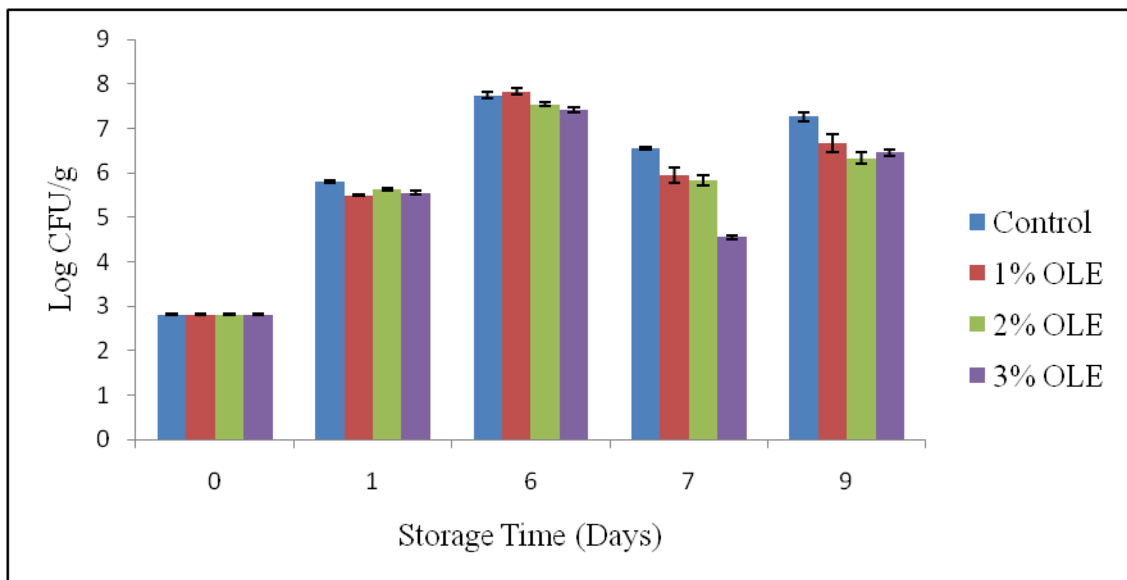


Figure 4.15. Effect of OLE on TCCs of beef cubes cold stored at 4ºC for 9 days.

TCC of both control and treated samples increased in first 6 days of cold storage of beef cubes. The lowest count value was obtained from the beef cubes treated with 3% OLE at the end of 6 days cold storage. Although all samples showed a decrease in TCC at seventh day of storage, the bacterial loads obtained from samples except beef cubes treated with 3% OLE were not significantly different. In addition, at the end of 9 days of cold storage, all treated samples showed inhibitory effect on TCC. Even though the lowest final TCC was obtained from the samples treated with 2% OLE, the difference among treated samples was insignificant.

4.2.1.3. Effect of OLE on Lactic Acid Bacteria Count of Beef Cubes

Effect of crude OLE on LAB growth of refrigerated stored beef cubes were investigated during 9 days storage (Figure 4.16 and Table B.3). Initial LAB load on fresh beef cubes was 3.93 log CFU/g beef on day 0.

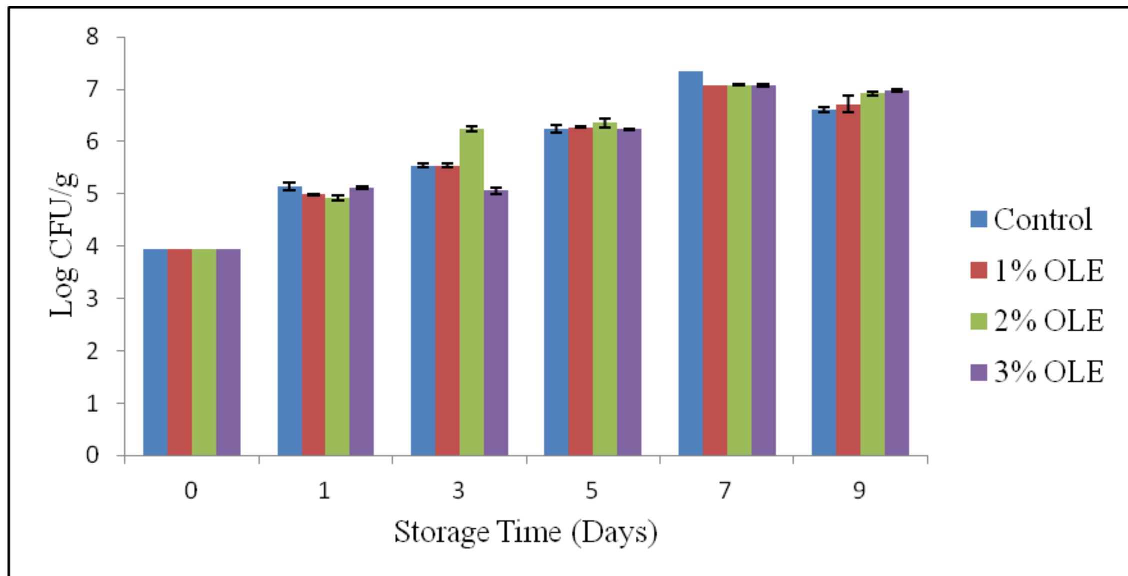


Figure 4.16. Effect of OLE on LAB count of beef cubes cold stored at 4ºC for 9 days.

LAB count of beef cubes treated with 1% and 2% OLE were lower from that of control and beef cubes treated with 3% OLE on the 1st day of storage. LAB count of control and treated samples gradually increased during 9 days of refrigerated storage. Although treatment with 3% OLE reduced the LAB count by 0.05 log CFU/g on the 3rd day, there was no significant difference detected between control and treated samples on the 5th day of storage. However, LAB counts of treated samples were lower than that of control samples at day 7. At the end of storage period, OLE did not show any significant effect on LAB and yet this overall result was not in agreement with previous studies in literature.

Growth of LAB in olive brines is a common issue that is faced frequently. Manzanilla variety, for example, do not allow the growth of LAB and this inhibitory effect is generally attributed to oleuropein and its hydrolysis products such as oleuropein aglycon and elenolic acid. It was also reported that the main phenolic responsible of inhibition of LAB growth was hydroxytyrosol followed by tyrosol,

verbascoside and oluropein (Medina et al., 2007). However they demonstrated that the main antimicrobial phenolics in olives against LAB as dealdehydic ester of decarboxymethyl elenolic acid and the latter substance linked to hydroxytyrosol (Medina et al., 2007, 2008a, 2008b).

In fact, as can be seen from Table B.3. and Figure 4.16, LAB counts obtained from beef cubes treated with 3% OLE showed a fluctuation between first and fifth days of cold storage of beef cubes. This observation may be attributed to the degradation or consumption of anti-LAB compounds within OLE during storage of beef cubes.

4.2.1.4. Effect of OLE on Oxidative Stability of Beef Cubes

Lipid oxidation is one of the most undesirable change that affect the quality of foodstuff during storage due to deterioration of polyunsaturated fatty acids (PUFA) (Pazos et al., 2008). The oxidative stability of beef cubes were examined by conducting thiobarbituric acid reactive substances (TBARS) assay. Absorbances obtained from these assays at 532 nm, were then used to calculate amount of TBARS. Results are given in Table B.4. and Figure 4.17 as mg malonaldehyde equivalent/kg beef. The initial TBARS value of fresh beef cubes were determined as 0.91±0.063 mg MDA/kg beef. Level of lipid oxidation was increased in all control and treated samples during refrigerated storage. Although treatment with 3% OLE reduced the lipid oxidation of beef cubes by 0.7, the best result against lipid oxidation was obtained from beef cubes treated with 2% OLE after 9 days of cold storage; 2% OLE kept TBARS value of beefs under consumption level of 2 mg MDA/kg during first 6 days of cold storage. This result may be attributed to the amount of hydroxyl groups within the phenolic structures of constituents present in crude olive leaf extract mainly hydroxytyrosol and oleuropein. It is assumed that inhibition of lipid oxidation and hydrogen donor ability is enhanced with the increasing amount of hydroxyl groups (McDonald et al., 2001). Phenolic compounds possessing at least two hydroxyl groups are considered as iron binding and reducing properties (Keceli and Gordon, 2002).

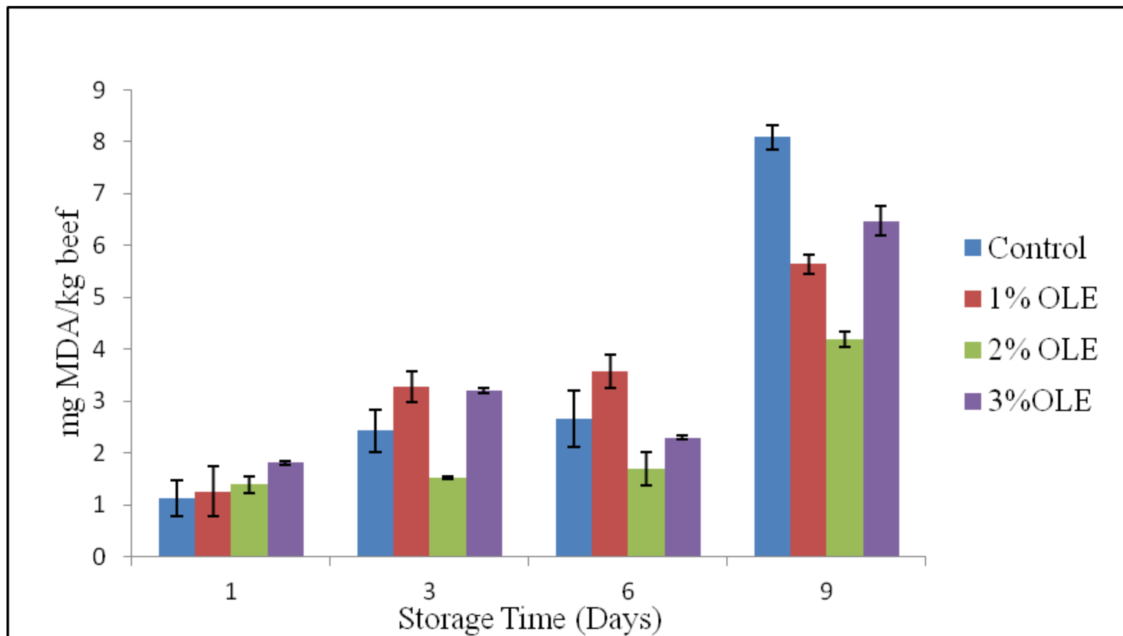


Figure 4.17. Effect of OLE on lipid oxidation of beef cubes stored at 4ºC for 9 days.

The amount of oxidation in samples treated with 1% and 3% OLE were considerably higher than that of beef cubes treated with 2% OLE throughout the cold storage. Higher level of oxidation in 1% OLE samples may be explained by the considerably lower concentration of antioxidant material within samples. Phenolics in crude 1% OLE solution may be enough to neutralize metal ions to some point. However, it may also reduce ions such as Fe(III) to their most active pro-oxidative state as Fe(II) (Keceli and Gordon, 2002), and there may not be any more substance with antioxidative in the media to neutralize these pro-oxidants.

Phenolics within crude OLE may also act as pro-oxidants themselves such as ascorbic acid and gallic acid (Yen et al., 2002). With same manner higher concentration of antioxidant may cause production of more reactive substances while reducing metal ions, and may not pace with this rapidity and end up with higher oxidation levels. This consideration may be the answer for why 2%OLE treatment gave better results than 3%OLE treatment in oxidative stability determination of beef cubes.

4.2.1.5. Effect of OLE on Color Attributes of Cold Stored Beef Cubes

The average L*, a*, b*, chroma and hue angle values for all samples during 9 days of cold storage are given in Figures 4.18-4.20. and Table B.5-B.6. Storage and each treatment had significant effect on color attributes of beef cubes.

Storage time increased the lightness of beef cubes while increasing concentration of olive leaf extract caused a decrease in lightness of beef samples.

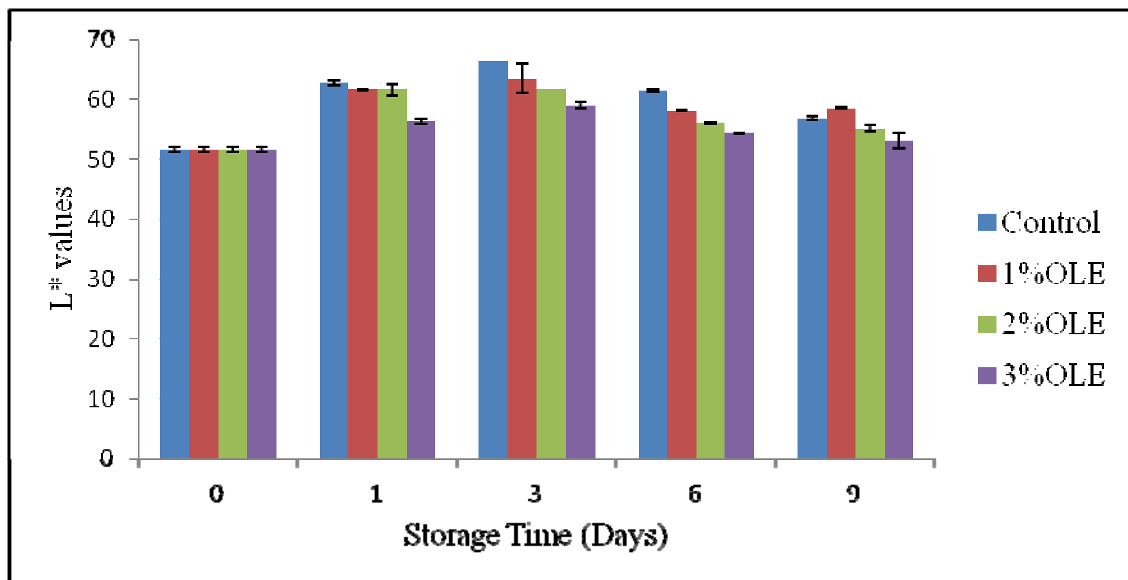


Figure 4.18. L* values of beef cubes cold stored at 4ºC for 9 days.

Redness of control samples was higher than that of treated samples during refrigerated storage. Yellowness of both control and treated samples decreased during storage. At the end of the 9 days of refrigerated storage, yellowness of beef cubes treated with 3% OLE was greater than that of control and samples treated with lower concentrations of OLE.

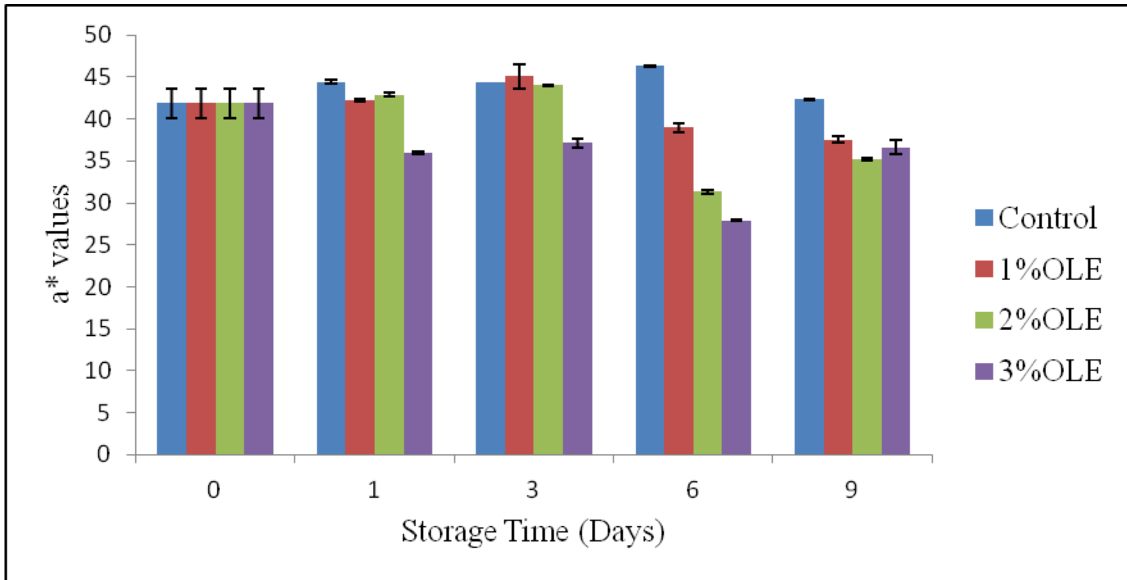


Figure 4.19. a* values of beef cubes cold stored at 4ºC for 9 days.

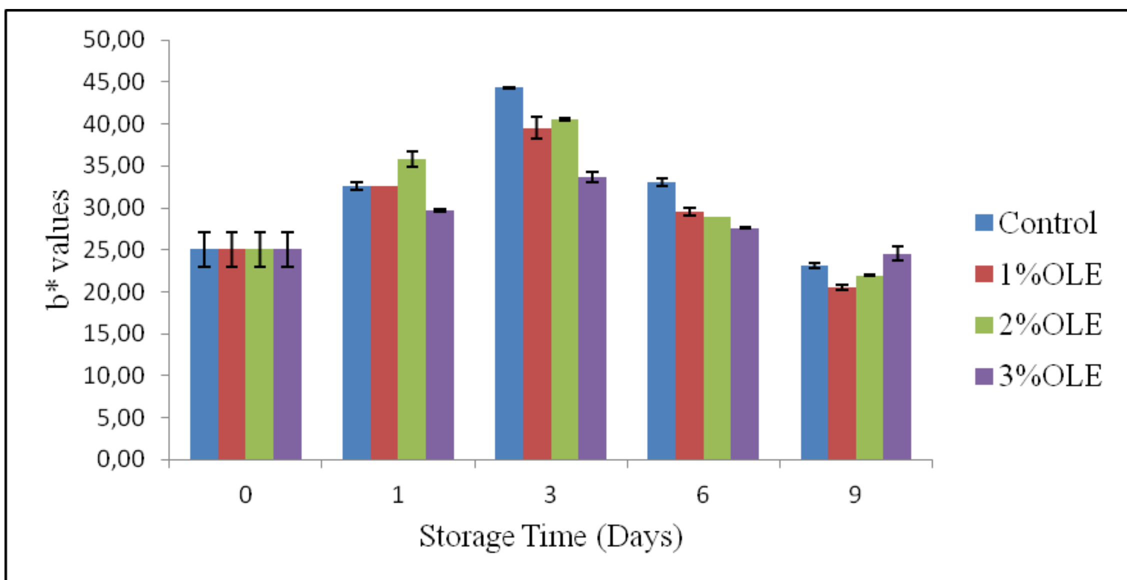


Figure 4.20. b* values of beef cubes cold stored at 4ºC for 9 days.

These changes in color attributes may be attributed to the natural yellow-brown color of the olive leaf extract used for the treatment of the beef cubes. There was no significant difference in chroma between control and treated samples during first three days of cold storage. Chroma of the treated samples was then decreased during storage and increasing concentration of OLE.

4.2.2. Antimicrobial and Antioxidant Activities of Olive Leaf Extract on Sardine Marination

Sardine (*Sardina pilchardus*) is the most abundant fish caught in Aegean Sea. Sardines are mostly consumed fresh, salted or canned as well as used as fishmeal or in production of fish oil. When excess amount of raw material is available, development of new products based on sardines such as marinated sardine. Due to their fat content, sardines are very proper for marination (Kılınç and Çaklı, 2004).

Marinades are fish products made of fresh and/or frozen fish processed by edible acids, mostly organic acids such as citric acid or acetic acid, and salt, then stored in sauces, creams or oil. Marinades are considered as semi-preservatives, acetic acid and salt may retard the bacterial or enzymatic actions, thus result in extended but limited shelf life (Gökoğlu et al., 2002).

Fresh sardines were cleaned and washed under tap water and placed into 10% (w/v) NaCl solution with a ration of 1:1 (fish:salt solution) for 1 hour. After this pretreatment, sardine samples were then drained and placed into marinade solutions including 0.5% (w/v) citric acid, 2% (v/v) acetic acid and 10% (w/v) salt. For OLE samples, concentration of crude OLE was adjusted as 300ppm. Marination process took place at 4ºC and lasted for 22 days.

4.2.2.1. Proximate Composition of Marinated Sardine Fillets

Changes in crude fat, protein, moisture, and ash contents and water activity of fresh and marinated fillets during marination process are shown in Table 4.4.

Table 4.4. Proximate composition of sardine fillets during marination process

	Storage Time (Days)					
	0		1		22	
	Control	OLE	Control	OLE	Control	OLE
Crude fat (%)	3.27±0.03	3.27±0.03	3.78±0.56	3.69±0.42	3.69±0.20	3.69±0.23
Crude protein (%)	19.90±0.02	19.90±0.02	21.52±0.62	21.57±0.44	20.01±1.43	20.67±1.55
Ash (%)	1.57 ± 0.13^{x}	1.57 ± 0.13^{x}	5.85 ± 0.05^{z}	5.77 ± 0.02^{z}	4.91 ± 0.05^{y}	4.88 ± 0.04^{y}
Moisture content (%)	75.96 ± 0.31^{x}	75.96 ± 0.31^{x}	69.44 ± 0.65^{ay}	73.59 ± 0.83^{by}	75.13 ± 0.41^{x}	75.09 ± 0.26^{x}
Water activity	0.91 ± 0.00^{xy}	0.91 ± 0.00^{xy}	0.91 ± 0.02^{y}	0.93 ± 0.01^{y}	0.90 ± 0.01^{y}	0.91 ± 0.01^{y}

$^{a-b}$: Means having different letters within each treatment differs significantly (p<0.01)
$^{x-z}$: Means having different letters within each storage time differs significantly (p<0.01) Data are given as mean ± S.D. (n=3).

According to the analyses, fresh sardine fillets contain 3.27±0.03% crude fat, 19.9023±0.024% crude protein, 1.57±0.13% ash and 75.96±0.31% moisture content. Chemical composition of fish fillets were in agreement with previous studies performed by Kilinç and Çaklı (2004). Water activity of fresh samples was detected as 0.91±0.00. Decrease of moisture content between day 0 and day 1 of marination could be attributed to the hypertonic marinade solution that caused fillets loose water to the bulk media. While the concentration equilibrium between flesh and marinade started forming, the moisture levels became normal.

4.2.2.2. Changes in pH of Sardine Fillets and Marinade

The pH value of raw sardine was 6.39. Gökoğlu et al., (2004) found that pH value for sardines as 6.3. They also reported that in previous studies Nunes et al., (1992); Marrackchi et al., (1990); Varlık (1994) and Gökoğlu et al., (1998) found pH of fresh fish fillets 6.1, 6.83, 6.35, and 6.2, respectively. Change in pH values of fillets and marinade solution are shown in Figures 4.21-4.22, Tables C.1-C.2, respectively.

After the fillets were placed into marinades, pH of samples decreased to 4.25 and 4.30 for control and OLE samples respectively. During marination period pH of fillets increased. Increase in pH of fillets was attributed to the decomposition of nitrogenous compounds in the post-mortem period (Shenderyuk and Bykowski, 1989).

However, increase in pH does not always indicate certain spoilage. Spoilage of fish can be detected by other chemical and sensory analyses.

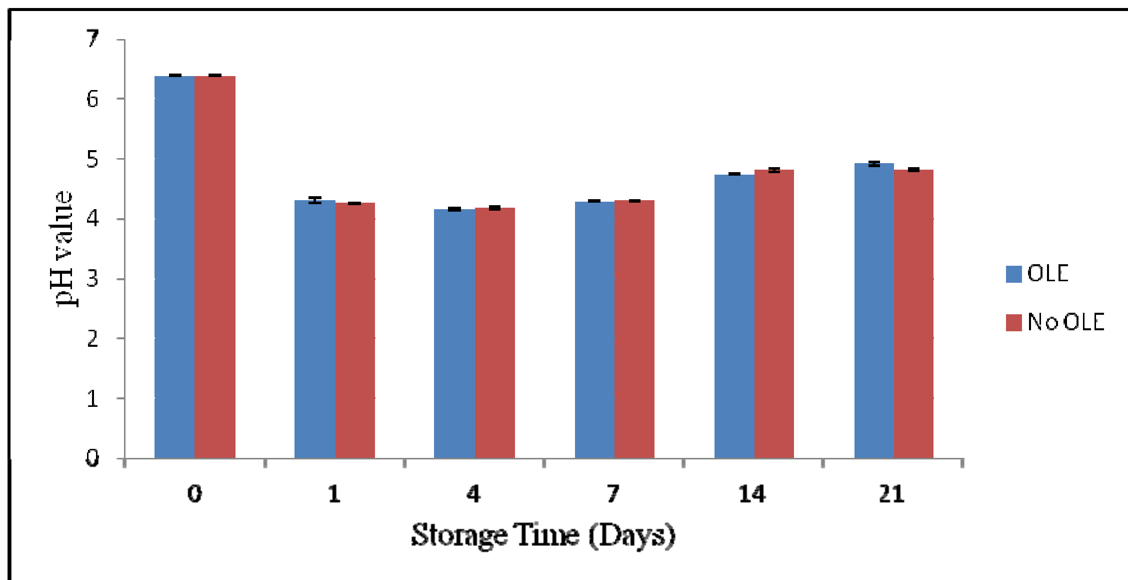


Figure 4.21. pH change in fish fillets during marination process

The pH of control and OLE marinades were 5.33 and 5.36 on day 1 and decreased to 5.11 and 5.08, respectively. Generally pH of marinade solution increases due to hetero-fermentative LAB actions such as degradation of amino acids. Thus, carbon dioxide and other carboxylation products can bind acetic acid and cause decrease in pH of marinade solution (Shenderyuk and Bykowski, 1989).

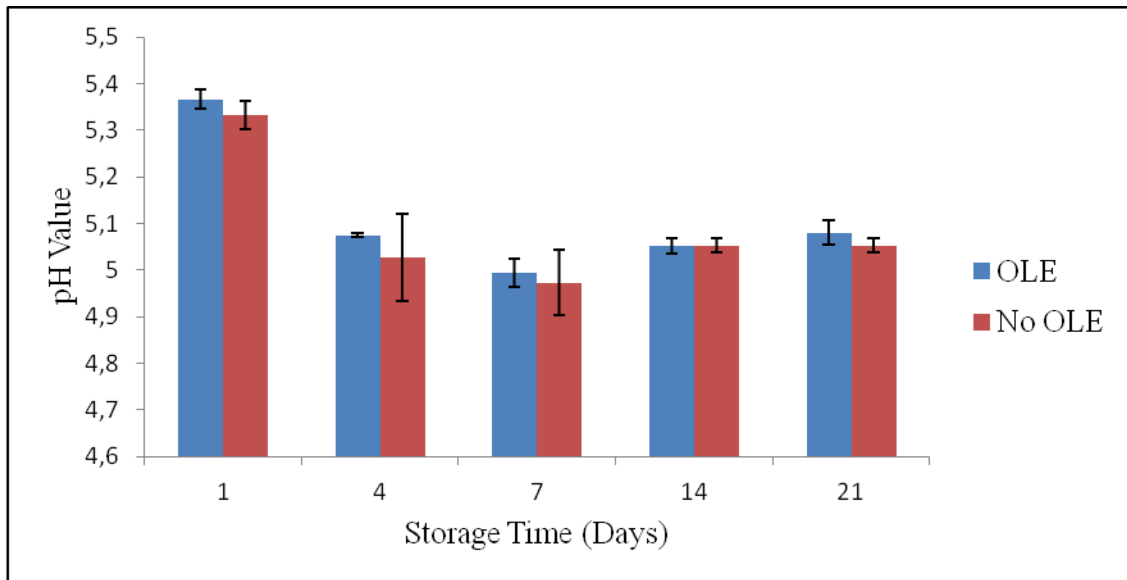


Figure 4.22. pH change in marinade solution during marination process

The slight fluctuation of pH during marination process could be explained by the diffusion of salt, acetic and citric acid through the fish fillets until an equilibrium point was reached.

4.2.2.3. Changes in Acidity of Sardine Fillets and Marinade

Initial acidity of control and OLE treated sardine fillets were 0.74 and 0.69, respectively. Although acidity in flesh increased between the 4th and 14th days due to the diffusion of citric and acetic acids through tissue, the final acidity values of both control and OLE treated fillets almost remained same as initial acidity. Changes in acidity of fillets and marinade are shown in Figures 4.23-4.24 and Tables C.3-C.4.

The difference between treatments was only significant on day 4. For control samples, no significant difference was detected during refrigerated storage. Changes in marinade acidity after the 4th day of marination were not significantly different either on time or sample basis. Same effect was also observed in flesh acidity. Based on these results we can say that acid concentration equilibrium was established after the 4th day of marination.

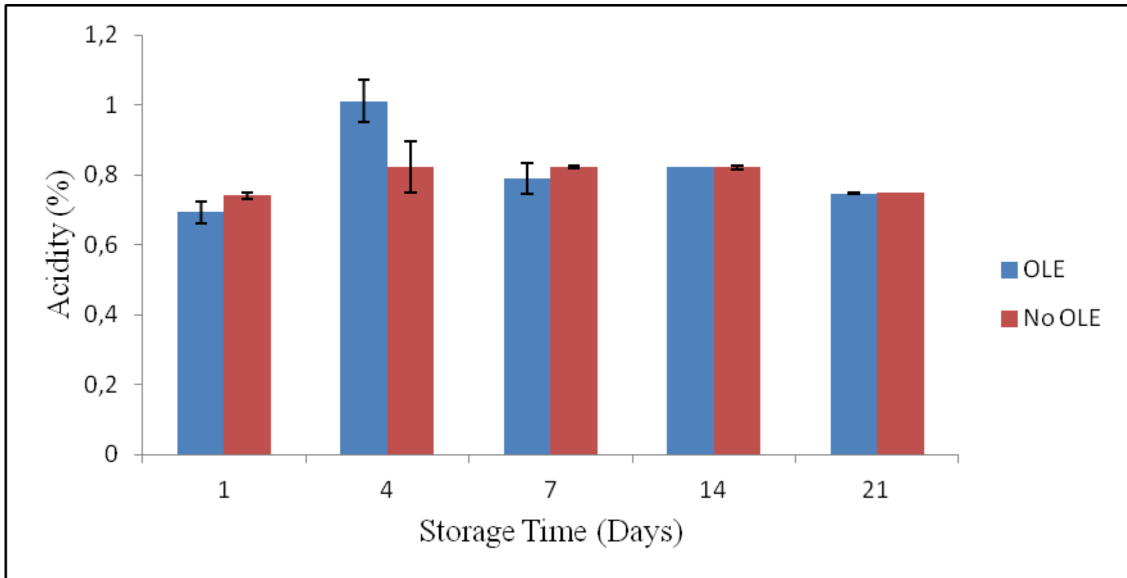


Figure 4.23. Acidity in fish fillet during marination process

The decrease and increase in acidity matches with fluctuation in pH of sardine fillets shown in section 4.2.2.2. This pattern may be explained either by formation of concentration equilibrium or deterioration of nitrogenous compounds such as proteins and amino acids.

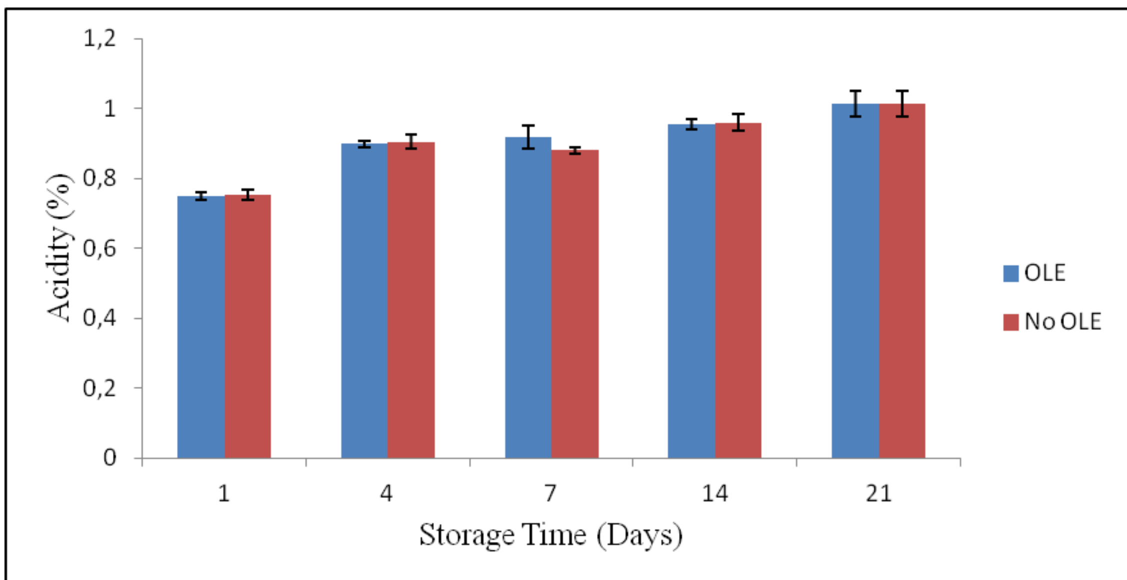


Figure 4.24. Acidity in marinade solution during marination process

4.2.2.4. Changes in Saltiness of Sardine Fillets and Marinade

Initial salt content of fresh sardine fillets was 3.72% and increased during marination in both control and OLE treated fillets due to diffusion of salt from marinade solution to fillets. As a response, salt content of marinade solution decreased due time. Changes in salt contents in sardine fillets and marinate are given in Figures 4.25-4.26 and Tables C.5-C.6.

As can be seen from Figure 4.25, there was no significant difference detected between two treatments. Salt content of sardine fillets increased during marination due to formation of salt equilibrium between the marinade and fillets.

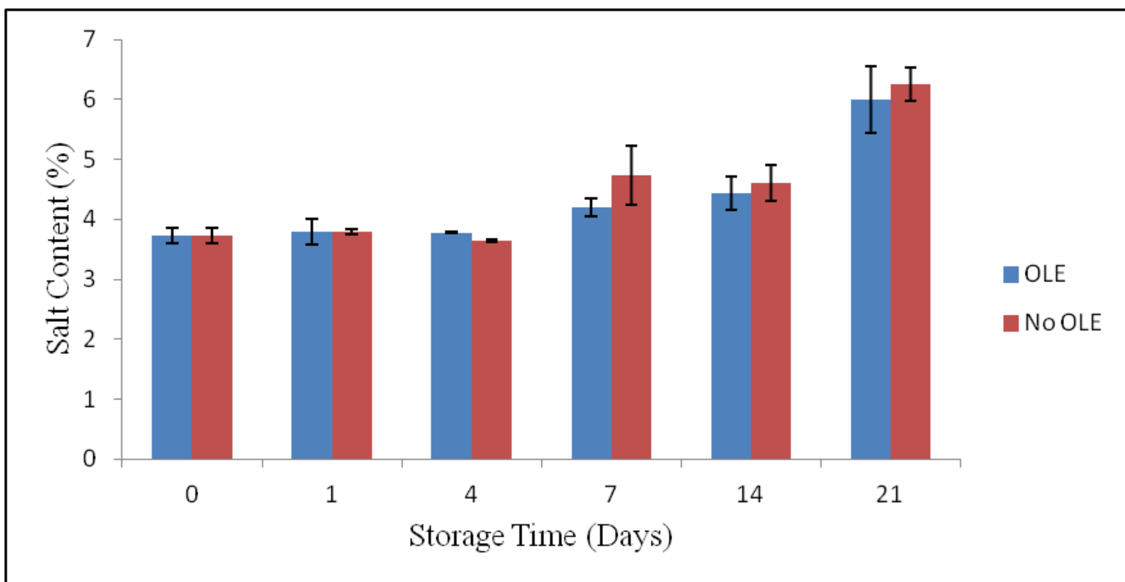


Figure 4.25. Changes in salt content of fish fillets during marination process

Only significant difference between marinade and fish fillets was observed at the first day of marination process. However, at the end point of marination there was no significant difference in salt content of both marinade solutions.

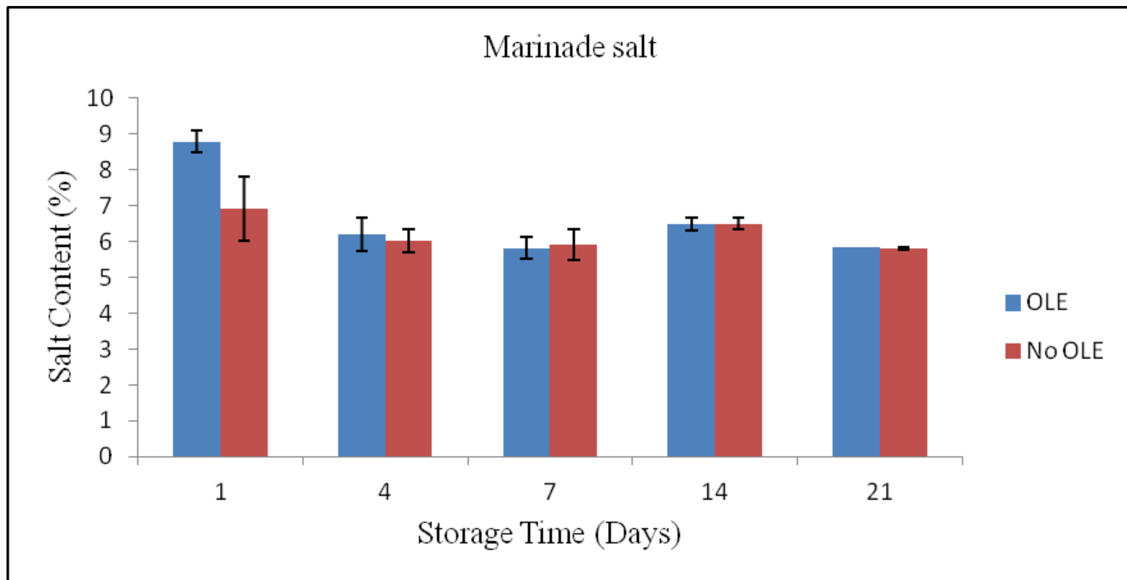


Figure 4.26. Changes in salt content of marinade solution during marination process

4.2.2.5. Effect of Olive Leaf Extract on Total Volatile Base Nitrogen Levels of Sardine Fillets

Total volatile base nitrogen (TVB-N) is produced by the degradation of proteins and other nitrogenous compounds within fish tissues and used to determine spoilage of fish muscle during storage period. Kılınç and Çaklı (2004) and Ludorff and Meyer (1973) reported that a maximum value of 35 mg/100 g fish is accepted as critical value for human consumption. TVB-N value is affected by type of species, catch season, sex and age of fish (El Marrackchi et al., 1990). The initial TVB-N value of fresh fillets was 10.35 mg/100 g. This value is in agreement with previous studies performed by Kılınç and Çaklı (2004); Gökoğlu et al., (2004), as 10.24 and 10.28 mg/100 g, respectively. Results are shown in Figure 4.27 and Table C.7.

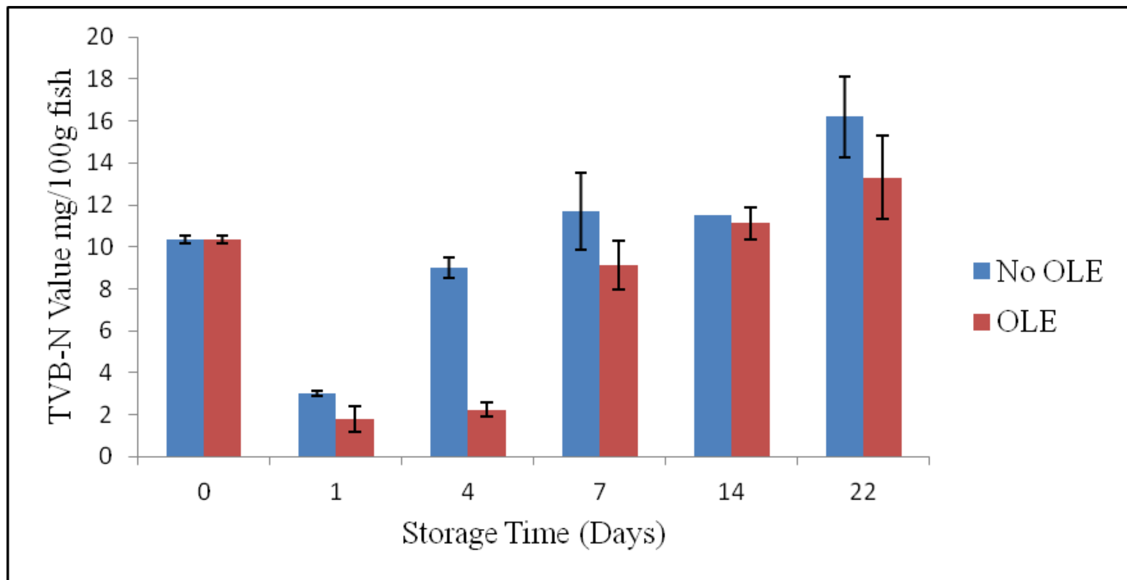


Figure 4.27. Changes in TVB-N value in sardine fillets during marination process

After sardine fillets placed into marinade, TVB-N value decreased to 3.02 and 1.48 mg/100 g in control and OLE treated fillets, respectively. This decrease could be attributed to the hypertonic marinade medium which leeches TVB-N components (Kılınç and Çaklı 2004). The TVB-N values of the fillets increased during marination process. At the end of marination process, TVB-N value for control reached to 14.92 mg/100 g while it was 11.92 mg/100 g for OLE sample. At the end of marination, difference between TVB-N values of control and OLE samples were significantly different. In addition, Gökoğlu et al., (2009) reported that TVB-N value of 8.3 mg increased to 15.1 mg/100 g; in marinated anchovy after 150 days (Aksu et al., 1997) and in another research, 9.8 mg/100 g TVB-N value increased to 14 mg/100 g in 8 months (Dokuzlu, 2000). The final value for OLE treated samples might be accepted as in agreement with the values of 11.2±2.80 mg/100 g which was obtained by Kılınç and Çaklı (2004) in sardines marinated with 7% acetic acid and 14% salt. This result points out the importance of acid and salt concentration on TVB-N formation within fish fillets. Since the hypertonicity of marinade solution used in this study is apparently not that effective on leeching TVB-N components from fish fillets, OLE might be considered as a protective agent against deterioration of nitrogenous compounds in fish flesh.

4.2.2.6. Effect of Olive Leaf Extract on Trimethylamine (TMA-N) Formation in Sardine Fillets

Formation of trimethylamine is caused by reduction of trimethylamine oxide by bacterial activity and partly by intrinsic enzymes and often used as index of freshness of marine fish (Villareal and Pazo, 1990). TMA-N value of fresh samples was found as 1.11 mg/100g which was found 0.88, 1.11 and 0.93 mg/100g by Kılınç and Çaklı (2004); Yapar (1998) and Gökoğlu et al., (2004) respectively. Results are shown in Figure 4.28. and Table C.8.

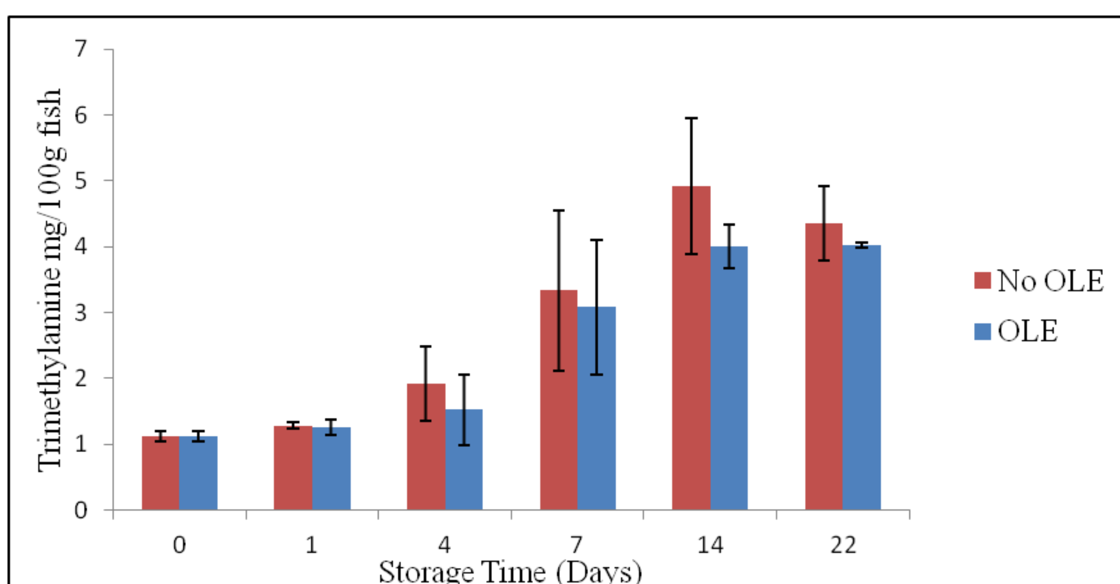


Figure 4.28. Changes in TMA-N amount in fish fillets during marination process

The TMA values obtained after 1 day of marination are also in agreement with corresponding studies. TMA-N values increased for both control and OLE treated samples due storage time and reached 4mg/100g. No significant difference between control and OLE treated fish fillets during marination. TMA-N values increased during refrigerated storage. Gökoğlu et al., (2009) reported that, 5-10 mg/100g as limit for acceptability of fisheries as previously expressed by Sikorski in 1989. Although results obtained at the end of marination process are below this limit, they are about 4 times greater than that of other studies. The highest amount reached by Yapar (1998) by 4.07 mg after 10 weeks of storage in presence of 2% acetic acid and 10% salt. Kılınç and

Çaklı (2004), found that increasing amount of acetic acid is effective on reduction of TMA formation in sardines.

4.2.2.7. Effect of Olive Leaf Extract on Oxidative Stability of Sardine Fillets

TBA analysis is a remarkable index of quality indicating lipid oxidation. Oxidative rancidity is a complex spoilage that occurs especially in fatty fish. TBA values of fresh sardine fillets was 1.84±0.06 mg malonaldehyde eq./kg. Kılınç and Çaklı had previously found the TBA value of fresh sardine 1.03±0.64 mg/kg. Kılınç et al., (2007) also reported that TBA value of fresh fish patties were 1.78±0.11 mg/kg. According to these results, TBA value of fresh fillets can be considered in agreement with these values. Values obtained from both control and OLE treated samples increased during marination. Results are shown in Figure 4.29. and Table C.9.

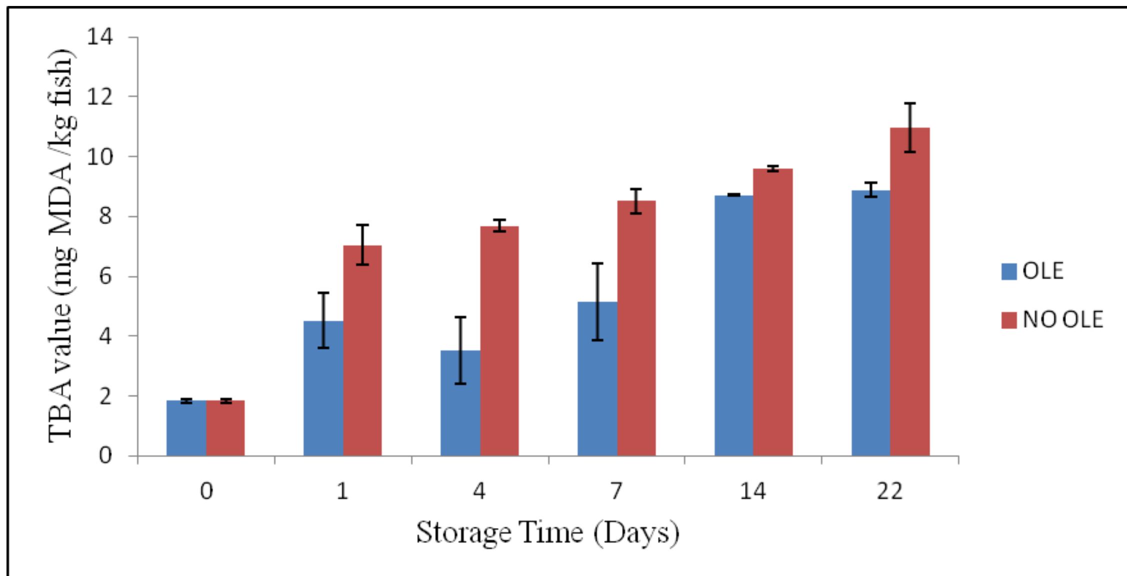


Figure 4.29. Changes in oxidative stability of fish fillets during marination process

In fish material with a perfect quality, TBA values should be less than 3 mg. Fish having TBA value between 3-5 mg MDA/kg are accepted as good quality and consumption level was determined as 8 mg/kg fish by Schormüller (1968, 1969), reported by Cadun et al., (2005). While control samples exceeded the consumption level of 8 mg in 7 days, it took OLE samples to exceed the value for 14 days. Although the

consumption level is exceeded, samples treated with OLE showed considerably better results than control samples at the end point of marination process.

4.2.2.8. Effect of Olive Leaf Extract on Microbial Counts of Sardine Fillets

Lactic acid bacteria counts (LAB), total viable counts (TVC), psychrotrophic bacteria counts (PCC) and mold and yeast formation (YF) data are shown in Table 4.5. Microbial loads of fish fillets during marination process decreased during process.

Table 4.5. Changes in microbiological analysis during mariartion

Bacterial Counts (logCFU/g fish)							
Storage Time (Days)							
	Treatment	0	1	4	7	14	22
LAB	Control	2.88	1.49	1.18	1.28	<1	<1
	OLE	2.88	<1	<1	1.91	<1	<1
TVC	Control	4.92	2.41	2.42	<1	<1	1.21
	OLE	4.92	1	1.47	<1	1.78	<1
PC	Control	4.40	<1	<1	1.36	<1	<1
	OLE	4.40	<1	<1	<1	<1	<1
YF	Control	3.71	<1	<1	ND	ND	ND
	OLE	3.71	<1	ND	ND	ND	ND

The initial LAB count of fish fillets was 2.88 log CFU/g fish. Cadun et al., (2005 and 2008), found that the initial LAB count of deepwater pink shrimps as 3.92 and 4.04 log CFU/g respectively and did not detected any LAB formation at the beginning/during marination process. Also, Kılınç and Çaklı (2004) reported that LAB counts of raw sardine as 3.62 logCFU/g and observed the decrease of LAB counts at first day of marination process. LAB counts remained under 1 logCFU/g during marination.

Total viable count of fresh sardine fillets were, 4.92 logCFU/g, which is in agreement with literature. TVC values of both samples decreased starting from the first day of marination. The decrease was more rapid in fillets treated with OLE than control

samples. At the end of marination process TVC values of both samples were approximately 1 log CFU/g.

In fresh fish fillets, psychrotrophic bacteria count was found 4.40 logCFU/g and immediately decreased to lover values than 1 logCFU/g. and remained almost constant during marination.

Yeast and Mold count of fresh sardine fillets was found 3.71 log CFU/g and also dropped lower than 1 log CFU/g after beginning of marination. For control samples, starting from 4th and 7th day till the end of the process, mold and yeast formation remained undetected for OLE and control samples, respectively.

According to these findings, it could be concluded that employing OLE in marinade solution slightly affected lactic acid bacteria count, total viable count and mold and yeast formation within marinated fish fillets. OLE did not show any additional effect on psychrotrophic bacteria counts during marination.

4.2.2.9. Effect of Olive Leaf Extract on Color Attributes of Sardine Fillets

Color measurements of marinated sardines are shown in Table 4.6. L* values of sardines in control group were 40.66 and 62.67 at the beginning and the end of marination process, respectively. The lightness of samples increased during marination. In samples treated with OLE, L* values were 40.66 and 62.66 in first and last day of marination. Same increase in lightness also observed for these samples. There was no significant difference between control and OLE treated samples at the end point of marination.

Table 4.6. Color attributes of sardine fillets during marination process

Color Values		Storage Time (Days)					
		0	1	4	7	14	22
L* (Lightness)	Control	40.66 ± 1.98^{w}	$60.95\pm1.16^{a,x}$	$59.21\pm4.28^{a,y}$	$64.46\pm2.66^{a,x}$	$59.85\pm4.34^{a,x}$	62.16 ± 3.41^{y}
	OLE	40.66 ± 1.98^{w}	$60.39\pm2.37^{a,xy}$	$60.78\pm5.18^{a,xy}$	$61.02\pm3.76^{b,xy}$	$58.69\pm2.32^{a,x}$	62.67 ± 2.86^{y}
a* (redness)	Control	4.97 ± 1.57^{z}	$2.41\pm0.46^{a,y}$	$2.26\pm0.84^{a,y}$	$1.21\pm0.63^{b,v}$	$1.71\pm1.05^{b,vy}$	1.09 ± 0.34^{v}
	OLE	4.97 ± 1.57^{z}	$2.34\pm0.81^{a,w}$	$3.32\pm1.58^{b,x}$	$2.24\pm1.31^{a,w}$	$4.97\pm1.48^{a,y}$	1.31 ± 0.32^{v}
b* (yellowness)	Control	3.98 ± 0.79^{w}	$2.92\pm0.78^{a,x}$	$2.81\pm0.73^{a,x}$	$7.02\pm2.26^{a,y}$	$6.79\pm1.50^{a,y}$	10.38 ± 3.39^{z}
	OLE	3.98 ± 0.79^{w}	$5.97\pm2.05^{b,x}$	$7.32\pm2.72^{b,xy}$	$8.70\pm2.63^{a,y}$	$7.89\pm1.83^{a,y}$	11.28 ± 1.94^{z}

$^{a-b}$:Means having different letters within each storage time differs significantly ($p<0.05$)
$^{w-z}$:Means having different letters within each treatment differs significantly ($p<0.05$)
mean ± S.D., (n= 30).

Both control and OLE treated samples show a decrease from 4.97 to 1.09 and 1.31 in redness (a*) values, respectively, during marination period. On the other hand yellowness (b*) both control and OLE treated samples increased due time of marination from 3.98 to 10.38 and 11.28 respectively. The higher yellowness value possessed by OLE samples may be attributed to the yellow-brown color of olive leaf extract. At the end of matination, no significant difference between L*, a* or b* values of both control and OLE treated samples.

CHAPTER 5

CONCLUSION

OLE is a potent source of polyphenols having antioxidant, antimicrobial, anti-inflammatory and antiviral properties. The aim of this study was to investigate these properties possessed by OLE and benefits in possible food applications. Amount of oleuropein, which is the most abundant polyphenols in olive leaf extract, was 12.18% in Gemlik type olive leaves which corresponds to 121.83 mg/g leaf. Relative antioxidant capacity of OLE obtained from these leaves was 966 µg ascorbic acid eq./mg OLE and total phenol content was 197.42 mg GAE/g OLE.

Microdilution tests were performed by 96 well microplate method for OLE against selected bacteria in presence of positive control penicillin. According to the results, the most susceptible bacteria were determined to be *E. coli, L. innocua* and *S. carnosus*.

The results clearly indicated that using of 2% and 3% OLE had the beneficial effect in controlling the microbial load (TVC and TCC) of beef cubes during 9 days of storage at 4ºC. However, using OLE on beef cubes showed no inhibitory effect on LAB. Two percent OLE treatment applied to beef cubes also delayed the oxidative deterioration compared to the other samples. On the other hand, no beneficial effects of OLE treatment were determined on surface color attributes, moisture content and pH values of beef cubes.

In sardine samples, employing 300 ppm OLE was effective in controlling TVC and LAB counts of sardine (*Sardina pilchardus*) fillets. In addition to this, OLE took a more rapid action against yeast and mold formation in sardine samples. Application of OLE on fillets delayed the oxidative deterioration, TVB-N formation. On the other hand, OLE had no beneficial effect on surface color attributes, TMA formation, levels of salt, acid, pH of marinade solution and/or sardine fillets; ash, crude protein, fat content and moisture of fish fillets.

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