EXTRACTION OF OLEUROPEIN FROM OLIVE LEAVES

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ABSTRACT

EXTRACTION OF OLEUROPEIN FROM OLIVE LEAVES

The aim of this study is to obtain oleuropein, which is the main phenolic compound content of olive leaf by soxhlet extraction. Oleuropein has been known in the health field for a long time and is found in high rates in our country. In this context, the effect of various solvent types (ethanol, methanol, acetonitrile and water), extraction time (4 cycles, 4 hours, 8 hours), particle size (250-500 μm and 900-2000 μm) and the pre-treatment applied to the olive leaf on the yield of oleuropein was investigated.

The amount of oleuropein in the liquid product was determined using a High Performance Liquid Chromatogram (HPLC). When the grain size of the raw material to be used in extraction was reduced, higher oleuropein was obtained. The use of solvents in an aqueous form resulted in a higher amount of oleuropein compared to pure solvents. Increasing the extraction time caused a significant increase in the amount of oleuropein. On the contrary, it was observed that the pretreatment applied to olive leaves caused a decrease in the yield of oleuropein. As a result of these findings, the highest oleuropein amount and extraction efficiency were obtained after 8 hours of extraction period by using olive leaf with 250-500 μm grain size and 80% methanol solution as the solvent. The highest oleuropein amount was found to be 13.35 oleuropein mg/g dry leaves and the highest extraction efficiency under these conditions was found to be 36 %.

ÖZET

OLEUROPEİNİN ZEYTİN YAPRAKLARINDAN ÖZÜTLENMESİ

Bu çalışmanın amacı, sağlık alanında uzun süredir bilinen ve ülkemizde yüksek oranda bulunan zeytin yaprağının ana fenolik bileşik içeriği olan oleuropeini sokslet ekstraksiyonu ile elde etmektir. Bu bağlamda, çeşitli solvent tiplerinin (etanol, metanol, asetonitril ve su), ekstraksiyon süresinin (4 döngü, 4 saat, 8 saat), parçacık boyutunun (250-500 μm ve 900-2000 μm) ve zeytin yaprağına uygulanan ön işlemin oleuropein verimi üzerine etkileri araştırılmıştır.

Sıvı ürün içindeki oleuropein miktarı Yüksek Performanslı Sıvı Kromatogram (HPLC) kullanılarak belirlenmiştir. Ekstraksiyonda kullanılacak hammaddenin parçacık boyutu küçüldüğünde daha yüksek oleuropein eldesi gözlemlenmiştir. Saf solventlere kıyasla sulu formdaki çözgenlerin kullanılması ile daha fazla miktarda oleuropein elde edilmesini sağlamıştır. Ekstraksiyon süresinin arttırılması, oleuropein miktarında önemli bir yükselmeyle sonuçlanmıştır. Bunun aksine zeytin yapraklarına uygulanan ön işlemin, oleuropein veriminde ve miktarında düşüşe neden olduğu gözlemlenmiştir. Bu deneyler sonucunda, en yüksek oleuropein miktarı ve ekstraksiyon verimi 250-500 μm parçacık boyutuna sahip zeytin yaprağı ve çözücü olarak 80 % metanol çözeltisi kullanılarak 8 saatlik ekstraksiyon süresi sonunda elde edilmiştir. En yüksek oleuropein miktarı 13.35 oleuropein mg/g kuru yaprak olarak bulunmuş ve en yüksek ekstraksiyon verimi bu koşullarda 36 % olarak bulunmuştur.

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To my dear family,

CHAPTER 1

INTRODUCTION

1.1. The Aim and the Importance of the Study

The importance of this study is to determine and compare oleuropein and extraction yield in a wide range of solvents in both pure and aqueous concentrations under various parameters.

The aims of this study can be listed as follows:

- To contribute to the economy of our country by obtaining extracts from the leaves of olive trees, which have a great commercial value in our country,
- To extract polyphenol oleuropein from olive leaves by soxhlet extraction with different solvents (water, ethanol, methanol and acetonitrile) and to examine the effects of various working parameters (extraction time, particle size and pretreatment) on extract and oleuropein yields.

In order to optimize the recovery of phenolic components, it is also among the aims of this thesis to examine the parameters affecting the extraction process in extraction of plant extracts by soxhlet extraction and to contribute to research on these issues.

1.2. The Importance of Herbal Extracts

Plant extracts are structures that have long been used in the health field and are obtained from the fruits or leaves of plants (Vinatoru 2001). Determination of phenolic compounds in plants is very important due to their natural drug activities (antimicrobial, antioxidative). Biophenols also protect living things against UV rays (Balasundram, Sundram, and Samman 2006; Rahaiee, Moini, and Hashemi 2015).

1.2.1. Phytochemicals (Plant Chemicals)

Plants produce various organic compounds (phytochemicals) and basic chemical components (carbohydrates, proteins, nucleic acids, etc.). Phytochemicals produced by plants are used for chemically developed compounds that play an active role in the structure of plants (Shahidi et al.2019).

In another definition, the word 'phyto-', which means plants in Greek, is the source of the word phytochemicals. For this reason, plant chemicals are also called phytochemicals. Although it cannot be detected in most of the fruits, vegetables, and grains that make up the structure of the plants, it is known that there are at least 5000 different plant chemicals (Liu 2004).

The health benefits of phytochemicals have been widely studied. Although they are called non-nutritional compounds, they are used as active ingredients in foods. The health benefits of plant chemicals can be listed as follows:

- To react in chemical structure for biochemical reactions (substrates),
- Ensuring that enzymes are active (cofactors) in enzymatic reactions,
- Adhering to undesirable structures in the intestine and destroying them
- Digesting essential nutrients such as carbohydrates, fat and protein,
- To create a growth environment for beneficial bacteria in the stomach and intestines,
- Detecting harmful bacteria in the gut and reducing its effectiveness (selective inhibitor) (Dillard and German 2000).

The role of non-nutritive plant chemicals (phytochemicals) in reducing the risk of disease of some foods obtained from plants is quite large. These chemicals have less potential compared to drugs. However, when consumed regularly and in the right proportions, it can be clearly beneficial (Garcı, Toma, and Espı 2007).

Phytochemicals, which are the main components of essential oils and found in the flowers of plants, consist of various polyphenol groups, terpenoids, alkaloids and compounds containing sulfur (Shahidi et al.2019). The general distribution of these herbal products is given in Figure 1.1

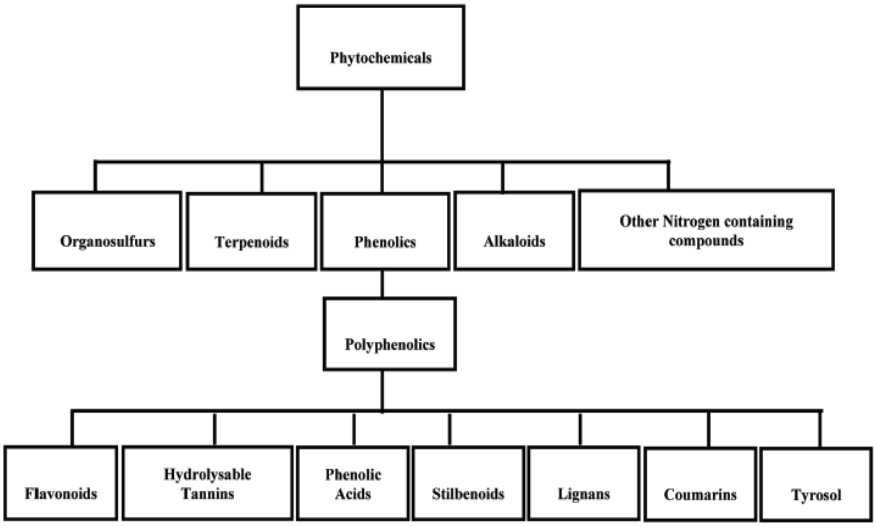


Figure 1.1. Phytochemical groups

(Source: Shahidi et al., 2019)

1.2.2. Functional Foods and Nutraceuticals

Nutraceuticals and functional foods are defined as food compounds that are more beneficial for health than essential nutrients needed (Sevilmiş, Olgun, and Artukoğlu 2017). The definition of "functional food" was first developed in Japan in 1984 (Hardy 2002; Kwak and Jukes 2001). Foods that help the body's human physiology and many metabolic functions and therefore strengthen the immune system are called functional foods (Bigliardi and Galati 2013; Kurt 2012).

While functional foods can be a nutrient that has not undergone any chemical changes, it can also appear as a genetically modified food with various methods (Krystallis, Maglaras, and Mamalis 2008).

Nutrients have a role in the development of the human body and the protection of its organs and systems. These foods are called nutritional functions. In other words, it can be said that it provides energy, vitamins and minerals produced from essential nutrients (proteins, carbohydrates and fats) required for a healthy body (Bigliardi and Galati 2013). Nutraceuticals are used in medical pharmaceutical forms (powders, capsules, pills, etc.) (Garcı, Toma and Espı 2007).

1.2.3. Phenolic Compounds

There are more than 200,000 chemicals in the world that are obtained differently from plants. These chemicals are called primary and secondary metabolites. Primary metabolites are essential for our basic food sources. This group of metabolites plays a role in maintaining the cell for carbohydrates, proteins, and various fatty acids. Secondary metabolites are important to plants, although they are not directly involved in respiratory and photosynthesis reactions. The structure and chemistry of the two main metabolites differ from each other. Secondary metabolites are more diverse and responsible for plant defense (Chikezie, Ibegbulem, and Mbagwu 2015). The role of secondary metabolites is to protect the plants from UV rays and oxidants. Secondary metabolites are classified according to their structure as follows: (1) terpenoids, (2) alkaloids and sulfur-containing compounds with nitrogen in their structure, (3) flavonoids called some phenolic and polyphenolic compounds. These compounds bind to primary metabolites through many reactions (Vora and Pednekar 2017).

Phenolic compounds, one of the secondary metabolites, are abundant in plants. Phenolic compounds play an important role in the growth, development, and reproduction of plants by fighting against pathogens and harmful microorganisms. It is also responsible for sensory properties such as color, bitterness, taste, and smell in vegetables and fruits (Chikezie, Ibegbulem and Mbagwu 2015).

Phenolic compounds are called structures that have one or more hydroxyl groups attached to the aromatic ring. Phenol is the lowest part of the whole group (Nicholson 2006). Its structural representation is shown in Figure 1.2.

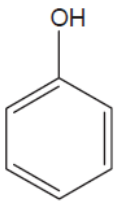


Figure 1.2. Structural representation of phenol
(Source: Nicholson 2006)

The structure of phenols can range from simple molecules to complex compounds. Those with high phenolic compound properties are in contact with one or more phenolic groups with mono- and polysaccharides. Phenolic compounds can also be added to the esters. They create structural diversity according to their bonding state, so there is a wide variety of polyphenols in nature. More than 8000 polyphenol structures are known as a result of existing research (Del et al. 2013). The most important phenolics are examined in three parts as flavonoids, phenolic acids and tannins (Heleno et al. 2015).

Flavonoids: Flavonoids make up the largest part of phenolic groups as they contain 6000 or more of the more than 8000 phenolic compounds found in plants. It has a structure of 15 carbons and has a low molecular weight (Gil-izquierdo 2008). It is formed by the combination of two aramotic rings, A and B, as three carbons with the aromatic molecule C, which has atoms other than carbon (Diane F. Birt 2013). Figure 1.3 shows the structure of a flavonoid molecule.

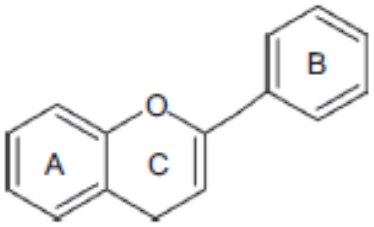


Figure 1.3. Structure of a flavonoid molecule
(Source: Diane F. Birt 2013)

Flavonoids bind to non-carbon (heterocyclic) molecules in their rings and differ in their bonding patterns. These differences cause flavonoids to be divided into subclasses (Merken and Beecher 2000). Figure 1.4. shows the major flavonoid classes and structures.

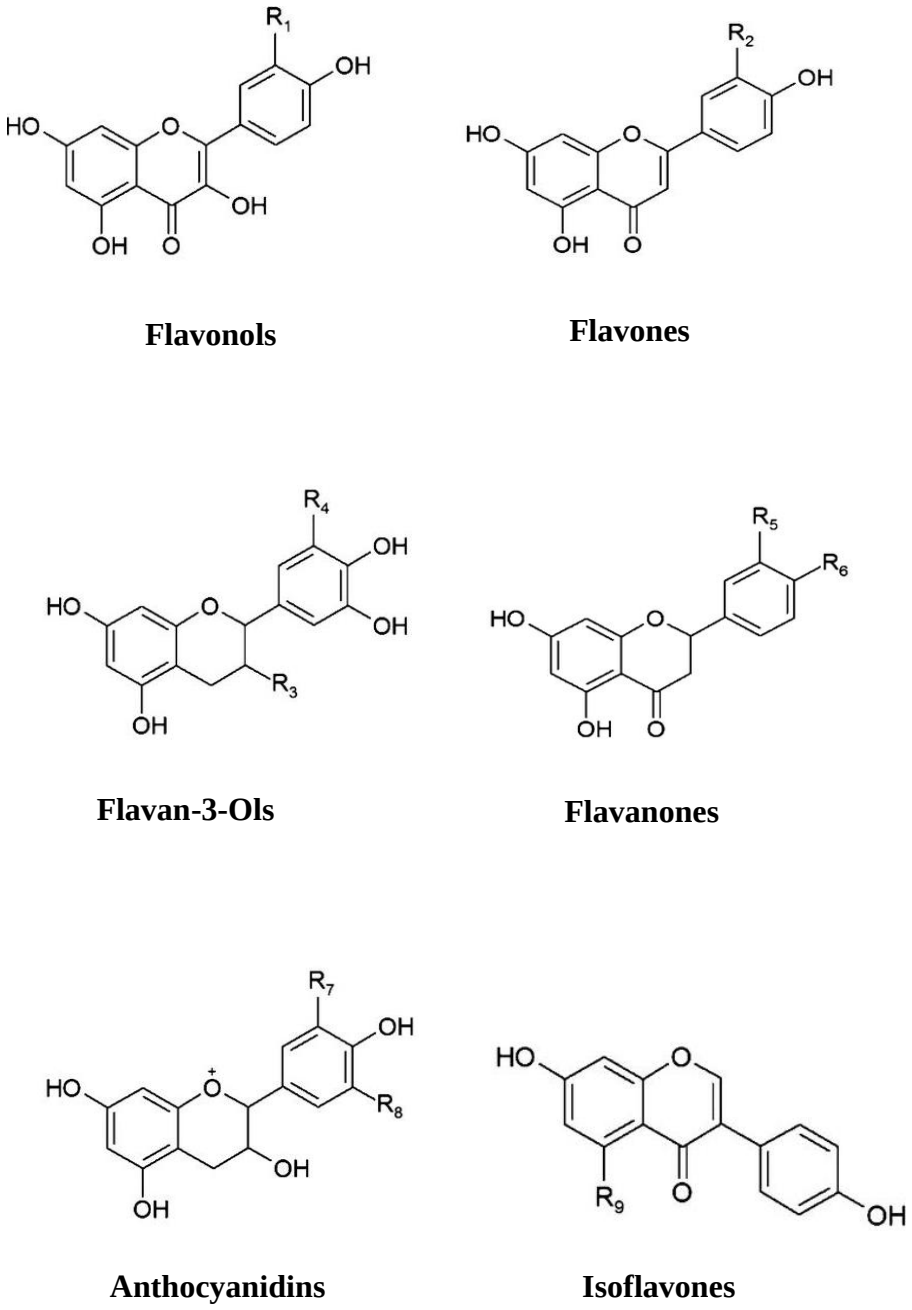


Figure 1.4. Generic structures of major classes of flavonoids
(Source: Diane F. Birt 2013)

Flavonoids are found in plants in the form of glucose molecules (glycosides) and aglycones. Instead of simple acidic carbohydrates, carbon atoms in its structure and hydrogen-oxygen atoms bonded by covalent bonds are used (Harborne and Williams 2000). The effect of this phenolic compound on health is being studied. Although it is

known that this effect is mostly caused by antioxidant function, it is estimated that functions other than antioxidants cause diseases such as heart and cancer (Shahidi 2018). Flavonoids are found in alcoholic beverages, herbs, dried grains, nuts, vegetables, and fruits (Faggio et al. 2017; Yeo and Shahidi 2017).

Phenolic Acids: Phenolic acids are found in plants, grains, seeds, and legumes. These acids can be used instead of hydroxybenzoic and hydroxycinnamic acids (Shahidi et al. 2019). Hydroxybenzoic acids are found in most plant species. Examples of these acids are gallic acid, p-hydroxybenzoic acid, and vanillic acid (Robbins 2003). The general structural representation of benzoic acid is given in Figure 1.5. Table 1.1 shows the types of hydroxybenzoic acids.

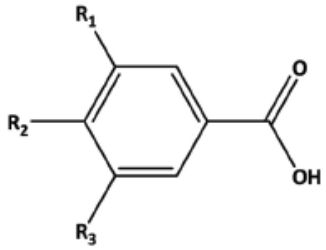


Figure 1.5. General structural of benzoic acid
(Source: Shahidi et al. 2019)

Table 1.1. Structures of common benzoic acid derivatives

Acid	**R_1**	**R_2**	**R_3**
p-Hydroxybenzoic	H	OH	H
3,4-dihydroxybenzoic	H	OH	OH
Vanilic	OCH_3	OH	H
Syringic	OCH_3	OH	OCH_3
Gallic	OH	OH	OH
Protocatechuic acid	H	OH	OH

Hydroxycinnamic acids have the structure of $C_6 – C_3$ and are linked together by double bonds. They are mostly found in foodstuffs as monomers, dimers or polymers. During food processing (sterilization, freezing or fermentation), they can obtain free forms (Factors, Rommel, and Wrolstad 1993). Hydroxycinnamic acids are found in nature as caffeic, ferulic, synaptic, p-coumaric and chlorogenic acids (Herrmann, Nagel, and Herrmann 2009). The general structure of cinnamic acid is shown in Figure 1.6. The types of hydroxycinnamic acids are given in Table 1.2.

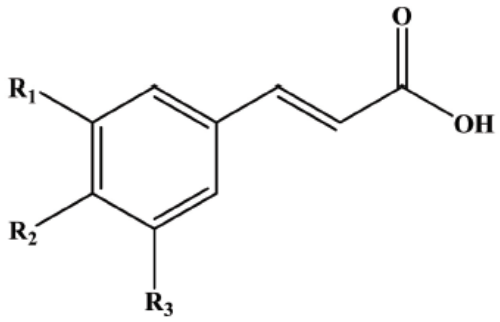


Figure 1.6. General structural of cinnamic acid
(Source: Shahidi et al. 2019)

Table 1.2. Structures of common hydroxycinnamic acid derivatives

Acid	**R_1**	**R_2**	**R_3**
p-Coumaric	H	OH	H
Caffeic	H	OH	OH
Ferulic	OCH_3	OH	H
Sinapic	OCH_3	OH	OCH_3

Hydroxycinnamatic acids are generally found in nature in fruits (peaches, strawberries, citrus fruits, plums, etc.). Caffeic and p-coumaric acids, which are types of these acids, are widely found in fruits (Archivio et al. 2007). As the name suggests, while caffeic acid is abundant in coffee, it is also found in grapes, pears, apples and oranges (Naczk and Shahidi 2006). Coumaric acid is found mostly in strawberries, although it can be found in other fruits. Sinapic acid is abundant in Brassica vegetables and grains, a genus of mustard family (Mattila 2005). Ferulic acid constitutes almost all of the content of wheat (Manach et al. 2004). Coffee beans are the most common food ingredient in clogenic acids such as caffeic acids (Clifford 1999). The structure of the chlorogenic acid is shown in Figure 1.7.

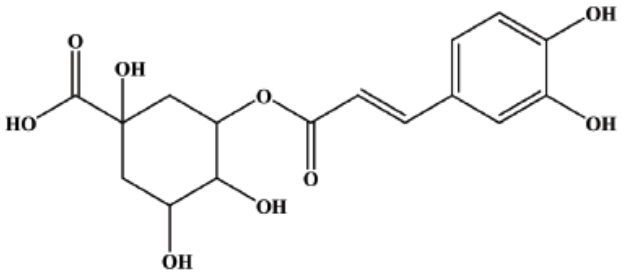


Figure 1.7. Structure of chlorogenic acid
(Source: Shahidi et al. 2019)

Tannis: Tannins are in constant interaction with plants and food webs. They are antimicrobial agents and can also act against grass-fed creatures. Its structural properties are that it can easily dissolve in water and form an insoluble bond with proteins. These acids consist of a large number of hydrogen and oxygen atoms that are structurally linked together by covalent bonds. They also contain functional groups and therefore exist in ester form. Tannins gain reactivity by forming intermolecular hydrogen bridges. Therefore, they impair the fatty ability of proteins and cause a bitter taste in most plants and fruits. The effectiveness of the interaction between protein and tannins depends on the molecular weight of the tannins. High molecular weight acids prevent the movement of fiber gaps in the structure of proteins and while forming a bridge between the two, they cannot interact with low molecular weight tannins (Ferrer et al. 2008).

Lignans: Lignans are the phenolic group derived from cinnamic acids and plants by combining two C_6-C_3 molecules. Its structural representation is given in Figure 1.8. Lignans were mainly studied in two main groups: These are secoisolarisinol and matairesinol. A structural representation is given in Figure 1.9. Lignan has many health benefits.

- It prevents cell division in humans and animals and causes the tumor to be destroyed.
- It treats hepatitis caused by viruses in China and is used for liver protection (Moss, Mary, and Road 2000).

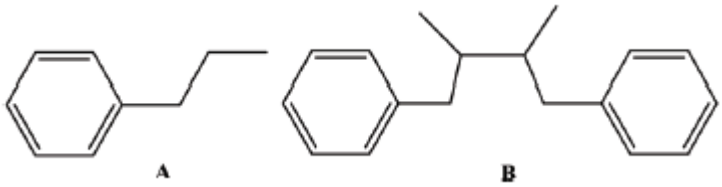


Figure 1.8. Parent structure of lignan (coupling (B) of two C_6-C_3 molecules (A)
(Source: Shahidi et al. 2019)

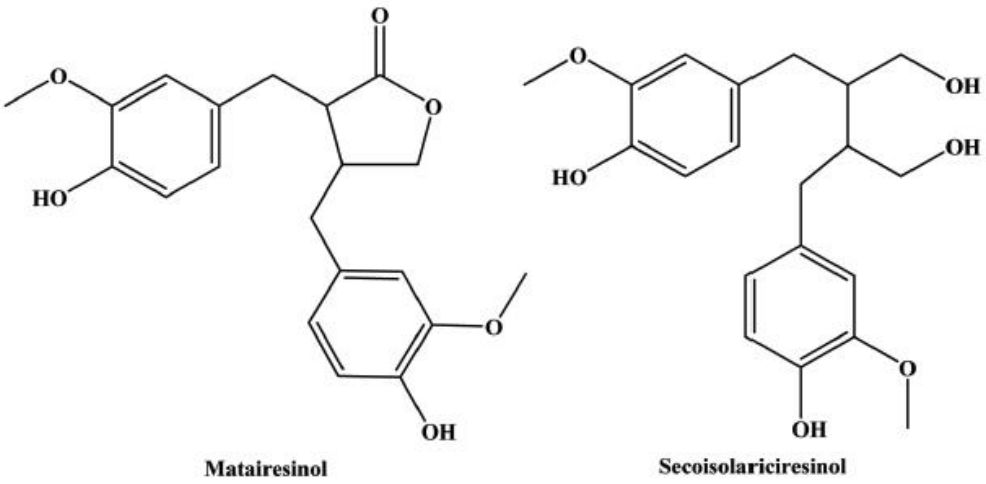


Figure 1.9. Structure of major dietary lignans
(Source: Shahidi et al. 2019)

Stilbenoids: Stilbenes are phenolics with a base-like carbon skeleton (C_6-C_2-C_6) containing 1,2-diphenylethylene as certain atom groups responsible for the characteristic chemical reactions of the molecules they contain. The structural representation of resveratrol (3,5,4'-trihydroxystylbene), which is a type of stilbene in free or glycoside structures and seen in some fruits, is given in Figure 1.10 (Nichenametla et al. 2007).

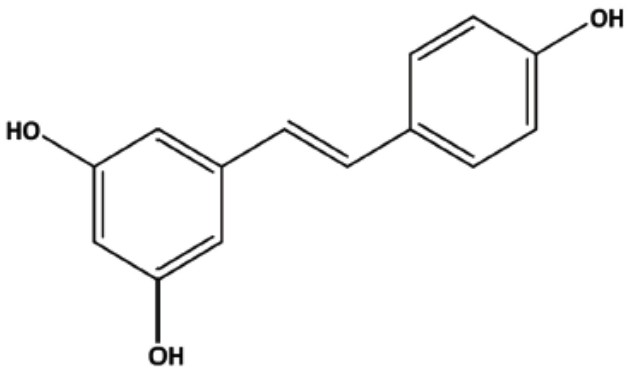


Figure 1.10. Structure of trans-resveratrol (trans-3,5,4-trihydroxystilbene)
(Source: Shahidi et al. 2019)

Hydroxytyrosol: It is known as the phenolic compound (hydroxytyrosol) in olive oil and wine has many health benefits such as reducing oxidative damage, eliminating inflammation in the body, treating the endothelial form, stopping tumor growth and protecting the central nervous system (Rodríguez-morató et al. 2016). The structural representation of hydroxytyrosol is shown in Figure 1.11.

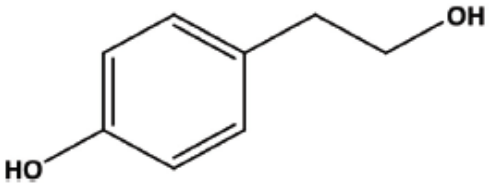


Figure 1.11. Structure of hydroxytyrosol
(Source: Shahidi et al. 2019)

1.2.4. Antioxidant Activity of Phenolic Compounds

1.2.4.1. Free Radicals

Free radicals can be defined as free molecules that are not attached to an electron. Since most free radicals do not bind to electrons, they can exhibit highly unstable properties. They can exchange electrons with molecules other than themselves. The reason why they act as oxidant or reductant is due to this electron exchange (Cheeseman and Slater 1993). Free radicals can be created by adding electrons to an uncharged atom or molecule, or by stealing electrons from that atom and molecule. Various energies are needed in this formation process. Heat, UV light and radiation are some of them (Halliwell 2001; Ima 2004). Because they do not pair with an electron, they tend to cling to electrons. In such cases, they go to compounds with nitrogen atoms such as proteins and nucleic acids. At the same time, they attack regions with excess electrons in double-bonded carbon fatty acids (Ima 2004).

Free radicals are known as reactive oxygen species (ROS) with oxygen radicals in the center and reactive nitrogen species (RNS) with nitrogen radicals in the center (Halliwell 2001). These types (ROS and RNS) can be distinguished according to whether they are radical or not. Compounds that exhibit radical properties are electron unpaired species and are free radicals in their own right. Species that do not show radical properties perform oxidizing reactions in living organisms (Durackova 2015; Phaniendra and Babu 2015). Free radicals fail to defend against any substance that harms living organisms. Therefore, it is known to have a negative role in organisms (Ková 2010). Free radical sources can be investigated inside (endogenous) or outside (exogenous) of the cell or organism being studied. For example, the (endogenous) sources formed inside the cell are found in mitochondria, endoplasmic reticulum and peroxisomes where oxygen is consumed a lot (Phaniendra and Babu 2015). Representation of endogenous sources is given in Figure 1.12.

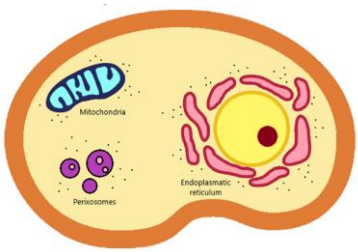


Figure 1.12. Typical endogenous sources of free radicals

(Source: Preedy 2020)

Sources occurring outside of the examined tissues and cells are given in Figure 1.13.

Figure 1.13. Most common exogenous sources of free radicals

(Source: Preedy 2020)

1.2.4.2. Oxidative Stress

Oxidative stress occurs most frequently in two situations. An excess of ROS, which is a kind of free radical, and a decrease in its antioxidant properties create oxidative stress. This situation harms human tissues and cells physically, chemically and psychologically and may lead to various diseases (Tian et al. 2007). Living things have developed a good defense system in response to this negative situation. Resources that include various defense systems such as physical and antioxidant defense try to overcome this negative oxidative stress (Valko et al. 2007).

Some diseases caused by oxidative stress are given below (Agarwal and Prabakaran 2005; Pham-huy, He, and Pham-huy 2008; Valko et al. 2007):

- Cardiovascular diseases (heart diseases, hypertension etc.)
- Neurological diseases (Alzheimer's disease, memory loss, parkinson etc.)
- Tumor formation and cancer (lung, breast, ovarian cancer, etc.)
- Chronic lung diseases
- Stomach and intestinal diseases (peptic ulcer, colitis, etc.).

1.3. Olive Leaf and Characteristic Properties

The olive tree (Olea europaea L.) comes from the *Oleaceae* family, which includes 900 evergreen species and 24 different genera. Trees of the same species as *Olea europaea L*. are shown in Table 1.3.

Table 1.3. Taxonomic classification of the olive tree
(Source: Omaki et al. 2003)

Kingdom	**Plantae**
Division	Magnoliophyta
Class	Magnoliopsida
Order	Lamiales
Family	Oleaceae
Genus	Olea
Species	*E. oleaster* *E. sativa*
Binomial name	*Olea europaea*

The fruit leaves and other parts of the olive tree (branches and flowers) contain significant amounts of biophenols. Olive leaves contribute to properties such as color and taste, depending on the variety and density of the fruit. In addition, the number of leaves creates a difference between various tissues (Bilgin and Selin 2013; Rahmanian, Jafari, and Wani 2015). Among olive tree products, the biophenol ratio of the leaf is

different from that of flowers, fruits and branches (Musa and Bertrand 2016). Known as the symbol of power and peace, olive leaf was first used as medicine in Egypt. It has been used in the treatment of many diseases (flu, cold, tooth and ear pain, etc.), especially in Mediterranean cultures. Doctors recommended olive leaf tea for common malaria in the mid-19th century (Gao et al. 2008; National 2014). Polyphenols are one of the phytochemical groups that form the most structure of plants (Gianmaria F. Ferrazzano et al. 2011). Polyphenols are protective against all kinds of disease-causing organisms, so they have both physiological and morphological importance in plants and trees (Astro 2006; Rahmanian, Jafari, and Wani 2015).

Polyphenols in olives constitute the secondary metabolites group in olives due to their properties such as water solubility and molecular weight. The phenolic compounds (polyphenols) found in the olive tree differ structurally. Phenolic compounds found in the fruit and oil of olives; It consists of four main parts as flavonols, phenolic acids, phenolic alcohols and secoiridoids (Ghanbari et al. 2012). The phenolic compounds found in olive leaves are specified in five groups. These are oleuropeosides, flavones, flavonols, flavan-3-ols and substituted phenols (Musa and Bertrand 2016). The names of the phenolic groups contained in the olive leaf and examples of these groups are shown in Table 1.4.

Table 1.4. Phenolic compounds found in olive leaves

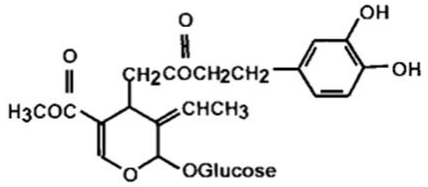
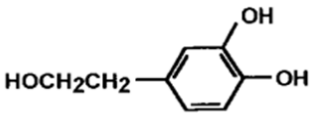
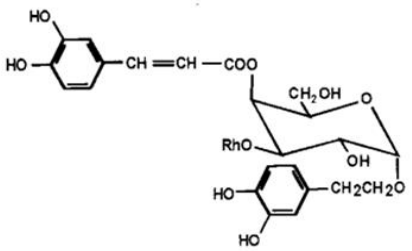
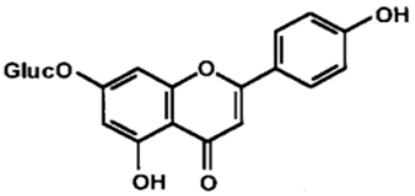
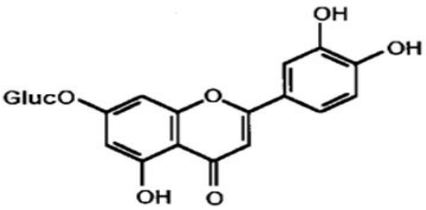
(Source: Benavente-García et al. 2000)

Group	Examples
Oleuropeosides	Oleuropein, Verbascoside
Flavones	Luteolin-7-glucoside, Apigenin-7-glucoside, Diosmetin-7- glucoside, Luteolin, Diosmetin
Flavonols	Rutin
Flavan-3-ols	Catechin
Substituted Phenols	Tyrosol, Hydroxytyrosol, Vanilin, Vanillic acid, Caffeic acid

Oleuropein is the most abundant phenolic compound in olive leaves. This is followed by the order hydroxytyrosol> luteolin> apigenin> verbascoside> floven-7-

glucosides (Musa and Bertrand 2016). The chemical structure of phenolics, which olive leaves contain the most, is shown in Table 1.5.

Table 1.5. Structural representation of the most abundant phenolics in olive leaves (Source: Benavente-García et al. 2000)

Phenolic compound	**Chemical formula**
Oleuropein	
Hydroxytyrosol	
Verbascoside	
Apigenin-7-glucoside	
Luteolin-7-glucoside	

1.4. Oleuropein and its Properties

1.4.1. Oleuropein Chemistry

Oleuropein is the most abundant biophenol in olive leaves. It is also known as the phenolic compound responsible for the bitterness of the olive aroma. Structurally, the glucoside consists of elenolic acid esterified with 2- (3,4-dihydroxyphenyl) ethanol (hydroxytyrosol) (Omar 2010; Soler-Rivas, Espiń, and Wichers 2000). Oleuropein has been examined in three main parts (hydroxytyrosol, monoterpene and glucose parts) as its molecular structure is shown in Figure 1.14.

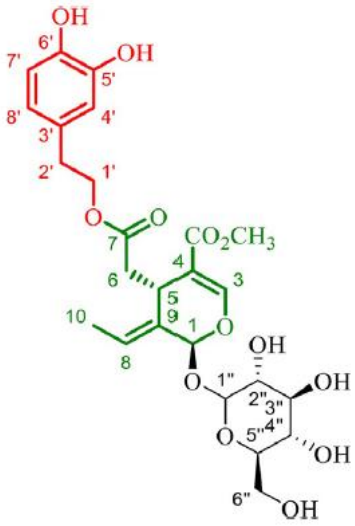


Figure 1.14. General molecular structure of oleuropein. Representation of the hydroxytyrosol (red), monoterpene (green) and glucoside (black) moieties (Source: Cavaca et al. 2020)

1.4.2. Biosynthesis of Oleuropein

Iridoids (A) are called monoterpenes that contain a heterocyclic ring attached to the cyclopentane ring from the cycloalkane group. Secoiridoids (B) are composed of 7

and 8 linked iridoids in the open structure of the cyclopentane ring, a cycloalkane group. Figure 1.15 shows their general structure. Oleuropein and other secoiridoids specific to oleocyte plants are defined as oleocytic secoiridoids or oleocytes. Secoiridoids consist of monoterpene synthesis and branching in the mevalonic acid pathway where the phenolic unit is joined (Obied et al.2008).

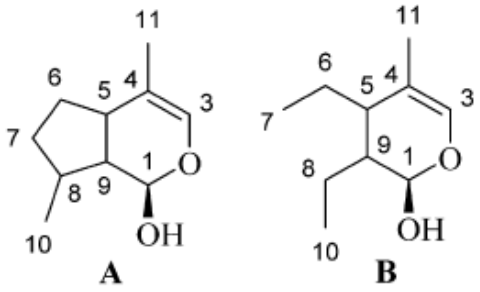


Figure 1.15. General representation of iridoids (A) and secoiridoids (B) molecular structures (Source: Cavaca et al. 2020)

Mevalonic acid consists of the condensation of three acetyl-SCoA molecules, which are the source of the HMG-CoA ester, which is formed after some hydrolysis and enzymatic reactions (Obied et al. 2008). The formation of oleuropein in *oleaceae* is shown by the biosynthetic pathway in Figure 1.16.

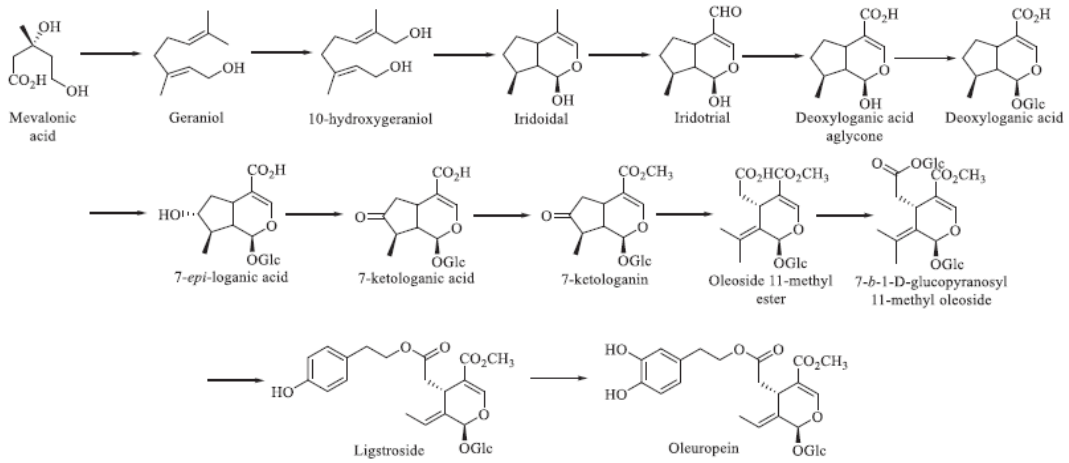


Figure 1.16. Proposed biosynthetic pathway for the formation of oleuropein (Source: Obied et al. 2008)

1.4.3. Health Effects of Oleuropein

Oleuropein has many beneficial properties in terms of health. Some of these can be listed as anti-inflammatory, anti-atherogenic, anti-cancer, antimicrobial and antiviral. The reason why it has an important place in the food and medicine industry in Mediterranean countries is that it has these pharmacological properties (Carluccio et al. 2003; Fredrickson 2000; Tripoli et al. 2005; Visioli, Bellosta, and Galli 1998; Visioli, Poli, and Galli 2002). Figure 1.17 shows the pharmacological effects of oleuropein.

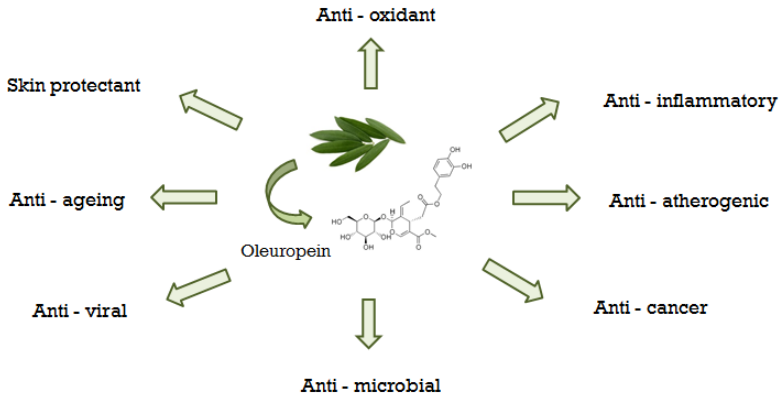


Figure 1.17. Pharmacological properties of oleuropein
(Source: Omar 2010)

Antioxidant activity: Oleuropein largely eliminates metal oxide formation (oxidation) of low density lipoproteins (LDL) (Visioli, Bogani, and Galli 2006; Visioli, Galli, and Galli 2002). Oleuropein can eliminate nitric oxide and contribute to the formation of nitric oxide synthase in the cell (De, Eugenia, and Domínguez 2001). The oxidative substance that damages proteins and enzymes in living things and produces in vivo in injured areas is called hypochlorous acid (HOCl). Oleuropein has the ability to destroy this acid (Visioli, Poli and Galli 2002).

Anti-inflammatory effect: Oleuropein increases nitric oxide (NO) production in macrophages, which are part of the immune system. Therefore, the activity of the immune system increases (Visioli, Bellosta and Galli 1998). At the same time, oleuropein is known to increase anti-inflammatory effects by destroying both

lipoxygenase enzyme and leukotriene B_4 production (Puerta, Gutierrez, and Hoult 1999).

Anti-cancer effect: Oleuropein aglycone (the sugar-free part of the glycoside) is known to be the most potent phenolic compound that reduces the activity of cells that make up breast cancer. SKBR3 cells that increase HER2 cancerous tissues and HER2 negative MCF-7 cells are known to be approximately five times more sensitive to oleuropein aglycone (Menendez et al. 2007). In later years, oleuropein aglycon and ligstroside aglycon determine the cell structures that makeup breast cancer and reduce the tympanic structures formed as a result of these structures. These two structures (oleuropein aglycon and ligstroside aglycone) eliminate the HER2 protein that causes cancer over time (Menendez et al. 2008).

Antimicrobial effect: Gram-negative, gram-positive, and anti-mycoplasma are strong antimicrobial properties of oleuropein (Bisignano et al. 1999; Maria et al. 2002). Phenolic compounds that are structurally similar to oleuropein disrupt the bacterial cell wall and enable it to gain antibacterial properties (Caturla et al. 2005).

Antiviral effect: The effect of oleuropein against the structure of HIV-1 gp41, a subunit of gclioprotein on the surface, by allowing HIV to enter and reproduce in healthy cells has been of interest. Despite these structures (HIV-1 gp41), studies continue to reduce the effect of oleuropein at the molecular level (Lee-huang et al. 2007).

1.5. Extraction Techniques

The solvent extraction technique is the oldest and most widely used separation process in history. The solvent extraction technique is commonly known as the process of transferring (separating) the phase containing the target compound or sample into a liquid phase that has undergone further processing (C. Poole, Mester, Miró, and Pedersen-bjergaard 2016). In solvent extraction, the treated phase is preferred as a liquid, and the sample is a gas, liquid or solid material that is slightly soluble in this phase. Samples consist of target compounds and unwanted matrix groups that are desired to be separated or dissolved. Therefore, the purpose of solvent extraction is to

separate desired target compounds from these matrix structures (Alexovič et al. 2018).

1.5.1. Liquid-Liquid Extraction

Usually, familiar materials such as separating funnels and bottles are used in liquid-liquid extractions. Different scaled volumes from the smallest liter value (microliter) to the liter range are placed in this equipment. All equipment used has a duty. For example, mixers are used to precipitate phases and separate them from each other (Alexovič et al. 2018; Chang et al. 2007).

1.5.2. Solid-Liquid Extraction

With the help of solvents, solids may not be able to fully penetrate the matrix. The uptake or separation of the desired (target) compounds depends on processes other than solvent. For these reasons, solids are not an easy phase for solvent extraction. The distribution of desired (target) compounds in the matrix, both the chemical and physical properties of the target compounds, and the properties of the matrix are procedures that affect the extraction rate (Huang, Boxin, and Prior 2005; Nilsson 2000; C. Poole, Mester, Miró, Pedersen-Bjergaard, et al. 2016). The extraction steps of target compounds composed of the porous structure are shown in Figure 1.18.

In solid-liquid extraction, the choice of solvent is extremely important, as the target compound is obtained with the help of a solvent. The things to be considered in the choice of solvent are listed below:

- The solvent chosen must be chemically stable.
- It should be easy to find and cost-effective
- It should behave inertly towards the materials used.
- It should have physical properties such as low viscosity, low freezing point, low evaporation temperature, and low explosion-flammability (Şahin 2011).

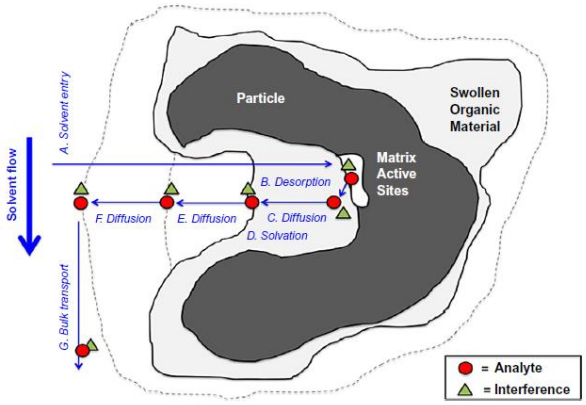


Figure 1.18. Extraction steps of target compounds composed of organic material and porous structure (Source: Poole 2020)

In solid-liquid extraction, the contact of the solvent with the material to be extracted is as important as the solvent selection. The diffusion phenomenon is directly proportional to the interface between the solid and liquid phases. Therefore, reducing the size of solids accelerates the extraction process. The cellular structure of every solid matter is different from each other. Vegetative tissue consists of cells surrounded by walls. The extraction of dried plant material with the aid of a solvent basically takes place in two stages. To help the substances (pores) found in plants to expand, it is first contacted with the selected solvent and soaked to swell the plant tissue. Then, the transition from enlarged pores to the solvent is achieved by diffusion (Vinatoru 2001).

The extraction process on vegetative tissue is shown in Figure 1.19.

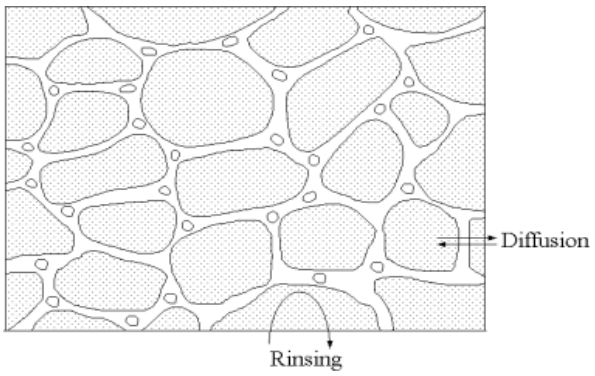


Figure 1.19. Schematic representation of plant cell tissue
(Source: Vinatoru 2001)

1.5.2.1. Soxhlet Extraction

Extraction is an important method for separating phenolic compounds and antioxidants from the plant matrix. Processes such as maceration, soxhlet, boiling, and soaking, known as traditional methods used for a long time, are widely used in extraction methods due to their low cost, easy working principle, and high extraction efficiency. Soxhlet extraction, one of these traditional methods, is known and preferred as an extraction method that provides much higher efficiency than other methods in obtaining the desired target compounds (antioxidant and phenolic compounds) (Orkan Dal, Duygu Şengün 2020). The Soxhlet device, defined in 1879 in history, is a highly preferred method to compare new methods (Azmin et al. 2016; Luque de Castro and Priego-Capote 2010). The setup of Soxhlet extraction is shown in Figure 1.20.

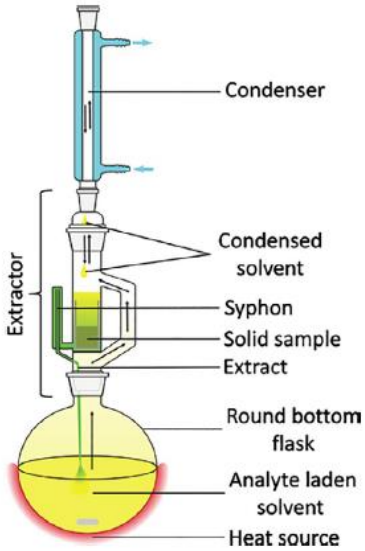


Figure 1.20. Soxhlet Extraction

(Source: Poole 2020)

The extraction solvent is heated by the heat source. The heated solvent evaporates and condenses over time. The concentrated solvent is filtered over the solid sample in the extraction thimble. The filtered solution accumulates in the thimble to a certain level, and then the solvent returns back to the boiling flask. This process works

according to the siphon principle. The suitable medium for Soxhlet extraction is at room temperature or close to the boiling point of the solvent used for different sample sizes. Usually, it is used as a 10 g solid sample and 50-250 ml solvent (Luque de Castro and Priego-Capote 2010).

The advantages of Soxhlet extraction can be listed as follows:

- The sample used in the extraction process is in constant contact with the solvent.
- The temperature of the system is always kept at the same value.
- Filtration is done after extraction and there is no need for extra filtration afterward.
- As the equipment used is less costly, parallel extraction can be done at the same time, thus increasing sample efficiency in a shorter time.

The most serious disadvantage of soxhlet extraction compared to other traditional solid-liquid extraction techniques is the long time required for the extraction process (C. F. Poole 2020).

1.5.2.2. Ultrasound-Assisted Extraction

Ultrasound-assisted extraction is used to speed up the extraction process of large-grained solids and to disrupt the cell structure of these solid samples. The probe device used in extraction provides better targeting of the solid sample in a high energy water bath. Several samples can run in parallel at the same time in sonic water baths. The ultrasound waves applied to the water bath create certain pressure waves in the liquid and cause collapse with bubble formation. This phenomenon is called cavitation. The bursting of these bubbles speeds up the bulk transport of target compounds. In this case, the interaction of the sample particles with the bubbles formed causes the particles to shrink in size and the material to deteriorate cellularly. Increasing temperature and pressure in the system causes bubble formation. This allows the resolution to be further increased. In ultrasonic extraction, recovery of target compounds is determined by sample type, just like soxhlet extraction. When comparing two different extractions, ultrasonic is faster and less time is required as extraction time. On the other hand, additional operations such as separating the sample from the solvent used and rinsing

are required after extraction (Picó 2013; Priego-Capote and Luque De Castro 2004).

1.5.2.3. Microwave-Assisted Extraction

In principle, microwaves are radiation that deals with the movement of ions and the interactions of molecules. Substances of the sample and solvent used in the microwave are directly proportional to its permeability. The energy of the microwave oven increases with temperature (Azmin et al. 2016; Luque de Castro and Priego-Capote 2010; Ridgway, Lalljie, and Smith 2007; Sun et al. 2012). With a low polarity (low permeability) solvent, the desired compounds (target compounds) in the sample can be extracted from the hot sample into the cold solvent. Two different apparatus are used in this extraction type. One of them is closed extraction vessels that can control temperature and pressure. The other is known as atmospheric pressure-sensitive microwave ovens. The temperature and pressure generated in these closed extraction vessels can be higher than the boiling point of the solvent used. Comparing the heating method and pressure systems, high pressure is more important than high temperature. It is an effective option for high-temperature microwave-assisted extraction. It increases the solubility of target compounds and reduces their interaction with matrices. Thus, the reduction of the solvent volume provides shorter and uninterrupted extraction. The extraction process takes less time compared to Soxhlet extraction (C.F. Poole 2020).

1.5.2.4. Pressurized Liquid Extraction

In the working principle, the sample in the solid phase is placed in the steel container. A certain level of solvent is pumped into this container. The container is heated to the specified temperature and the solvent is transferred to another container. The incoming solvent is washed with fresh solvent and purged with nitrogen gas. In this extraction, the sample (target compound) and the solvent are in contact for a longer time. For these reasons, the extraction time is important (Subedi et al. 2015).

1.5.3. Gas-Liquid Extraction

Gas-liquid extractions are used to separate volatile organic compounds from gas-phase solid adsorbents that are difficult to recover. Solvent-based colliders and bubbles participate in this extraction (Szulejko and Kim 2014). Although the colliders do not ensure that the gas and solvent are completely mixed as they pass through the selected solvent, the solvent creates a favorable environment for the separation of gases and vapors of great interest for optimally reactive compounds. Although the bubbles allow the solvent to mix well with the gas, the gas flow rates are very low (Yu et al. 2016; Zheng and Yao 2017).

CHAPTER 2

LITERATURE SURVEY

It has been known by many researchers that the olive tree leaf has therapeutic properties. The most important reason for having this feature is oleuropein, the highest phenolic compound found in the leaf. The widespread use of oleuropein in the healthcare field has led researchers to investigate this phenolic component from all sides. According to the researches, olives and the phenolic components in them vary according to many different factors such as the environmental conditions in which they are grown, the harvest season, the pre-treatments applied while extracting the extract, and the extraction methods. Therefore, in this section, a literature review is given on the composition of olives, the properties of oleuropein, experimental results for oleuropein extraction, and the effects of different extraction parameters on oleuropein yield.

2.1. Factors Affecting Oleuropein and Phenolic Levels

2.1.1. Environmental Conditions and Resource

Lujan et al. (2008) studied the identification and quantification of phenolic compounds in extracts obtained from olive tree materials (olive oil, alperujo known as waste material in oil production, leaves, olive stones and branches). Biophenols were extracted using different methods depending on the materials used. Liquid-liquid extraction for oil, micro-assisted filtration for leaves, stones and branches, and pressurized liquid filtration for alperujo were applied. According to the research, the highest phenolic compound in olive tree materials was determined as oleuropein with a concentration of 2% (w / w) in olive leaves. The oleuropein concentration was found to be approximately 19000 mg / g in the leaves and 600 mg / g in the branches. For this

reason, olive leaves have been identified as an excellent source for oleuropein (Japón Luján et al. 2008). The concentrations of phenolic compounds present in the materials are shown in Table 2.1.

Table 2.1. Concentration of olive phenolic compounds derived from olive oil and olive tree materials (n.d.: not detected) (Source: Japón Luján et al. 2008)

Sample/ biophenol (mg/kg)	**Hydroxytyrosol**	**Luteolin-7-glucoside**	**Apigenin-7-glucoside**	**Verbascoside**	**Oleuropein**
Olive oil	3.0 ± 0.2	n.d.	n.d.	0.08 ± 0.02	n.d.
Alperujo	831 ± 22	14.3 ± 2.3	6.2 ± 0.9	20.2 ± 2.8	37 ± 4
Olive leaves	n.d.	155 ± 10	207 ± 10	1428 ± 46	19050 ± 880
Olive branches	22.2 ± 2.0	175 ± 8	10.9 ± 0.8	1560 ± 50	673 ± 34
Olive stones	18.1 ± 1.9	6.2 ± 0.8	0.09 ± 0.01	0.15 ± 0.03	0.06 ± 0.02

Pereira et al. (2007) studied the biophenols in the leaf using an aqueous olive leaf extract. HPLC-DAD analysis was used for phenolic compound determination. These compounds; Caffeic acid, rutin, oleuropein, luteolin 7-O-glucoside, apigenin 7-O-glucoside, verbascoside, and luteolin were identified as 4'-O-glucoside. The study revealed the highest amount of phenolic oleuropein compound in the olive leaf extract. Oleuropein was found to be 73% of the total compounds and 1% of caffeic acid (Pereira et al. 2007). The quantities and comparison of the seven phenolic compounds are shown in Table 2.2.

Table 2.2. Determination of phenolic compound by HPLC-DAD analysis in olive leaf extract (Source: Pereira et al. 2007)

Compound	**mg/kg**
Caffeic acid	220.5 ± 23.3
Verbascoside	966.1 ± 18.1
Oleuropein	26471.4 ± 1760.2
Luteolin 7-O-glucoside	4208.9 ± 97.8
Rutin	495.9 ± 12.2
Apigenin 7-O-glucoside	2333.1 ± 74.7
Luteolin 4'-O-glucoside	1355.9 ± 75.9

Ranalli et al. (2006) evaluated the effect of environmental conditions on the oleuropein content in olive leaves. In this study, characteristics such as the picking time of the leaves, the color, and the age of the leaves were emphasized. Olive leaf extracts examined in the study were analyzed by repeated high-resolution gas chromatographic quantitation method and the HPLC method. The samples were taken from seven different Italian olive varieties. Regardless of the olive variety, it was observed that the oleuropein content in the dense green leaves was quite high. It was noted that green-yellowish leaves and yellow leaves near the falling level caused a significant decrease in oleuropein level. The effect of this color change negatively affected the amount of oleuropein in the leaf with the aging of the olive tree (Ranalli et al. 2006).

Zun-qui et al. (2015) examined the interaction of oleuropein during the harvest season. In this study, it was aimed to compare the oleuropein yields in leaves collected at different times. According to the results obtained, it was determined that oleuropein decreased during the differentiation of flower bud and ripening of olive fruit. It was determined that the month with the highest oleuropein content was January (19.58%) and the lowest was July (1.56%) (Zun-qiu et al. 2015).

Mitabolique et al. (1989) and Ramirez et al. 2016) examined the reasons for the decrease in oleuropein levels during the ripening of olive fruits and the reactions that occur during this period. During ripening, a decrease in oleuropein levels was observed in olive fruits, while glucosylated oleuropein derivatives such as elenolic acid glucoside (A) and demethyloleuropein (B) were accumulated. That is, these compounds (A and B)

were found to cause an increase in esterase activity while lowering oleuropein levels. It has been stated that this enzyme helps break down oleuropein and form aglycon forms (C, D, and E) (Mitabolique, Appliquie, and Index-olea 1989; Ramírez et al. 2016). Figure 2.1 shows the steps in which oleuropein is converted to aglycone forms with the aid of esterase.

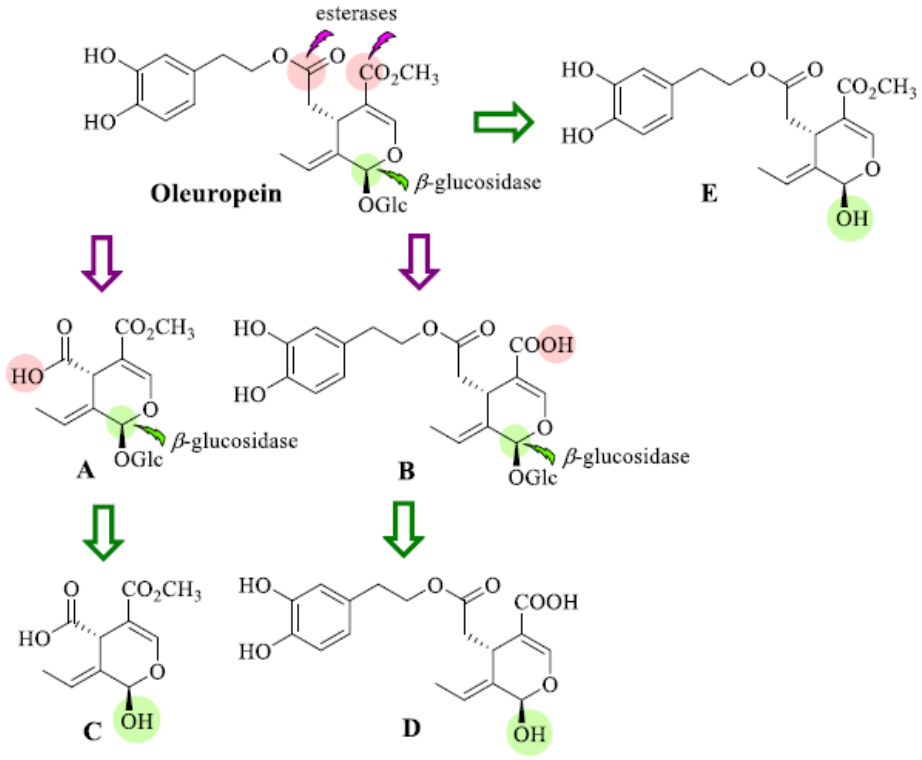


Figure 2.1. Molecular structures of elenolic acid glucoside (A) and demethyloleuropein (B), formed by esterase action, and aglycone forms (C and D) and oleuropein aglycone (E) by β-glucosidase action (Source: Cavaca et al. 2020).

2.1.2. Values in Oleuropein Concentration According to Soxhlet Extraction Method

The recovery of phenols depends on many parameters. Many important factors such as pre-processing of the sample, drying, storage conditions, extraction method,

purity of the standards used in the preparation of the calibration curve can be listed. In this section, literature researches which studied the effects of various parameters on oleuropein content by soxhlet extraction method are included. The compilation of oleuropein content values under various reaction conditions by the Soxhlet extraction method is shown in Table 2.3.

Table 2.3. Literature examples of oleuropein concentration according to Soxhlet extraction method

Reaction Conditions	**Solvent Types**	**Oleuropein (mg/g dry leaf)**	**Oleuropein Quantification**	**References**
10 g of dried and ground leaves (0.9-2 mm), 24h, 250 ml solvent	Water 100% ethanol 100% methanol 100% hexane	3.83 ± 0.05 0.29 ± 0.01 37.55 ± 1.43 0.01 ± 0.00	HPLC, LC/MS	Şahin, Bilgin, and Dramur 2011
15 grams of olive leaves, 4h, 300 ml solvent	80% ethanol 20% acetonitrile 100% ethyl acetate	15.6 ± 0.16 19.0 ± 0.21 0.19 ± 0.01	HPLC	Afaneh, Yateem, and Al-Rimawi 2015
15 grams of olive leaves sample, 4h, 300 ml solvent	80 % ethanol 20% acetonitrile	19.0 ± 0.66 15.6 ± 0.32	HPLC	Yateem, Afaneh, and Al-Rimawi 2014
10 g of dry olive leaves, 4h, 150 ml solvent	60% methanol	65.57 ± 0.70	HPLC-DAD	Lama-Muñoz et al. 2019
10 g of dry olive leaves, 4h	60% ethanol	49.1 ± 0.8	HPLC	Lama-Muñoz et al. 2020

2.2. Total Phenolic Content

Karakulak (2009) found the total phenolic values of olive leaf extracts obtained by soxhlet extraction using different solvent types (pure acetone, methanol, ethanol, and water) at 765 nm wavelength and compared them with each other. Experiments were carried out with 20 g of dried and ground olive leaves and 200 ml of solvent for 8 hours (Karakulak 2009). The mg gallic acid equivalents of gram dry leaves of the samples are shown in Table 2.4.

Table 2.4. Gallic acid equivalents in mg of extracts from Soxhlet extraction

Solvent Type	**Total Phenolic Content (mg GAE/g)**
Acetone	30.32
Ethanol	72.19
Methanol	75.16
Water	59.08

Lama-Munoz et al. (2019) investigated the amount of phenolic compound in the olive leaf using different extraction methods (soxhlet and ultrasound-assisted) with 60% ethanol. The phenolic compound value of the sample made by Soxhlet extraction was found to be higher. (Lama-Muñoz et al. 2019). The amount of phenol content of the two extraction methods is given in Table 2.5.

Table 2.5. Comparison of both extraction methods used in the study

Response	**Soxhlet Extraction**	**Ultrasound-Assisted Extraction**
TPC (mmol GAE/kg)	251.91 ± 2.13	210.25 ± 3.55
Oleuropein (mg/g)	65.57 ± 0.70	69.91 ± 1.53

CHAPTER 3

EXPERIMENTAL STUDY

3.1. Materials

3.1.1. Plant Materials and Chemicals

The olive leaves, which were used as a raw material source in experimental study, were collected from the olive trees in the campus of Izmir Institute of Technology. All chemicals used in this study were of analytical grade and were used without any purification. The list of these chemicals are given in Table 3.1 Moreover, de-ionized water was used to prepare all solutions and cleaning of olive leaves.

Table 3.1. List of chemicals used in the experiment

Name	**Producer**
Oleuropein	Sigma Aldrich, 98%
Methanol	Merck, 99.8%
Ethanol	Merck, 99.9%
Acetonitrile	Merck, 99.9%
Acetic acid	Merck, 99%
Folin Ciocalteu Reagent	Merck
Sodium carbonate	Sigma Aldrich, 99.5%
Gallic acid	Merck, 97,5%

3.1.2. Experimental Apparatus

Olive leaves were dried in a vacuum oven JSR JSVO-60T. After grinding of olive leaves, experiments were carried out by soxhlet extraction (Wisd, DH. WHM 12295) and ultrasonic extraction (WUC-D06H, WiseClean). The Soxhlet extraction set-up consists of a 500 ml solvent container, an extractor, a condenser and a heating system. In the ultrasonic extraction set-up consists of an ultrasonic bath which has a capacity of 6 L and its ultrasonic frequency is 40 kHz. At the end of the extraction, the extracted product was separated from the solvent with the aid of a Heidolph Laborota 4001 rotary evaporator. The phenolic compound (oleuropein) of olive leaf crude extract was analyzed by HPLC Agilent Technologies 1100 Series. Thermo's Multiscan UV spectrophotometer was used to find the total phenol content of the extract.

3.2. Experimental Procedure

In the first stage of this study, soxhlet extraction method was applied to obtain extracts from dried and ground olive tree leaves and then, the oleuropein content of obtained extracts were analyzed by HPLC. By using Soxhlet extraction method, the effect of different parameters (different solvent types, different particle size, extraction time, pre-treatment applied to ground olive leaves) over the conversion of olive leaf and yield of oleuropein were investigated. Liquid and solid products were obtained at the end of the extraction. The remaining solid residue was placed in a vacuum oven (Jsr jsvo-60T) to remove moisture for 24 hours at conditions set at 50°C. The overall conversion of olive leaf was calculated based on the initial amount of dry olive leaf according to the following equation:

$$Overall\ conversion\ (wt.\%) = \frac{Olive\ leaf\ (initial\ amount - solid\ residue)}{Olive\ leaf\ (initial\ amount)} \times 100 \quad (3.1)$$

After extraction step, firstly, the liquid product was evaporated in the rotary evaporator to seperate the solvent and extracted product. Then, HPLC was then used to determine the amount of oleuropein in the remaining extract. The amount of oleuropein in olive leaves identified by HPLC analysis was determined by the following equation:

$$Amount\ of\ Oleuropein\ (mg/g\ dry\ leaf) = \frac{Oleuropein\ in\ the\ sample\ \left(\frac{mg}{L}\right) \times Extracted\ volume\ (L)}{The\ initial\ amount\ of\ dry\ leaf\ (g)} \quad (3.2)$$

Also, total phenolic content was monitored by Folin Ciocalteu Method. The general diagram of the experimental procedure is given in Figure 3.1.

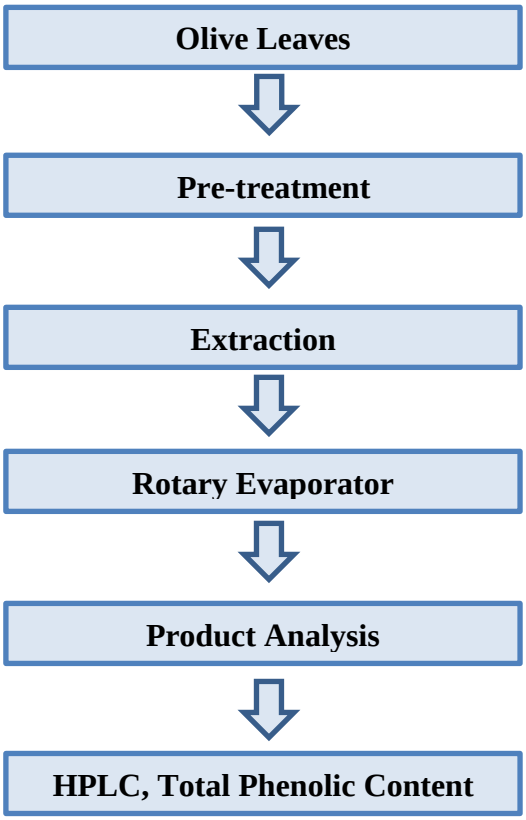


Figure 3.1. The general diagram of experimental procedure

3.2.1. Pre-treatment of Olive Leaves

Olive leaves were washed and cleaned with deionized water and dried in a vacuum oven for 24 hours at 55^{o} C. Figure 3.2 shows the drying process of olive leaves in the oven. After drying step, the mill was used for grinding of leaves and until the leaves became a fine powder of 250-2000 microns. Grinding process was carried out at intervals of 5 minutes to prevent frictional heating of the sample. In addition, in this study, the ground olive leaves were pre-treated for 1 hour in ultrasonic extraction before soxhlet extraction. The highest amount of oleuropein was obtained using two different solvents which are methanol (80%) and ethanol (80%) and these solvents were used for pre-treatment of olive leaves.

Figure 3.2. Drying process of olive leaves in the oven

3.2.2. Extraction of Olive Leaves

The experiments were carried out in a soxhlet extraction set-up branded Wisd, DH.WHM 12295. In this extraction method, to achieve the highest oleuropein yield; the effects of various parameters which are the solvent type (methanol, ethanol, acetonitrile, water), and their concentrations, extraction time (4 cycles, 4h, 8h), particle size (250-

500 μm, 900-2000 μm) were investigated. Additionally, the effect of pre-treatment of olive leaves was examined. In the first stage, olive leaves were ground with a laboratory scale grinder. 10 g of ground olive leaves were weighed into filter paper and placed in the extractor. A volume of 250 ml of solvent was poured into the solvent container. Soxhlet extraction ready for experiment is shown in Figure 3.3. When the solvent started to evaporate at its boiling point, it concentrated and filled the extractor with the help of a steam condenser. When the solvent level reached the siphon level, the extracted product and solvent were poured into the solvent bottle. In this way, a cycle is completed.

At the end of the extraction, the extract was separated from the solvent by rotary evaporator (Laborota 4001, Heidolph). The temperature of the water bath in the rotary evaporator was set at 40^{o}C and the rotation frequency at 60 rpm. The pressure in the condenser was adjusted with a vacuum valve to evaporate the solvent faster. The evaporating solvent was concentrated via a condenser. Finally, olive leaf raw extract was obtained to be used in future studies.

Figure 3.3. The picture of soxhlet extraction unit

Experiments were carried out at different extraction conditions to comprehend the effect of various parameters over the obtained amount of oleuropein and the experimental conditions are given in Table 3.2.

Table 3.2. Experimental study of olive leaf soxhlet extraction

Experimental Number	Solvent Types	Extraction Time	Particle Size (µm)
1	100% MetOH	4 cycles	900-2000
2	100% EtOH	4 cycles	900-2000
3	80% EtOH	4 cycles	900-2000
4	Water	4 cycles	900-2000
5	100% MetOH	4 cycles	250-500
6	100% EtOH	4 cycles	250-500
7	80% EtOH	4 cycles	250-500
8	70% EtOH	4 cycles	250-500
9	Water	4 cycles	250-500
10	100% MetOH	4h	900-2000
11	100% EtOH	4h	900-2000
12	80% EtOH	4h	900-2000
13	70% EtOH	4h	900-2000
14	100% MetOH	4h	250-500
15	80% MetOH	4h	250-500
16	50% MetOH	4h	250-500
17	100% EtOH	4h	250-500
18	80% EtOH	4h	250-500
19	70% EtOH	4h	250-500
20	50% EtOH	4h	250-500
21	20% EtOH	4h	250-500
22	20% Acetonitrile	4h	250-500
23	Water	4h	250-500
24	100% MetOH	8h	900-2000
25	100% EtOH	8h	900-2000
26	100% MetOH	8h	250-500
27	80% MetOH	8h	250-500
28	50% MetOH	8h	250-500

(cont. on the next page)

Table 3.2 (cont.)

29	100% EtOH	8h	250-500
30	80% EtOH	8h	250-500
31	70% EtOH	8h	250-500
32	50% EtOH	8h	250-500
33	20% EtOH	8h	250-500
34	20% Acetonitrile	8h	250-500
35	Water	8h	250-500
Pre-treatment with ultrasonic extraction (1h, 40 kHz, 25^{o}C)			
36	80% EtOH	8h	250-500
37	80% MetOH	8h	250-500
38	80% EtOH	4h	250-500
39	80% MetOH	4h	250-500

3.3. Product Analysis

3.3.1. HPLC Analysis of Phenolic Compounds

High performance liquid chromatography (HPLC) analysis was performed to determine the amount of oleuropein in olive leaf extract. HPLC analysis was performed in the Environmental Development Application and Research Center at IZTECH. The HPLC system is equipped with a C_{18} Inerstil column (5 µm, 250 mmx4.6 mm) and (DAD) Agilent 1100 Series detector. The mobile phase, acetonitrile/water (20:80, v/v) containing 0.1% acetic acid, was used at a flow rate of 1 ml/min and the temperature of the column was set at 30^{o} C. The features of the HPLC analysis used in this study are shown in Table 3.3 and the schematic image of the HPLC analysis is shown in Figure 3.4.

Figure 3.4. Schematic representation of the HPLC system

Table 3.3. The properties of HPLC system

Property	**Value or Names**
Column	Column C18 Inertsil
Column Length	250 mm
Column Diameter	4,6 mm
Particle Size	5 µm
Mobile Phase	Mobile Phase A: acetonitrile/water (20:80, v/v) Mobile Phase B: 0.1% acetic acid
Flow Rate	1 mL/min
Temperature	30^{o}C
Detector	Diode Array Detector (DAD) Agilent 1100 Series
Absorbance	280 nm

The oleuropein stock solution was prepared to generate the calibration curve to determine the amount of oleuropein in olive leaf extract. Oleuropein was dissolved in deionized water in six different concentrations (50 ppm, 75 ppm, 100 ppm, 200 ppm,

500 ppm, 1000 ppm) and these solutions were analyzed by HPLC. Oleuropein amounts were calculated using areas corresponding to each different concentration analyzed in HPLC and a calibration curve was obtained. Thus, the amount of oleuropein extracted in the experiments was determined using this linear calibration curve. The amount of oleuropein in a sample determined by HPLC analysis is shown in Figure 3.5. The calibration curve of oleuropein is given in Figure 3.6.

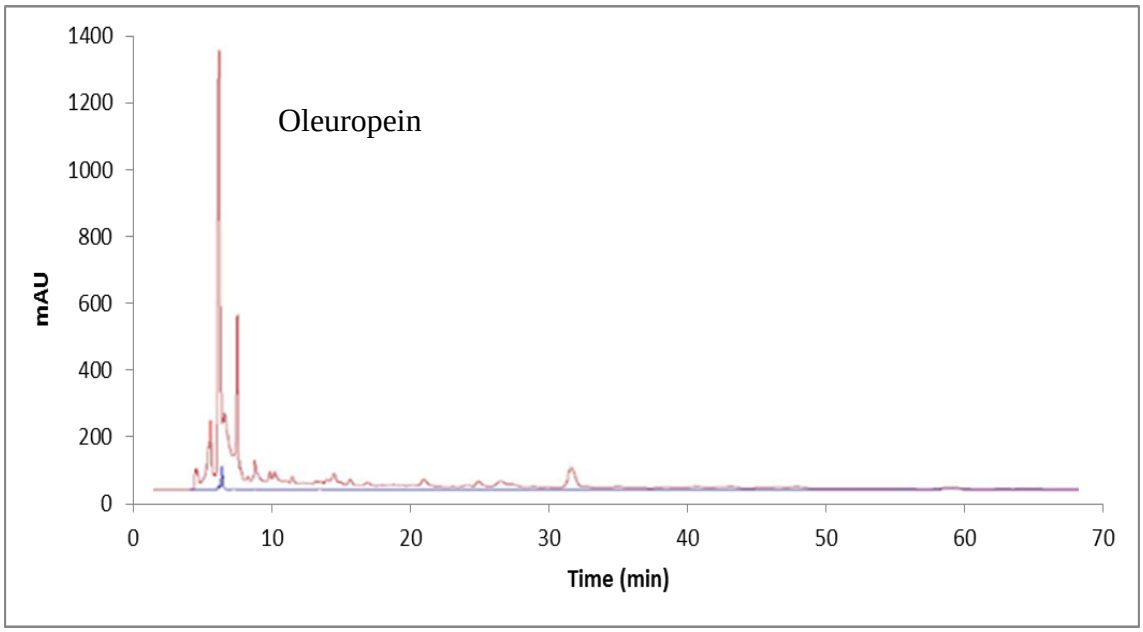


Figure 3.5. HPLC chromatogram of olive leaf

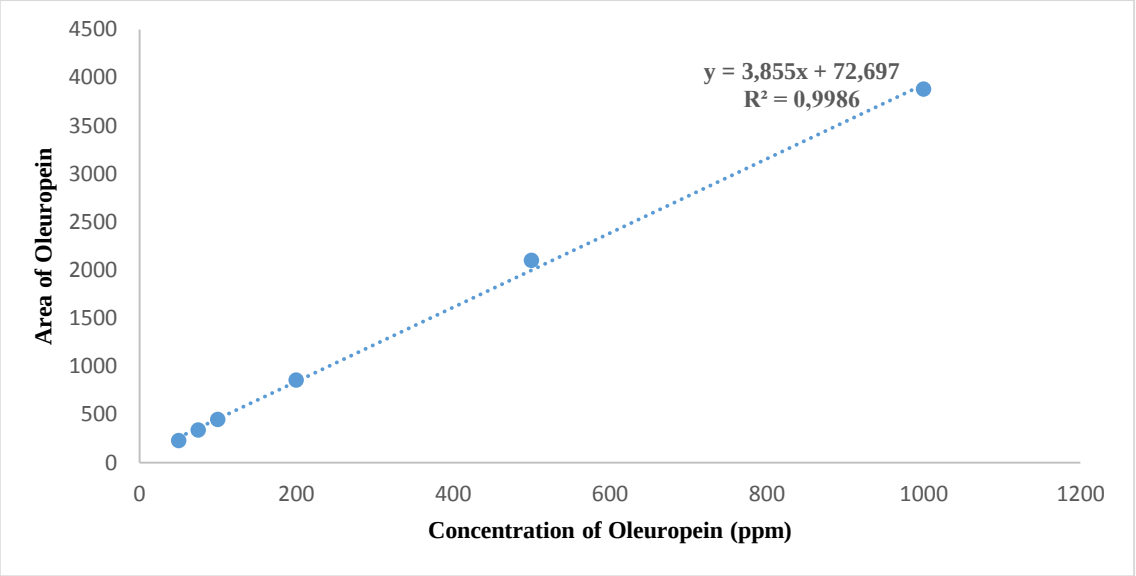


Figure 3.6. Calibration Curve of Oleuropein

3.3.2. Total Phenolic Content (Folin Ciocalteu Method)

The total phenolic content of the liquid extract was determined using the Folin Ciocalteu method. In this method, gallic acid stock solution was initially prepared. 50 mg of gallic acid was weighed out and diluted in 100 ml of distilled water. Standard solutions were prepared at various concentrations (0.02-0.04-0.06-0.08-0.1 and 0.12 ppm) to obtain the calibration curve of gallic curve. Then, a gallic acid calibration curve was formed using the standard solutions prepared based on Folin Ciocalteu Method. The calibration curve of gallic acid is shown in Figure 3.7.

Folin Ciocalteu reagent was diluted 10-fold with distilled water and 7.5% (75 g/L) of sodium carbonate (Na_2CO_3) solution was prepared with distilled water. 0.5 ml of Folin Ciocalteu reagent, 0.5 ml of liquid product and 1 ml of saturated Na_2CO_3 solution were mixed and then volume adjusted to 10 ml with distilled water. After mixing, the mixture was left in the dark at room temperature for 45 min. The absorbance was measured at 765 nm and distilled water was used as blank sample.

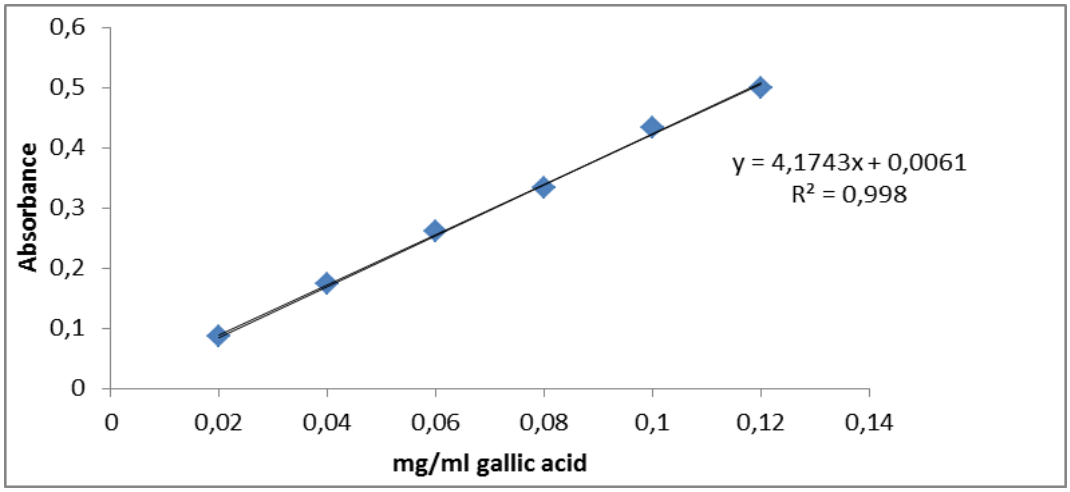


Figure 3.7. Calibration Curve of Gallic Acid

CHAPTER 4

RESULTS AND DISCUSSION

The effects of solvent types (ethanol, methanol, water, acetonitrile) and the ratio of solvent solution, reaction time (4 cycles, 4h and 8h), partical size ranges (250-500 μm and 900-2000 μm) over the yield of oleuropein by extracting olive leaves were investigated. Additionally, the effect of pre-treatment was also investigated.

4.1. Effect of Extraction Solvent on Conversion of Olive Leaf and Yield of Oleuropein

Experimental results of soxhlet extraction process to investigate the effect of solvent type on conversion and oleuropein yield are shown in Figures 4.1. and 4.2. The experiments were carried out at the following conditions; 10 g dried olive leaf with a particle size of 250-500 μm, in the presence of various solvents (100% ethanol, 80% ethanol, 70% ethanol, 50% ethanol, 20% ethanol, 100% methanol, 80% methanol, 50% methanol, 20 % acetonitrile and water) and extraction duration of 8 hours.

The selectivity of the solvent has a great importance in extracting processes to obtain the desired compound (target compound) from the plant material. To obtain high yield of desired compound in extraction process, the extracted compound and solvent must show similar polar properties. Oleuropein, which is the most abundant phenolic compound in olive leaf extract, is a polar compound so that it is necessary to choose a solvent with high polarity in order to obtain oleuropein with high efficiency (Le Floch et al. 1998; Lee et al. 2006). The polarity and boiling point values of the solvent types used in the analysis are shown in Table 4.1.

Table 4.1. Polarity and boiling point values of the solvent types

Solvent Type	**Boiling Point (^{o}C)**	**Polarity Value**
Acetonitrile	81.6	0.460
Ethanol	78.5	0.654
Methanol	64.6	0.762
Water	100	1.000

The effect of the solvent type on the amount of oleuropein in the olive leaf extraction performed by Soxhlet method for 8 hours is shown in Figure 4.1 and extraction yield in Figure 4.2.

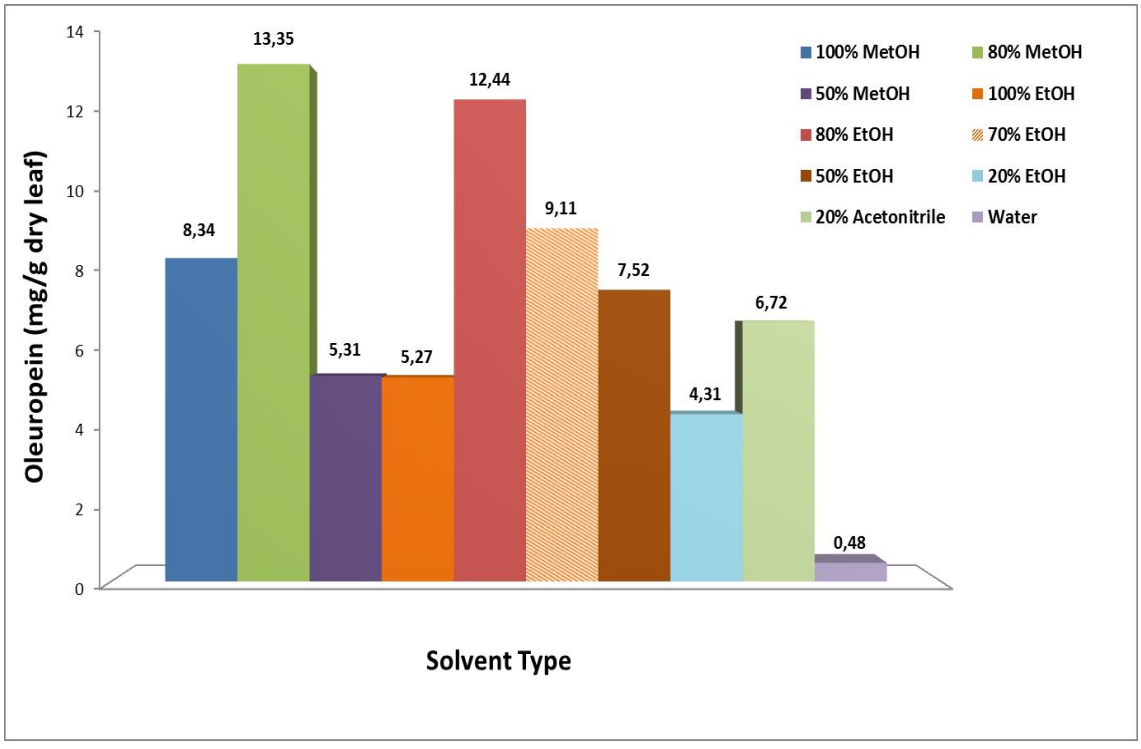


Figure 4.1. Effect of solvent type on the extracted amount of oleuropein per gram of dried leaf by the Soxhlet extraction for 8h

The oleuropein content was varied between 0.48 and 13.35 mg/g dried olive leaf. The amount of oleuropein obtained in the experiments where methanol was used as a solvent was generally higher than other solvents. The highest oleuropein amounts were found as 13.35 mg/g dry leaf with 80% methanol solvent, and 12.44 mg/g dry leaf with 80% ethanol solvent, respectively. Şahin et al. (2011) aimed to examine the oleuropein content of the solvents used in experiments with the soxhlet extraction method. Hexane, water, ethanol, methanol and methanol/hexane (3/2:v/v) mixture were used as solvent in extraction experiments. According to the results obtained from the experiments, the highest oleuropein yield was obtained by using methanol solvent and found as 37.55 mg / g dry leaves. The highest oleuropein value after methanol was found as 18.58 mg / g dry leaves with methanol / hexane solvent. In this study, it was determined that the oleuropein content values of ethanol, water and hexane solvents were very close to each other and they did not show a good performance in oleuropein yield (Şahin, Bilgin, and Dramur 2011).

Garcia-Castello et al. (2015) aimed to find the appropriate solvent type in the study of extraction of phenolic compounds. It was stated that methanol and ethanol were the most suitable solvents for separating phenolic compounds. It was stated that the polarity levels of methanol and ethanol were lower compared to water. Hence, it led to a reduction in the dielectric constant of the solvent, thus facilitating the solubility and diffusion of the desired target compounds (phenolic compounds) in the solvent. Using solvents in their pure form causes plant cells to dehydrate and collapse. It also causes denaturation of proteins and phenolic compounds in the cell wall. For these reasons, the extraction of phenolic compounds becomes difficult (Garcia-Castello et al. 2015).

As the polarity of the solvents used increases, the amount of oleuropein generally increases. In this study, a higher oleuropein yield was obtained in experiments where methanol was used as a solvent. This can be explained by the fact that methanol has a higher polarity value compared to other solvents. There is a big difference in oleuropein yield between solvents in pure form (ethanol and methanol) and their aqueous solvents. This may be due to the higher polarity value of aqueous solvents compared to pure solvents. Therefore, it could resolve the olive leaf better. Although water is the most polar solvent used, it appears that it does not perform well in extracting oleuropein. The reason for this may be that the prolonged boiling period of the soxhlet method at high temperatures degrades the oleuropein content of the extract.

The absence of such a situation in methanol extracts can be explained by the fact that the boiling point of methanol (64.7^{o} C) is lower than that of water.

Altiok et al. (2008) aimed to evaluate the isolation of polyphenols from olive leaves. The extraction efficiency of oleuropein and rutin was determined based on the solvent type and concentration of ethanol. The highest oleuropein yield in the study was provided using 70 % aqueous ethanol solution. Thereafter, a decreasing performance on the oleuropein yield was observed in the presence of 50 % ethanol and 20 % ethanol aqueous solution, respectively (Altiok et al. 2008). Based on the results of this study, the best oleuropein yielding was obtained using 80 % ethanol, 70 % ethanol and 80 % methanol.

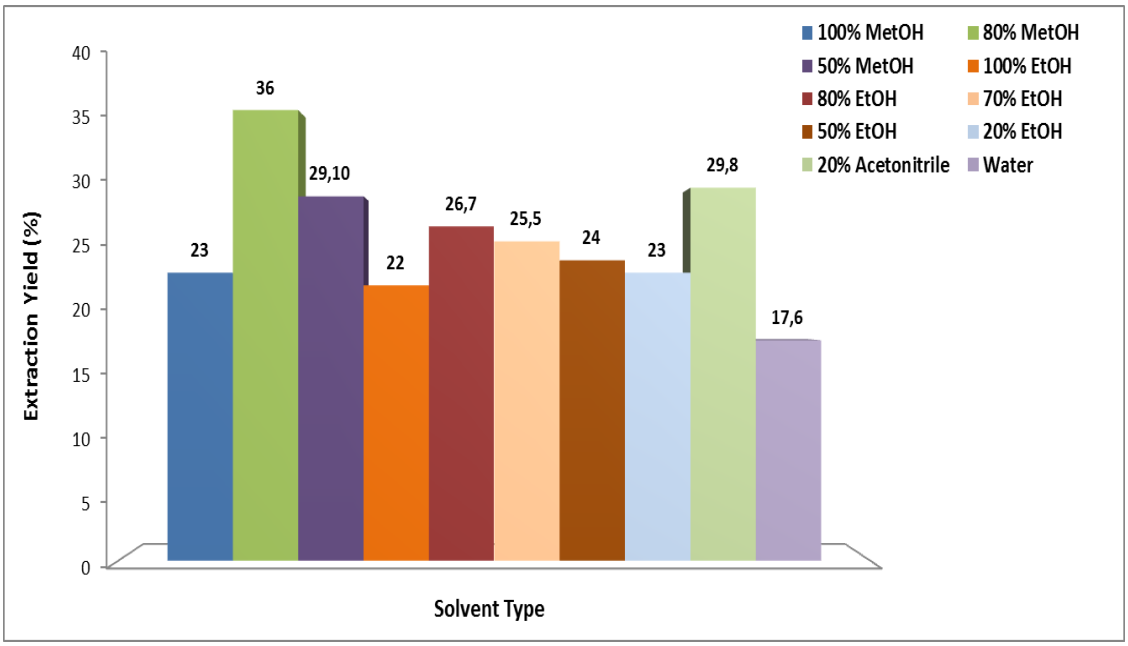


Figure 4.2. The effect of the type of solvent used in olive leaf extraction on the extract amount performed for 8 hours by the soxhlet extraction

As seen in Figure 4.2, the lowest extraction efficiency was found as 17.6% in water, while the best extraction efficiency was obtained as 36% and 29.8% in 80% methanol and 20% acetonitrile in volume, respectively. Pure ethanol and its aqueous solutions gave almost the similar results. These values can be explained by the polarity of the solvents. Solvents with polar properties (ethanol and methanol) are characterized

as polar protic solvents and release hydrogen into the environment (Mazaheri et al. 2010). Polar protic solvents (which are polar properties) feed the environment with hydrogen thanks to -OH bonds and thus provide higher extraction efficiency (Demirkaya et al. 2019).

4.2. Effect of Extraction Time on Conversion of Olive Leaf and Oleuropein Yield

Extraction time is one of the most important parameters to be examined in the extraction process in order to minimize energy and cost. In the experiment, 10 g of olive leaves were extracted at 250-500 μm size in 4 hours and 8 hours by soxhlet extraction. Data were obtained by using 100% methanol, 80% methanol, 50% methanol, 100% ethanol, 80% ethanol, 70% ethanol, 50% ethanol, 20% ethanol, 20% acetonitrile and water for 4 h and 8 h. These data are shown in Table 4.2. Using these data, oleuropein and extract yield graphs (Figure 4.3 and Figure 4.4, respectively) are shown.

Table 4.2. The effect of extraction time (4h and 8h) on extraction yield and oleuropein in olive leaf extraction by soxhlet method

Solvent Type (250 ml)	**Extraction Time**	**Extraction Yield (%)**	**Oleuropein Yield (mg/g dry leaf)**
100% MetOH	4h 8h	16 23	4.13 8.34
80% MetOH	4h 8h	27 36	8.79 13.35
50% MetOH	4h 8h	19 29.1	2.05 5.31
100% EtOH	4h 8h	14.6 22	3.70 5.27
80% EtOH	4h 8h	30 26.7	10.60 12.44

(cont. on the next page)

Table 4.2 (cont.)

70% EtOH	4h 8h	32.3 25.5	6.61 9.11
50% EtOH	4h 8h	26 24	3.44 7.52
20% EtOH	4h 8h	20 23	1.28 4.31
20% Acetonitrile	4h 8h	22.9 29.8	6.40 6.72
Water	4h 8h	14 17.6	0.33 0.48

The effect of extraction times on the amount of oleuropein in the extraction of olives is shown in Figure 4.3. and the extraction yield is shown in Figure 4.4.

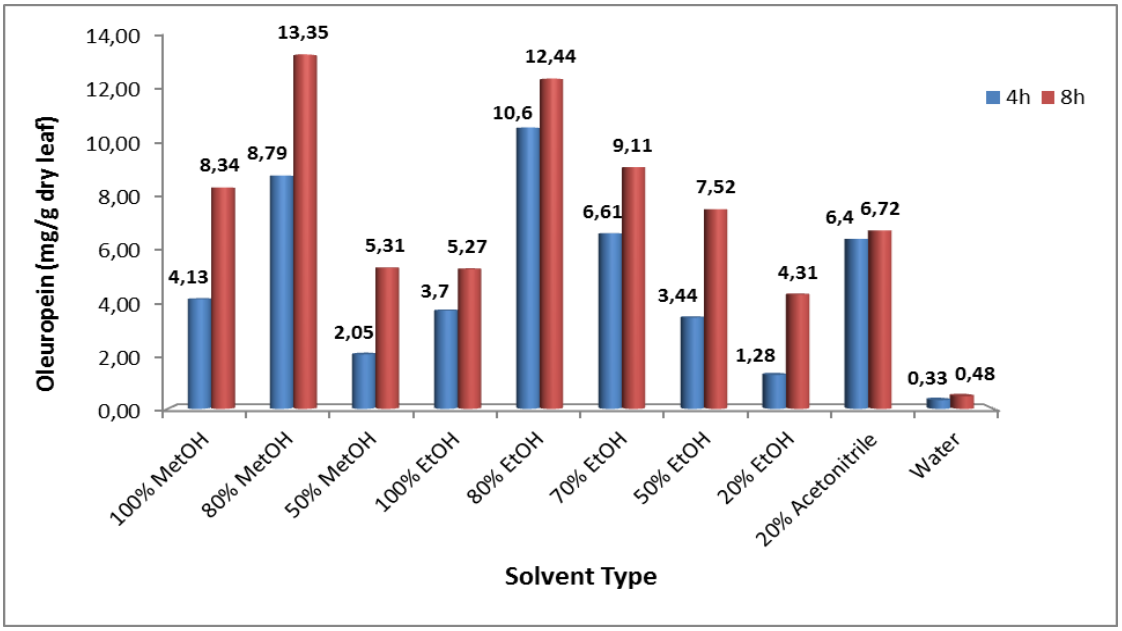


Figure 4.3. Effect of extraction time on the extracted amount of oleuropein per gram of dried leaf in the soxhlet method for 8h and 4h

As seen in Figure 4.3, the highest oleuropein value was obtained with the experiment in which the solvent was methanol in 8 hours extraction. The highest oleuropein value was recorded as 13.35 mg/g dry leaves after 8 hours of extraction. The highest oleuropein amount after 80% methanol was found to be 12.44 mg / g dry leaf using 80% ethanol solvent after 8 hours of extraction. When comparing the extraction times, it is seen that experiments that usually lasted 8 hours had a much higher oleuropein yield than experiments for 4 hours. In the experiments performed at each different time, it was seen that methanol had better performance than ethanol (oleuropein value 100% methanol 4.13 mg / g dry leaf for 4 hours, oleuropein value 100% ethanol 3.7 mg / g dry leaf). It was analyzed that less oleuropein was extracted from olive leaves from pure solvents than aqueous solvents. Xie et al. (2013) examined different proportions of ethanol and water mixture on oleuropein extract yield. The maximum oleuropein yield was found in the range of 55-75% ethanol, and it was stated that the mixture of ethanol and water was a good solvent. This property is considered extremely important as it combines polarity and penetration properties. It has also been suggested that the affinity of the solvent and solute and the increased surface area of the solvent-solute contact are the effect of increasing the yield for the target compound. Therefore, it was observed that the oleuropein yield and ethanol concentration decreased between 75-85% and remained unchanged at the value between 85-95%. As a result of the observations, the ethanol concentration increased while its polarity decreased. It was stated that this feature was not beneficial for the oleuropein yield and the optimum ethanol concentration was determined as 75% (Xie et al. 2015).

Both 80% methanol and 80% ethanol achieved higher yield of oleuropein for different experimental conditions. The amounts of oleuropein in 20% acetonitrile solvent were almost the same for different extraction times. For example, 6.4 mg oleuropein/g dry leaf was obtained using 20% acetonitrile for 4 hours, while 6.72 mg oleuropein/g dry leaf was obtained using 8 hours. The oleuropein amount of 20% acetonitrile solvent gave better results than 20% ethanol solvent in 4 hour and 8 hour extraction trials. Although acetonitrile performs well in the amount of oleuropein, it has not been used in other experiments due to its high boiling point and being an expensive solvent. The boiling temperature of acetonitrile is 81.6^{o} C and similar oleuropein yields have been obtained since it is close to the boiling point of water. Since the boiling points of ethanol or methanol are lower than the boiling temperature of water, higher

oleuropein yields have been observed in the presence of these solvents (Şahin, Bilgin, and Dramur 2011).

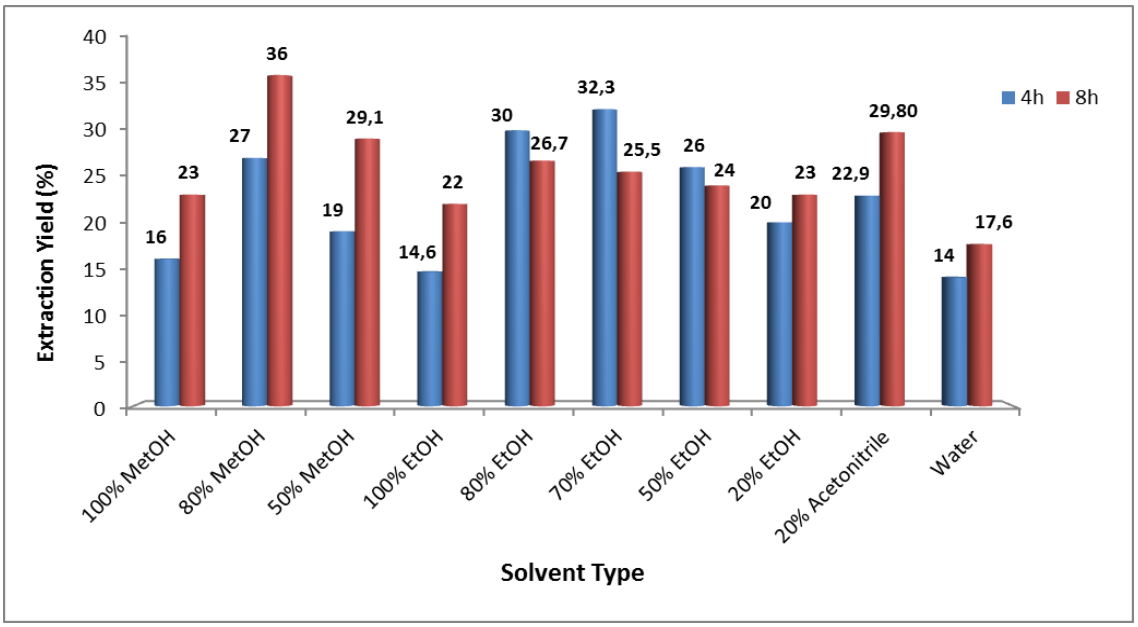


Figure 4.4. The effect of the extraction time used in olive leaf extraction for by the soxhlet method on the extraction yield

The highest extraction efficiency was found as 36% using 80% methanol for 8 hours. The following highest values were determined using 80% ethanol (30%) and 70% ethanol (32.3%) for 4 hours. According to the results obtained, the extraction efficiency was 30% in 4 hours using 80% ethanol as solvent, while the yield in 8 hours was 26.7%. The extraction yields at two different extraction times were also very close in the presence of 50% ethanol.

4.3. Effect of Particle Size on Olive Leaf Conversion and Oleuropein Yield

Pretreatments such as separation techniques or particle size reduction could be applied to raw materials to achieve a great improvement on the extraction efficiency of

phenolic compounds (target compound) (Medina-Torres et al. 2017). Nagy (2008) examined the effect of particle size and moisture content of chilli peppers in supercritical fluid extraction. In the study, the application of smaller particle size in extraction resulted in higher extraction yields. By reducing the particle size of the sample, the mass transfer surface area was increased and the amount of soluble fraction on these surfaces increased (Nagy 2008).

In the extraction of herbal extracts, physical processes such as drying and grinding has a great importance. In this section, it was aimed to find oleuropein and extraction yield values in different particle size ranges. 10 g of olive leaves were extracted with solvents of 100% methanol, 100% ethanol, 80% ethanol and water in 4 cycles (1 cycle = 45 minutes for methanol, 40 minutes for ethanol, 75 minutes for water) in the soxhlet extraction. The operating conditions are kept as the same in all experiments, the particle sizes of olive leaves (250-500 µm, 900-2000 µm) were changed to comprehend the effect of particle size over oleuropein yield. The oleuropein and extraction yields determined as a result of soxhlet extraction of particles in different sizes are shown in Figure 4.5 and Figure 4.6, respectively.

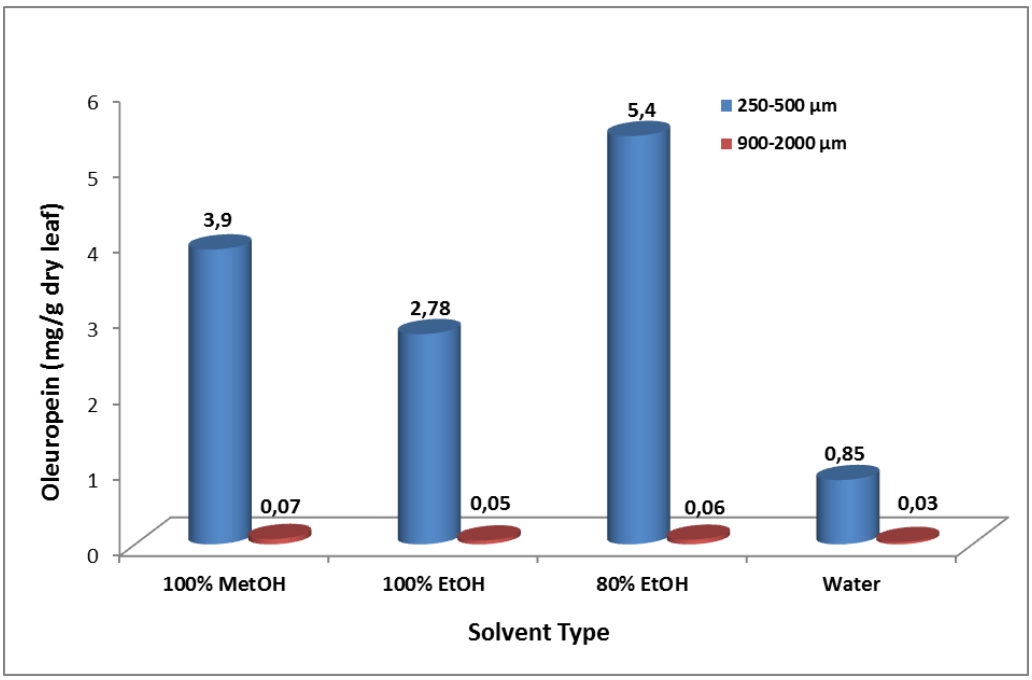


Figure 4.5. Effect of partical sizes on the extracted amount of oleuropein per gram of dried leaf in the soxhlet method for 4 cycles

As seen in Figure 4.5, it was found that the particle size clearly affects the oleuropein yield. The oleuropein yield in the extract obtained with the size of 250-500 µm in 80% ethanol solvent was approximately 5 times higher than that obtained using the size of 900-2000 µm. The highest oleuropein yield was 5.4 mg/g dry leaf (80% ethanol, 250-500 µm) whiles the lowest oleuropein yield was 0.03 mg/g dry leaf (water, 900-2000 µm). This can be explained by the fact that the surface area increases with the reduction in size and thus the olive leaves are extracted much more easily and efficiently.

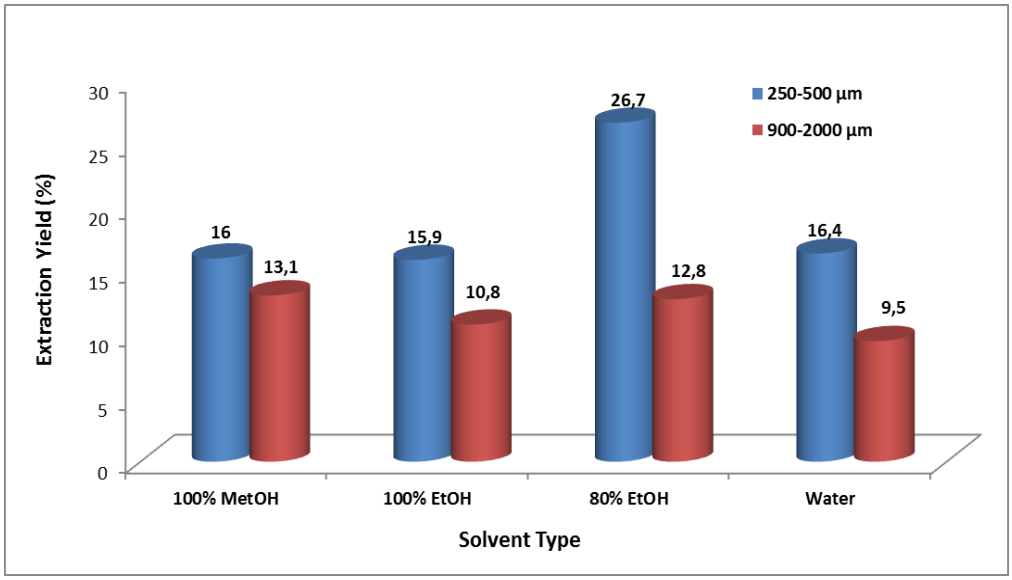


Figure 4.6. The effect of the particle sizes used in olive leaf extraction on extraction yield by the soxhlet method

The effect of particle size on extraction yield is shown in Figure 4.6. The highest yield (26.7%) was found using 80% ethanol solvent and with 250-500 µm particle size whereas the lowest yield (9.5%) was obtained using water with 900-2000 µm particle size of olive leaves. The oleuropein yield increased as the particle size decreased and it can be explained by the fact that the particles can be extracted more easily in solvents by increasing the surface area. In this study, it was observed that the solvents (ethanol, methanol and water) used for particles of 250-500 µm size were higher in extraction efficiency.

4.4. The Effect of Pre-treatment on Oleuropein Yield and Extraction Efficiency

In this section, effect of pre-treatment on the amount of oleuropein in the extract and the extraction efficiency were investigated. In this study, the extraction parameters leading to the highest oleuropein yield were used, taking into account previous experiments. Particle size was chosen in the range of 250-500 μm, and 80% methanol and 80% ethanol were chosen as the solvent. The solvents selected for pretreatment (80% ethanol and 80% methanol) were adjusted to 100 ml volume. 10 grams of olive leaves were placed in the prepared solvent. Treatments were carried out in an ultrasound bath at 40 kHz to 25-30 ° C for 1 hour. After 1 hour pre-treatment with ultrasonic bath, olive leaves were placed on filter paper and placed in soxhlet extractor. For the Soxhlet extraction, 80% ethanol and 80% methanol were adjusted to 250 ml volume. Extraction took place in 4 hours and 8 hours.

Irakli et al. (2018) aimed to investigate the effects of ultrasound assisted extraction conditions (solvent type, solvent concentration, extraction time and temperature) on the yield of oleuropein and hydroxytyrosol. Extraction times were studied between 10 min, 30 min, 60 min, and 120 min. According to their studies, the highest recovery of oleuropein, the main phenolic components in olive leaves, was obtained in the first 10 minutes of extraction and its value was found to be 10.65 mg/g dry leaf. A steady decrease in oleuropein yield was noticed when extraction times longer than 10 minutes were applied in the experiment (Irakli, Chatzopoulou, and Ekateriniadou 2018). Xie et al. (2013) investigated the effects of several important factors such as the type and concentration of extraction solvent, extraction time, extraction temperature, ultrasonic power, liquid/solid ratio and extraction pressure on oleuropein extraction yield from olive leaves. In this experiment, olive leaves were extracted by ultrasound assisted and low pressure extraction methods. For the extraction time, which is one of the experimental parameters, 1, 2, 3, 4, 5, 10, and 15 minutes were chosen. Maximum oleuropein yield was achieved after 3 minutes. Oleuropein extraction efficiency increased up to 3 minutes, but then stabilized with increasing time. These observations showed that oleuropein extraction was over in 3 minutes. Therefore, the 3-minute extraction time was chosen as the optimum extraction condition (Xie et al. 2013).

To examine the effect of pre-treatment on oleuropein yield, the results were compared with soxhlet extraction experiments performed under the same conditions (250-500 μm, 80% MetOH (4 h and 8 h), and 80% EtOH (4 h and 8 h)). Comparing the experiments with the pretreatment and the soxhlet extraction method, the decrease in the amount of oleuropein was clearly seen. This situation can be explained by the degradation of phenolic compounds in the olive leaf with 1 hour sonication process. These data are given in Table 4.3. Among the pretreatment experiments, the highest oleuropein content was found as 5.57 mg / g dry leaf using 80% MetOH for 8 hours, while the lowest oleuropein amount was 3.17 mg / g dry leaf using 80% EtOH for 4 hours, respectively.

Table 4.3. Effect of ultrasonic pre-treatment on the yield of oleuropein

Solvent Type (250 ml)	Extraction Time	Oleuropein Yield (mg/g dry leaf)
80% MetOH (un-pretreatment)	4h 8h	8.79 13.35
80% MetOH (pre-treatment)	4h 8h	4.85 5.57
80% EtOH (un-pretreatment)	4h 8h	10.6 12.44
80% EtOH (pre-treatment)	4h 8h	3.17 5.06

4.5. Total Phenolic Content Assay (Folin Ciocalteu Method)

Folin-Ciocalteu method is used for the determination of total phenolic in plants. This method is one of the oldest known methods (Roginsky and Lissi 2005).

The medium must be basic for phenolic compounds or target compounds to react with Folin-Ciocalteu Reagent (FCR). Sodium carbonate provides this basicity to the environment. As a result of polyphenols losing an H^+ in basic environment,

phenolate anion is formed. This formed anion reduces the molybdate in the structure of FCR to its blue color. The intensity of the blue color seen in the samples is directly proportional to the total amount of phenolic matter in the sample (Huang, Boxin, and Prior 2005; Roginsky and Lissi 2005).

Phenolic content was analyzed at 765 nm and results represented as gallic acid equivalents (mg GAE / ml) from which the constants were obtained from the calibration curve previously given in Figure 3.6. (Eqn. 4.1). The effect of reaction time (4h and 8h), size of 250-500 µm, on total phenolic content of extracted with pure and aqueous solvents were given in Figure 4.8.

$$Total\ phenolic\ content\ (mg\ of\ GAE/ml) = \frac{Absorbance - 0.0061}{4.1743} \quad (4.1)$$

The effect of pure ethanol and its aqueous solutions (80%, 70%, 50% and 20%) and extraction times (4h, 8h) on total phenolic content were examined and results were given in Figure 4.7.

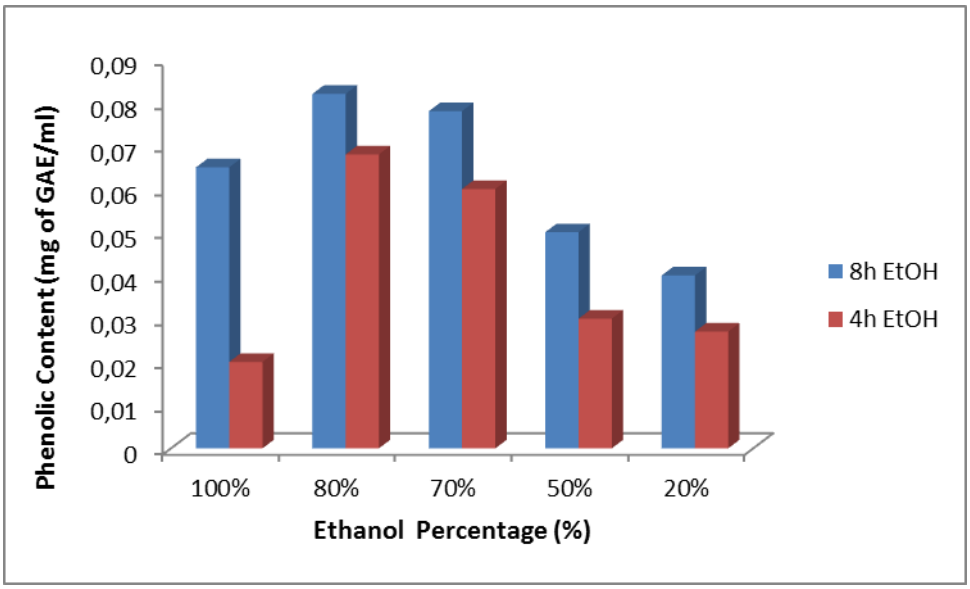


Figure 4.7. The effect of extraction time (4h, 8 h) and ethanol percentage on the total phenolic content

The highest phenolic content was observed using 80% ethanol for 8h (0.082 mg GAE/ml). Extraction for 4 hour with pure ethanol was resulted in the lowest value for phenolic content (0.02 mg GAE/ml). In both the oleuropein amounts and the total phenol content experiments, pure ethanol was not found to be a suitable solvent for extracting olive leaves. 20% ethanol appears to perform better results in terms of both oleuropein and total phenolic content than pure ethanol. It reveals that water is necessary to increase the diffusion of the polyphenols to be extracted from the plant tissues, thus the extraction could take places easily and more efficiently.

Altiok et al (2008) examined the total phenolic content of olive leaves by using pure acetone and its aqueous solutions, pure ethanol and aqueous solutions. The best phenolic content was observed using in 90% acetone solution, while the highest value for ethanol solutions was observed using 70% ethanol solution (Altiok et al. 2008).

Comparison of extraction with ethanol and methanol on phenolic content is shown in Figure 4.8.

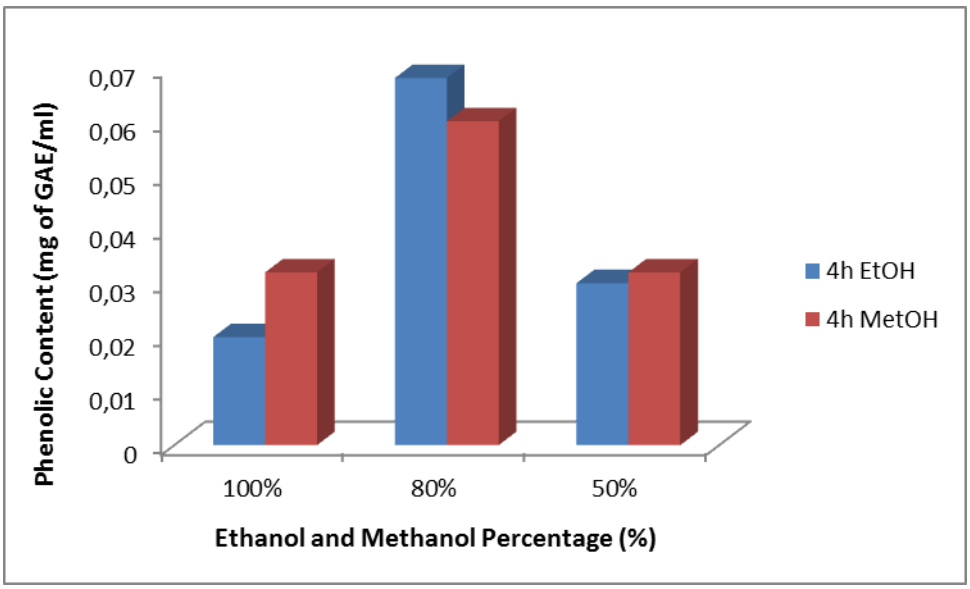


Figure 4.8. The effect of solvent type (ethanol, methanol) on the total phenolic content

The total phenolic content in terms of the effect of the solvent type and its aqueous solutions showed parallel results to the amount of oleuropein. The highest yield was achieved with using 80% ethanol solution and the phenolic content was found as 0.068 mg of GAE/ml.

CHAPTER 5

CONCLUSIONS

In this study, it was aimed to obtain oleuropein, which is the basic phenolic compound content of olive leaf by Soxhlet extraction. Oleuropein has been known for a long time in the health field and is a very important raw material with high availability in our country. In this context, the effects of various parameters on the oleuropein amount and extraction yield were investigated and these parameters were solvent type and aqueous solutions (ethanol, acetonitrile, methanol and water), extraction time (4-8 hours), particle size (250-500 μm and 900-2000 μm) and pre-treatment of the olive leaf.

The use of solvents in an aqueous form resulted in a higher amount of oleuropein compared to pure solvents. This is explained by the fact that water inflates the cells of plants and facilitates diffusion. When the particle size of the raw material to be used in extraction was reduced, a higher oleuropein yield was observed. Increasing the extraction time and using 80% methanol as solvent resulted in a significant improvement in oleuropein yield. In contrast, the use of pre-processed olive leaves in the extraction process resulted in a serious decrease in oleuropein yield. According to the experimental study, the highest oleuropein amount and extraction efficiency were obtained in olive leaves with a grain size of 250-500 μm during 8 hours of extraction and when using 80% methanol solution as a solvent. Under these conditions, the highest oleuropein amount was found to be 13.35 mg / g dry leaves and the highest extraction efficiency 36%.

Finally, this study is important in terms of investigating oleuropein, the highest phenolic compound of olive leaf, with soxhlet extraction method with a wide variety of solvent types and parameters. It also contributes to the valorization of olive leaves by extraction polyphenols from it.

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