AUGUST 2019 M.Sc. in Food Engineering SÜHEYLA İLGÜN BİLTEKİN

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EXTRACTION OF TRITERPENIC ACIDS FROM WET OLIVE POMACE BY USING CLOSED MICROWAVE EXTRACTION SYSTEM

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FOOD ENGINEERING

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Supervisor

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Süheyla İlgün BİLTEKİN

ABSTRACT

EXTRACTION OF TRITERPENIC ACIDS FROM WET OLIVE POMACE BY USING CLOSED MICROWAVE EXTRACTION SYSTEM

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In this study, triterpenic acids (maslinic and oleanolic acid) were extracted from wet olive pomace (51.5 moisture content/100 g wet pomace) under pressure by using closed vessel microwave extraction system with a nonpolar solvent, hexane. Effects of microwave power (150-300 Watt), extraction time (4-20 min) and solvent to solid ratio (5:1-10:1) on extraction efficiency were examined. Optimum conditions which provide the highest efficiency were found as 250 Watt, 12 min and 10:1 for microwave power, time and solvent to solid ratio, respectively. Total amount of triterpenic acid extract which obtained under optimum conditions was 26 mg/g dry pomace. The identification and quantification of obtained triterpenic acids were performed by using HPLC, TLC and ESI-MS. The percentages of the acids present in the extract were 80 % maslinic acid and 20% oleanolic acid. The acids present in the extract was separated from each other by using column chromatography with hexane:ethyl ether:acetic acid (65:34:1) mixture. The antioxidant and antimicrobial activity for extract and acid fractions were determined. According to the DPPH (% inhibition 22.87) and FRAP (263.087 μM TE) results the maslinic acid fraction had shown the highest antioxidant activity. In addition, it was observed that the maslinic acid fraction had almost as much antimicrobial effect on the studied microorganisms as amoxicillin.

Key Words: Microwave Assisted Extraction, Maslinic Acid, Oleanolic Acid, Olive Pomace

ÖZET

TRİTERPENİK ASİTLERİN KAPALI SİSTEM MİKRODALGA İLE YAŞ PİRİNADAN EKSTRAKSİYONU

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68 sayfa

Bu çalışmada yaş pirinadan (51,5g su/100g yaş pirina) kapalı sistem mikrodalga uygulamasıyla basınç altında apolar çözücü hekzan kullanılarak triterpenik asitlerin (maslinik ve oleanolik asit) ekstraksiyonu gerçekleştirilmiştir. Bu kapsamda mikrodalga gücü (150-300 Watt), mikrodalga uygulama süresi (4-20 dk) ve çözücü:pirina oranının (5:1-10:1) ekstraksiyon verimi üzerindeki etkileri incelenmiştir. En yüksek verimin alındığı optimum koşullar sırasıyla mikrodalga gücü, süresi ve çözücü:pirina oranı için 250 Watt, 12 dk ve 10:1 olarak belirlenmiştir. Bu optimum koşullarda elde edilen toplam triterpenik asit ekstrakt miktarı 26 mg/g kuru pirina olarak bulunmuştur. Elde edilen triterpenik asitlerin tanımlanması ve miktarlarının belirlenmesi HPLC, TLC ve ESI-MS kullanılarak gerçekleştirilmiştir. Elde edilen triterpenik asit ekstraktındaki asitlerin yüzdesi, % 80 maslinik asit ve % 20 oleanolik asittir. Daha sonra elde edilen triterpenik asit karışımı kolon kromatografisi ile hekzan/etil eter/asetik asit (65:34:1) karışımı kullanılarak birbirinden ayrıştırılmıştır. Triterpenik asit ekstraktının, ayrıştırılan maslinik asit ve oleanolik asit fraksiyonlarının antioksidan ve antimikrobiyal aktiviteleri belirlenmiştir. DPPH (% 22,87 inhibisyon) ve FRAP (263,087 µM TE) sonuçlarına göre maslinik asit en yüksek antioksidan aktivite göstermiştir. Ayrıca, maslinik asitin çalışılan mikroorganizmalar üzerinde neredeyse amoksisilin eşdeğerinde etki gösterdiği görülmüştür.

Anahtar Kelimeler: Mikrodalga Ekstraksiyonu, Maslinik Asit, Oleanolik Asit, Pirina

To my beloved family…

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ATCC	American Type Culture Collection
CE	Centrifuge extraction
CVD	Cardiovascular Disease
DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	Dry weight
EMS	Electromagnetic spectrum
ESI-MS	Electrospray ionisation mass spectrometry
GAE	Gallic acid equivalents
HPLC	High performance liquid chromatography
MA	Maslinic acid
MAE	Microwave Assisted Extraction
MIC	Minimum inhibitory concentration
OA	Oleanolic acid
OP	Olive pomace
SE	Soxhlet Extraction
SPSS	Statistical package for social sciences
TLC	Thin Layer Chromatography
TPC	Total Phenolic Content
TTA	Triterpenic acids

CHAPTER 1

INTRODUCTION

Olive oil is a nutritionally important food and it is extracted by using different methods. Olive pomace (OP) is obtained as a waste during producing of oil. OP includes vegetation water and olive oil, also it has some valuable compounds. Quantities of these compounds are depend on olive variety. OP contains inorganic compounds, pectic polysaccharides, phenolics, fatty acids and triterpenic acids (TTA) (Alburquerque et al., 2004; Clemente et al., 1997).

Maslinic acid (MA) and oleanolic acid (OA) are types of triterpenic acids that are found in OP. MA and OA have biological effects such as antimicrobial, anticancer, antitumor and anti-inflammatory. These triterpenic acids are used in cosmetic, pharmaceutical and food industry due to its medicinal properties (Lozano-Mena et al., 2014). MA and OA are obtained from different types of sources such as olive oil, olive leaves, fruits and bark etc. by using different types of extraction methods. Classical or modern methods are used such as microwave assisted extraction (MAE), ultrasound assisted extraction, accelerated solvent extraction, pressurized liquid extraction, supercritical and subcritical extraction, solid-liquid extraction and soxhlet etc. (Azmir et al., 2013; Jäger et al., 2009).

Extraction by using conventional methods takes long times, needs high labor cost and shows low extraction efficiency. Microwave assisted extraction method eliminates these drawbacks and some studies prove that it provides better extraction results. Solvent selection, volume of solvent, solvent to solid ratio, microwave power and extraction time are important parameters which affect extraction efficiency (Eskilsson et al., 2000).

The aim of this study is to determine optimum extraction parameters (microwave power, extraction time and solvent to solid ratio) for triterpenic acids by using closed vessel microwave assisted extraction method. Additionally, this study aims to

seperate triterpenic acids from each other to get MA and OA with high purity and to determine antioxidant and antimicrobial properties of them.

CHAPTER 2

LITERATURE REVIEW

2.1 Olive Fruit

Olea europaea is the botanical name of olive fruit. Genus of olive is *Olea* and family is *Oleaceae*. *Olea europaea* L. is the only edible species among 30 species. Olive tree is a small and slow-growing plant and grows naturally in Mediterranean area which has appropriate climate conditions (Luchetti, 2002).

The ninety percent of olives which are cropped all around the world are used for olive oil extraction and the rest is used for table olives. In the world, Spain is the leader country both having the highest number olive trees and being the biggest olive oil producer. Italy is the second country for having olive trees and producing olive oil. Greece is third country for having olive trees but it stay behind of Turkey about producing olive oil. In Turkey, production of olive and olive oil is done generally in Aegean, Marmara, Mediterranean and Southeast Anatolia regions. The biggest production in Turkey is done at Aegean and Marmara region (Saydam, 2015).

2.1.1 Structure of Olive Fruit

Olive is an oval-shaped fruit and has a kernel in it. It has three parts which are epicarp (skin), mesocarp (flesh) and endocarp (kernel). The structure of olive is shown in Figure 2.1. Epicarp is outer layer of olive fruit and has a protective role against the mechanical damage, fungal and pests attack. Skin is covered with wax and has too much chitin which is impermeable to water. Epicarp contains chlorophylls, carotenoids and anthocyanins that account for the color of fruit. During maturation its color changes from the bright to light-green, straw yellow, purple pink and finally to the black (Bianchi, 2003).

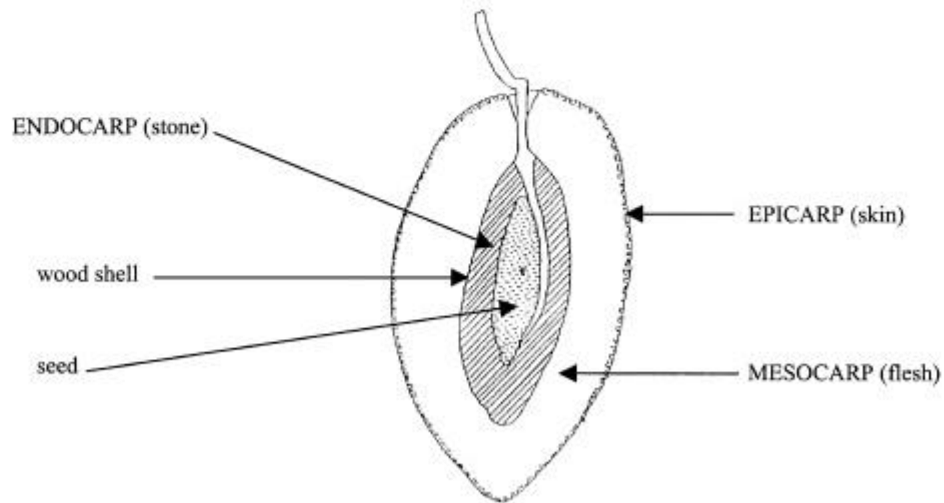


Figure 2.1 Structure of olive (Bianchi, 2003)

Mesocarp is 60-80 % of whole olive fruit and contains edible part. Moreover, olive fruit has water (50%), protein (1.6%), oil (22%), carbohydrate (19.1%) (glucose, fructose, sucrose and mannitol), cellulose (5.8%), inorganic substances (1.5%) and phenolic compounds (1–3%). Other important compounds present in olive fruit are pectin, organic acids (oxalic, succinic, malic, and citric acids), and pigments (Bianchi, 2003; Boskou, 1996).

Endocarp surrounds a kernel which has different weight (18-22 %) for each species. The size and weight of kernel and its easy separation from flesh are important quality parameters of olive fruit. Kernel contains 22-27 % of the oil (Bianchi, 2003).

2.2 Olive Oil Extraction and Olive Pomace

Olive pomace is a solid by product obtained after extraction of olive oil. There are woody particles, olive parts which are skin, pulp, seed in olive pomace and 80 % of total weight composed of these. Moreover, it contains small amount of residual olive oil and vegetable water (Bouknana et al., 2014).

Olive oil can be extracted by using batch (traditional press) or continuous system. The origin of olive oil extraction date back thousands of years. The oldest extraction method based on traditional press system in which olives are grounded by using millstones and then pressed to separate oil from olive paste. The low capital cost is

one of the most important advantages of this batch system (Figure 2.2). However, traditional press system has some disadvantages such as low production capacity, high labor cost and time consuming. In order to overcome these disadvantages the continuous extraction system has been developed. Two types of continuous extraction systems, two phase and three phase centrifugation, are available (Azbar et al., 2004).

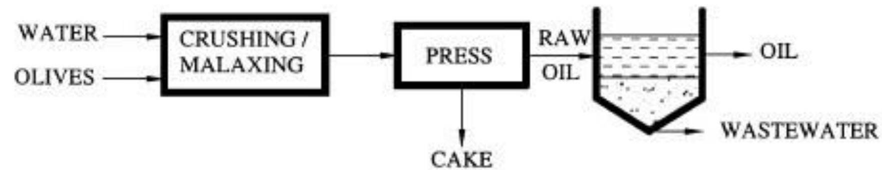


Figure 2.2 Process of traditional press (batch system)

Three-phase centrifugation system (Figure 2.3.a) has been introduced to increase production capacity and decrease labor costs. Although this system has some advantages, there are negative aspects such as high investment cost, energy and water consumption. Additionally, high amount of wastes are generated in three-phase centrifugation system (Roig et al., 2006). Moreover, the uses of high amount of water in three phase centrifugation cause environmental pollution and reduce the amount of natural antioxidants in olive oil (Di Giovacchino et al., 2001).

Two-phase centrifugation system (Figure 2.3.b) has been developed to decrease disadvantages of three-phase centrifugation, particularly to reduce the amount of wastes. In two-phase system two streams are yielded; oil and waste (solid+water) (Azbar et al., 2004). Semi solid by product of this system is olive pomace and known as alperujo (Roig et al., 2006). Phenolic content of olive oil increases by using two-phase system instead of three-phase (Di Giovacchino et al., 2002). Therefore two phase system does not give damage to environment as much as three phase system.

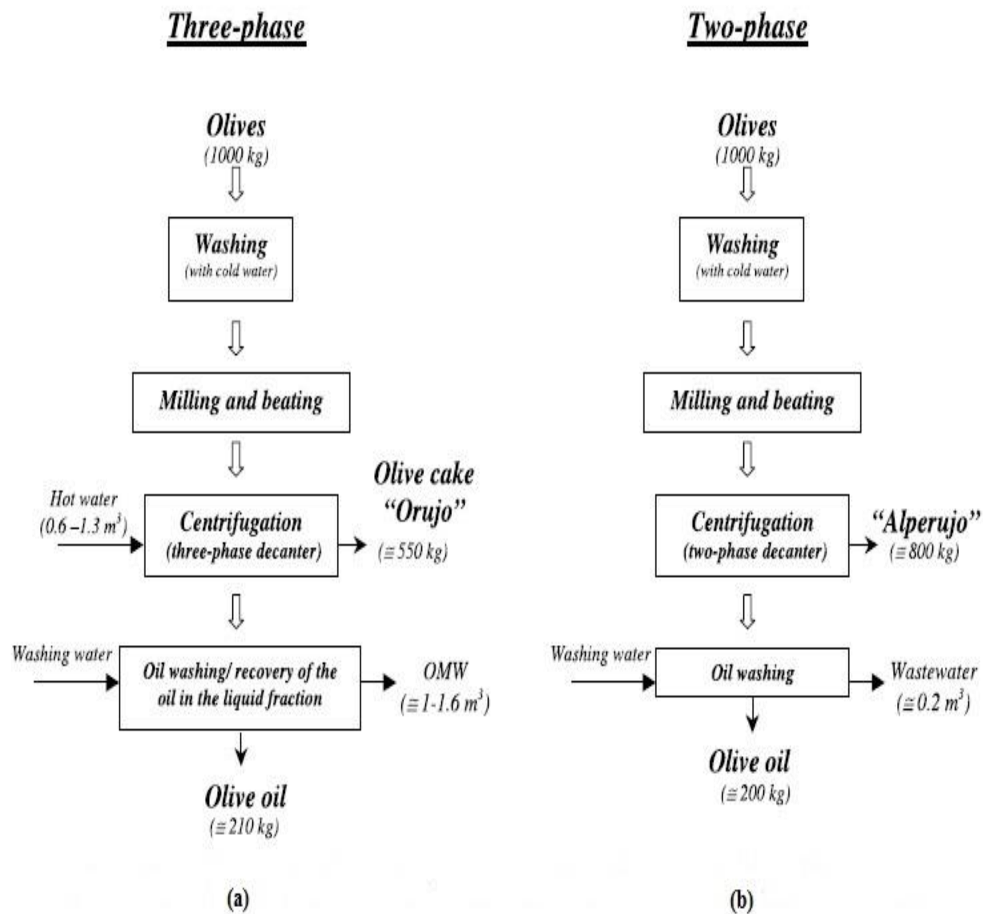


Figure 2.3 Comparison of the three and two-phase centrifugation systems for olive extraction (Alburquerque et al., 2004).

2.2.1 Major and Minor Components of Olive Pomace

Olive pomace has nutritionally valuable compounds and bioactive materials which have medicinal and benefits. Distribution, kinds, structure and quantity of these compounds vary depending on different parameters including climatological conditions, level of maturation and cultivar of olives. Moreover, efficiency of these valuable compounds are related with used extraction methods (Nasopoulou et al., 2013; Uribe et al., 2013).

Sugars, tannins, nitrogenous compounds, volatile and aromatic compounds, polyalcohols, fatty acids, polyphenols, coloring pigments (chlorophylls and carotenoids), pectins, tocopherols, phytosterols and squalene are valuable components which are found in olive pomace (Clemente et al., 1997; Rubio-Senent,

et al., 2015). The element, such as Zn, P, Na, K, Cu, Mg, Fe, Ca and Mn are also present in olive pomace (Alburquerque et al., 2004).

Olive pomace contains considerable quantities of pectic polysaccharides and hemicellulosic polymers. Galacturonic acid is found abundantly in pomace. Arabinose, mannose, xylose, galactose and glucose are other sugars that present in olive pomace (Rubio-Senent et al., 2015).

Fatty acid profile of olive pomace shows that unsaturated oleic and linoleic acids, saturated palmitic acids present in high amount. These fatty acids have beneficial effects for human health (Clemente et al., 1997; Uribe et al., 2013).

OP contains about 98% of olive fruit phenolic compounds which can be divided in several classes: simple phenols (e.g., tyrosol (2-(4-hydroxyphenyl)ethyl alcohol) and hydroxytyrosol (HT) (2-(3,4-dihydroxyphenyl)ethyl alcohol)); cinnamic acid derivatives (e.g., caffeic acid and verbascoside); flavonoids (e.g., apigenin, luteolin and rutin (quercetin-3-rutinoside)); and secoiridoids (e.g., oleuropein, oleuropein aglycone and de(carboxymethyl)oleuropein aglycone isomers) (Obied et al., 2007a). They have high antioxidant capacity, radical scavenging and pharmacological activities (Alu'datt et al., 2010; Alhamad et al., 2017). Studies show that they have antiulcer, antiallergic, anti-inflammatory and antidiarrheal property (Yahyaoui et al., 2014).

Pentacyclic triterpenic acids are another important group of compound which present in olive pomace. Pentacyclic triterpenic acids have a wide variety of biological effects. Main triterpenic acid types found in olive pomace are maslinic acid and oleanolic acid (Garcia et al., 2008; Romero et al., 2010).

2.3 Terpenes

Terpenes are one of the important classes of organic compounds. This class is large and has different structural and functional groups. Terpenes exist abundantly in nature; both in plants and in organisms. Terpenes can be found in the form of free, esters and glycoside conjugates (Lesellier et al., 2012).

The terpenes are biosynthetically constructed from isoprene (C_5H_8) units. Therefore, they are also known as isoprenoids. Carbon skeleton of terpenes forms according to

‘Isoprene rules’. Terpenes are classified according to their number of isoprene units and carbon atoms. Table 2.1 shows classification of them (Reddy et al., 2009).

Table 2.1 Types of Terpenes

Number of Isoprene Units	Chemical Formula	Class
1	C_5H_8	Hemiterpenes
2	$C_{10}H_{16}$	Monoterpenes
3	$C_{15}H_{24}$	Sesquiterpenes
4	$C_{20}H_{32}$	Diterpenes
5	$C_{25}H_{40}$	Sesterterpenes
6	$C_{30}H_{48}$	Triterpenes
7	$C_{35}H_{56}$	Sesquarterpenes
8	$C_{40}H_{64}$	Tetraterpenes(Carotenoids)
N	$(C_5H_8)_N$	Polyterpenes

2.3.1 Triterpenes

Triterpenes exist in nature as triterpene alcohols, aldehydes, esters, ketones and acids forms in plants and some organisms. Triterpenes have six isoprene units and 30 carbon atoms. Over the 100 skeleton structure have been produced by different combination of these isoprene units. These structures can be obtained from enzymatic reactions or natural sources. Triterpenes are classified according to their number of rings present. Squalene (Figure 2.4) is the simplest triterpene which has no ring in its structure and other triterpenes are derived from it (James et al., 2009).

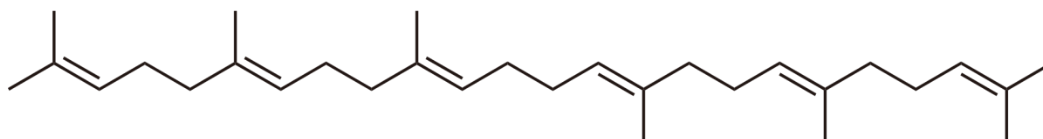


Figure 2.4 Structure of squalene

Number of rings which present in the structure of triterpenes can be two, three, four or five. In this case triterpenes are named as acyclic (squalene), monocyclic, bicyclic, tricyclic, tetracyclic or pentacyclic triterpenes (Xu et al., 2004) (Figure 2.5.a). These rings can be five or six membered. For example cholesterol is an important tetracyclic triterpenoids and has three six-membered rings and one five-membered ring.

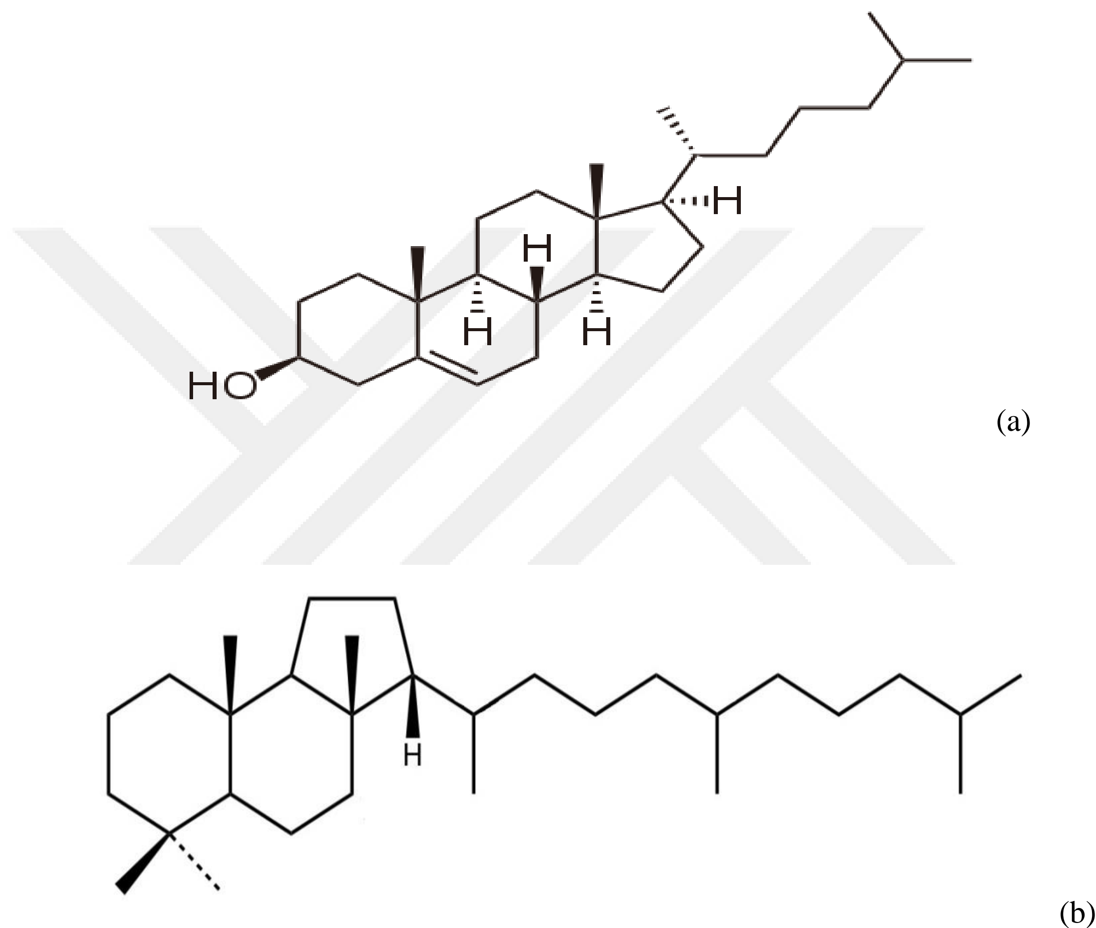
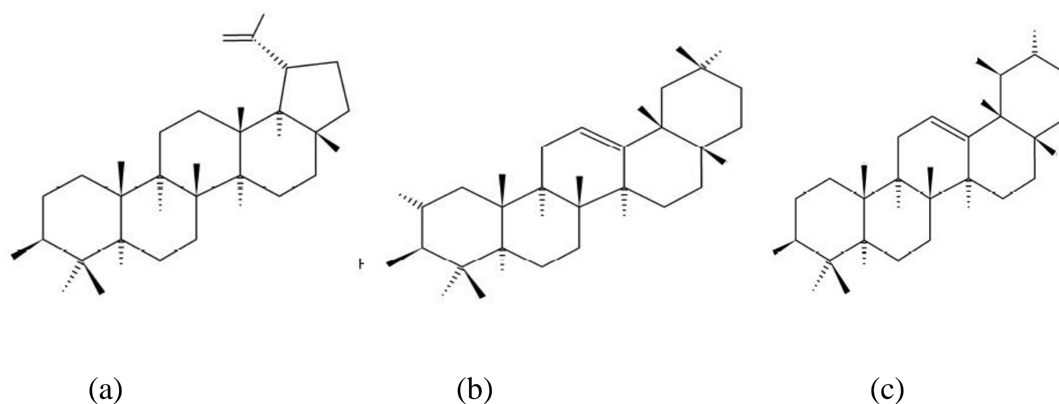


Figure 2.5 (a) Structure of cholesterol (Tetracyclic), (b) Structure of tricyclic

Pentacyclic triterpenes are divided into subgroups: hopanes, ursanes, lupanes, gammaceranes etc. according to their carbon skeletons. Ursanes and oleanes have six-membered carbon ring. Lupanes and hopanes are composed of 4 six-membered rings and 1 five-membered ring (Figure 2.6). Oleane, lupane and ursane are important groups of pentacyclic triterpenes. Maslinic acid, oleanolic acid, erythrodiol and β-amyrin are oleane type triterpenes. Lupeol, betulinic acid and betulin are

lupane type triterpenes. Uvaol and ursolic acid are ursane type triterpenes (Jäger et al., 2009; Muffler et al., 2011).



(a) (b) (c)

Figure 2.6 Structure of (a) lupane, (b) oleane, (c) ursane

2.3.1.1 Oleanolic acid

Oleanolic acid (3b-hydroxyolean-12-en-28-oic acid) is an oleane type pentacyclic triterpene and exist in many plant in nature. Oleanolic acid is found in olive pomace, bark, leaves and fruit (Jäger et al., 2009; Szakiel et al., 2012). Oleanolic acid and ursolic acid are isomers (Figure 2.7 a,b) and found in nature in the form of free or as an aglycone of triterpenoid saponins (Pollier et al., 2012). Oleanolic acid and its isomer also its derivatives are important members of phytochemicals which have diverse pharmacological activities (Sultana et al., 2008).

Oleanolic acid have therapeutic effects on neuroinflammatory/neurodegenerative diseases such as multiple sclerosis, celebral ischemia, Parkinson’s and Alzheimer’s disease. The results of studies (in vitro&vivo) have been shown that oleanolic acid either provide a treatment or prevent formation of disease (Caltana et al., 2015; Martin et al., 2012).

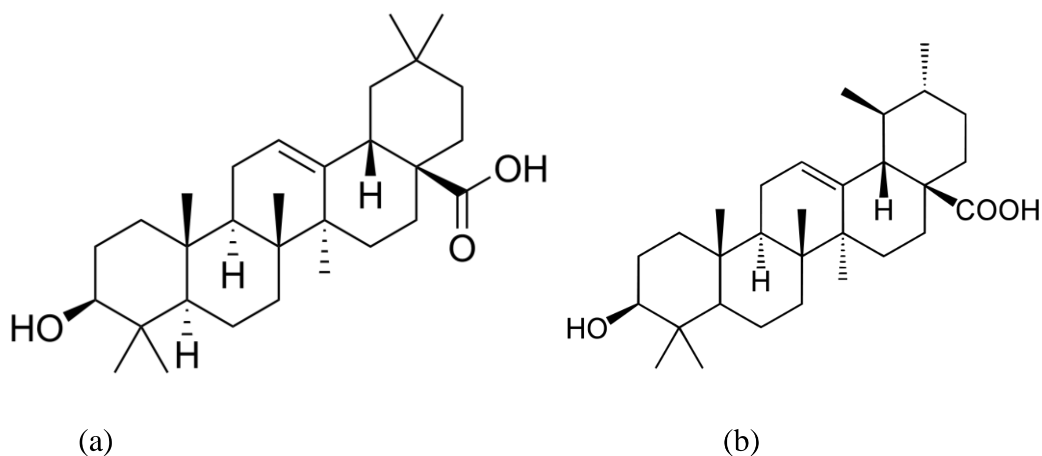


Figure 2.7 Structure of (a) Oleanolic acid and (b) Ursolic acid

Oleanolic acid has antitumor properties. It inhibits formation of tumor or prevent growth of tumor which is at initial or advance step (Laszczyk, 2009). Healing effects of oleanolic acid has been confirmed in vitro&vivo studies at various cancer lines such as liver (Bao et al., 2015), skin (Liu, 1995), pancreatic (Wei et al., 2012), and breast (Allouche et al., 2011; Allouche, et al., 2010).

Oleanolic acid has anti-hyperglycemic activity. Hyperglycemia, other name is diabetes, is a disease that causes blood glucose levels to rise higher than normal (Castellano et al., 2013). Oleanolic acid regulates insulin levels in the blood and helps to treat Type 2 diabetes (Sato et al., 2007).

Drugs or some chemicals intake to body give damage to liver and cause liver injury. This type of illness is called as hepatotoxity. Researches done on mice have been reported that oleanolic acid protects liver of mice from disorder (Reisman et al., 2009). Oleanolic acid also show healing effect in vivo due to anti-ulcer properties (Rodriguez et al., 2003).

Hypertension is major factor of heart diseases. If blood floods with a high pressure gives damage to wall of blood vessels. Oleanolic acid has antihypertension properties also it has antihyperlipidemic effect (Somova et al., 2003).

2.3.1.2 Maslinic Acid

Crategolic acid is other name of maslinic acid ((2α,3β) Dihydroxyolean-12-en-28-oic acid). It is an oleane type natural pentacyclic triterpene and maslinic acid has one more hydroxyl group than oleanolic acid. (Jäger et al., 2009) Figure 2.8 shows chemical structure of maslinic acid.

Maslinic acid presents abundantly on the surface of olive leaves and olive fruits (Romero et al., 2010; Sanchez-Avila et al., 2009). Maslinic acid protects the fruit from insects and it acts as an antimicrobial agent (Pungitore et al., 2005). Maslinic acid is used in medicine and cosmetic area due to pharmaceutical properties (Hashmi et al., 2015). Recent researches show that maslinic acid is an important compound because of some bioactive properties (Lozano-Mena et al., 2014).

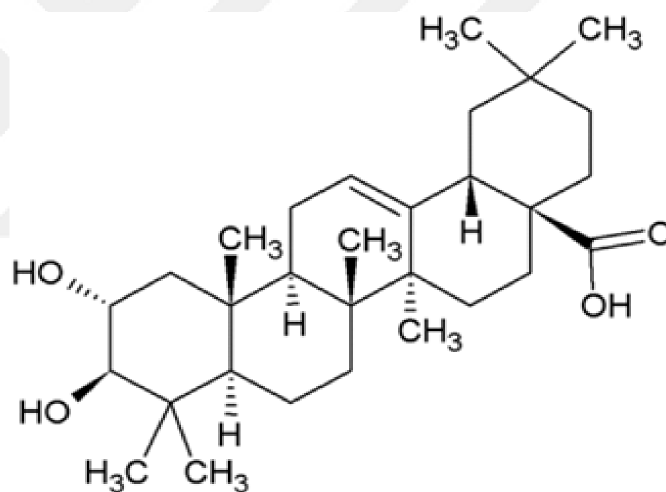


Figure 2.8 Structure of maslinic acid

Cancer is a disease causes death and it is widespread worldwide. Because of that scientists make researches to find cancer drugs. Maslinic acid is used for this purpose due to its apoptosis effects. Apoptosis means ‘programmed cell death’. Maslinic acid uses this property and causes death of cancer cells (Rufino-Palomares et al., 2013). Researches show that maslinic acid and its derivatives trigger apoptosis for various cancer types such as colon cancer (Juan et al., 2008), esophagus and stomach cancers (Lin et al., 2014), skin cancer (Parra et al., 2011).

Maslinic acid is used as food additive in diet of gilthead sea bream (a type of fish). Experiment results show that maslinic acid enhances growth, whole body and total white-muscle weight (Rufino-Palomeres et al., 2012).

Alcohol-related liver disease occur due to drinking too much alcohol. Cirrhosis is the final stage of this disease. Maslinic acid protects the liver from damage of alcohol (Yan et al., 2014).

Glutamate is neurotransmitter, sends signals from cell to another cell, and it is released by nerve cells in the brain. It is very important protein for brain at normal level, however it gives damage when it is found high concentrations. Nerve cells are overexcited and killed or damaged. This situation is called as excitotoxicity. Maslinic acid is neuroprotective agent and regulates glutamate concentration as well as prevents brain from damages (Qian et al., 2011).

Cardiovascular diseases (CVD) are first rank of the cause of death in the world. CVD include heart and blood vessels disorders. High lipid level in blood gives damage to blood vessels and causes CVD which is hyperlipidemia. Maslinic acid regulates cholesterol level of blood, so it supports the health of the heart and blood vessel and hence helps to prevent CVD (Allouche et al., 2010b).

2.4 Extraction Methods of Bioactive Compounds

Extraction can be called with different words in literature; fractionation, isolation, purification, partition etc. Whatever its name, the objective of these processes is to recover target single or a class of compounds from whole. Primary or secondary metabolites can be extracted. In food industry; extraction is used to obtain bioactive compounds such as alkaloids, phenolic compounds, terpenes and terpenoids. These active compounds are used as ingredients in cosmetic, chemical, pharmaceutical and food industry (Azmir et al., 2013).

Bioactive molecules can be extracted directly from plants such as roots, leaves, barks, flowers, fruits, herbs or wastes of food industry. Extraction can be performed from solid to liquid or liquid to solid and so on. The target compounds have different properties. Hence, extraction methods gain importance. Obtaining high amount of yield in a short time with a suitable method is very crucial (Baiano, 2014; Cheok et al., 2014; Sasidharan et al., 2011).

Extraction methods are divided into two groups; traditional extraction and modern extraction. Maceration, soxhlet, solid-liquid or liquid-liquid extraction techniques are under traditional methods category. The main principle of these techniques is relied on the solubility of solute from the source into solvent. Because of that, high volume of solvent is used. Therefore, traditional methods are time consuming methods. Mechanical stirring or shaking may enhance the migration of solutes to solvent. Increasing the temperature may also decrease extraction time, however exposure to heat may cause degradation of thermally labile compounds. Conventional methods are not efficient due to these disadvantages (Azmir et al., 2013; Azwanida, 2015).

Modern extraction methods are designed and developed in recent decades due to disadvantages of traditional method. Some of the modern methods are microwave assisted extraction, ultrasound assisted extraction, accelerated solvent extraction, pressurized liquid extraction, supercritical and subcritical extraction etc. These methods are also called green extraction methods due to using less energy and less solvent. They are automated, so they are under control. Reduction of operational time, higher efficiency and lower cost are some of the other advantages (Azmir et al., 2013; Cheok et al., 2014).

All these techniques have some common processes. They need some pretreatment steps such as maceration, grinding, milling, homogenisation, mechanical stirring, drying etc. for preparing samples to extraction stage. Additionally, all extraction methods require isolation and purification process after extraction to obtain desired component (Cheok et al., 2014).

2.4.1 Microwave Assisted Extraction

2.4.1.1 Microwave and Heating Principle

Electromagnetic spectrum (EMS) consist of classification of electromagnetic waves according to their various wavelengths and/or frequency. EMS ranges from longer wavelengths (ultraviolet, X-ray and gamma rays) to shorter wavelengths (infrared, microwave and radio waves) (Figure 2.9). If wavelength of waves is shorter than 1 m (up to 1 mm), they are called as ‘microwave’. Microwave, within the frequency range of 300 MHz-30 GHz, is placed between infrared and x-rays (Kaufmann et al., 2002; Letellier et al., 1999).

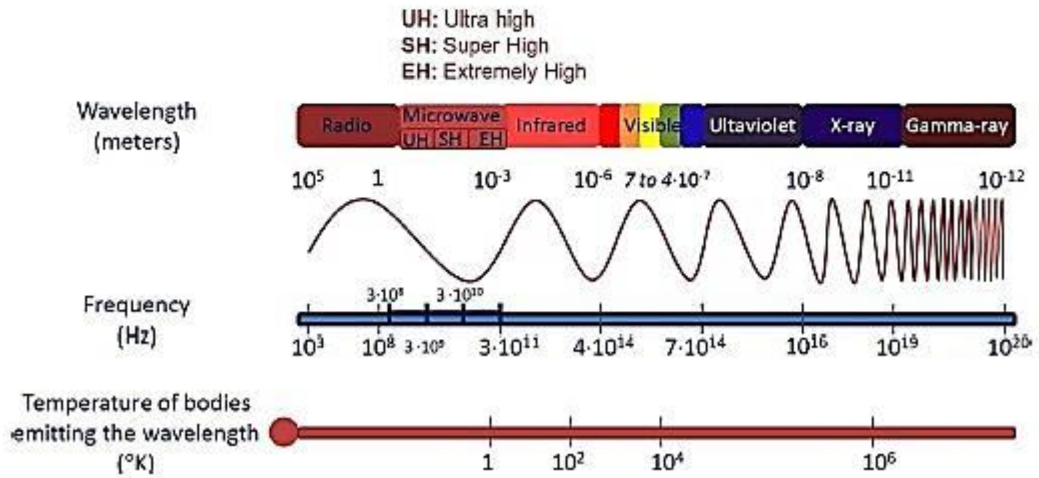


Figure 2.9 Electromagnetic Spectrum

As shown in Figure 2.10, microwaves are electromagnetic waves emerged as a result of oscillation of two fields (electric field and magnetic field) which are perpendicular to each other.

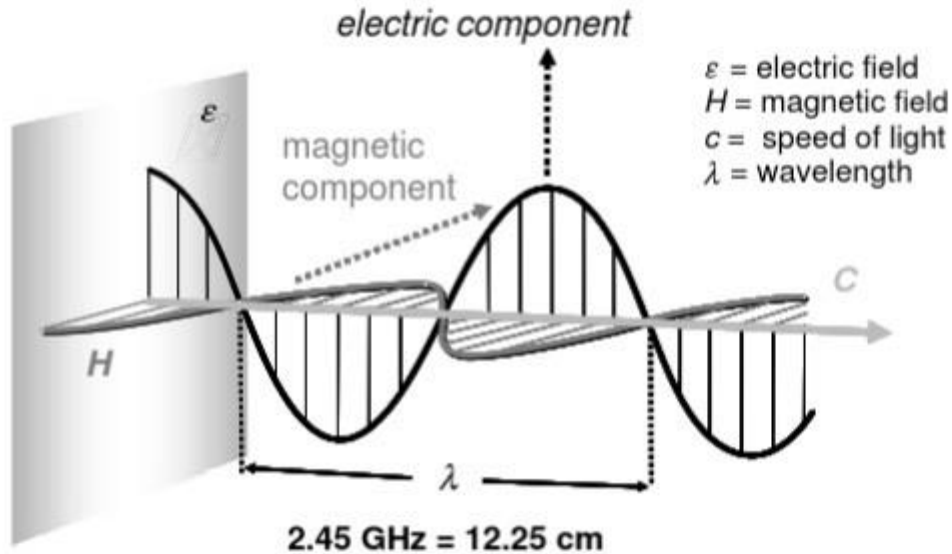


Figure 2.10 Electric and magnetic field components in microwaves

Microwave techniques and applications are developed firstly for military purposes; however, todays it is used at different areas such as communication, medicine, space, food etc. In food industry, generally, heating property of microwave is used. For

example, it is used in sterilization and pasteurization, thawing, drying and cooking of foods (Chandrasekaran et al., 2013; Eskilsson et al., 2000).

Heating mechanism of microwave depends on dipolar motion and ionic conduction which are caused by oscillation of electric field. When microwave is applied, ionic conduction is occurred because of electrophoresis and ions migrate through the medium under influence of electric field. The migration of ions causes collisions between molecules and creates frictions. The direction of ions changes many times as the field changes sign. So, heat is generated due to the resistance of medium to ion flow. Also, dipole molecules are try to divert themselves to the oscillating electric field pole which is shown at Figure 2.11. These rotation of molecules creates heat (Eskilsson et al., 2000; Letellier et al., 1999).

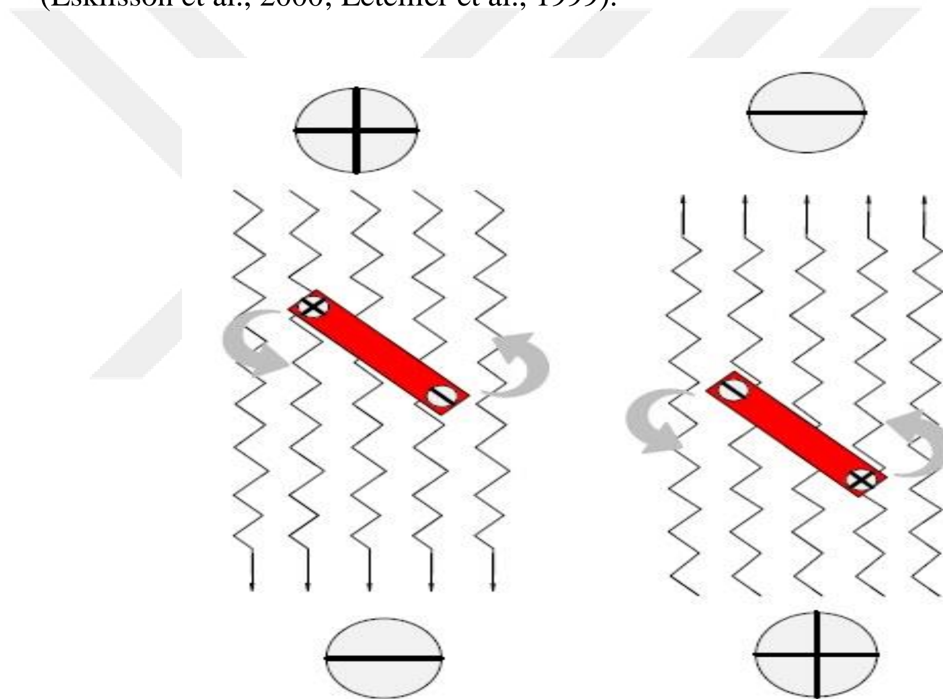


Figure 2.11 Mechanism of microwave heating, dipolar rotation (Zhou et al., 2017)

Microwaves can be reflected, transmitted or absorbed by materials. Materials, which are able to absorb microwave energy and convert it to heat, must be used for creating heat in systems that microwaves are applied. Dielectric constant (ε') expresses the ability of absorbing microwave energy by molecules. Dielectric constant of some solvents are shown at Table 2.2. It is seen that polar molecules (e.g. water) has higher dielectric constant, namely they can absorb microwave energy highly and create

more heat. Not only absorbing energy but also converting it to heat is very important. Efficiency of converting microwave energy into heat is expressed with dielectric loss factor (ε"). These parameters are taken into consideration before microwave application and then suitable solvent is selected to obtain high extraction yield (Camel, 2000; Chandrasekaran et al., 2013; Kaufmann et al., 2002).

Table 2.2 Dielectric constant of some commonly used solvents (Kaufmann et al., 2002)

Solvent	Dielectric constant (20°C)
Hexane	1.89
Toluene	2.4
Dichloromethane	8.9
Acetone	20.7
Ethanol	24.3
Methanol	32.6
Water	78.5

Microwave creates heat with energy transfer not heat transfer, namely this heating can be called as non-contact heating. Microwave heating is different from conventional heating with this aspect. In conventional heating the temperature gradient is from outside to inside and temperature increases slowly due to interaction of outer surface with environment. However, microwaves increase temperature of whole volume of sample simultaneously from inside to outside. Heating effect of microwave is homogeneous, therefore temperature increases faster than conventional heating. Although microwave heats molecules faster, it is not strong enough to induce chemical reactions due to lack of power to breakdown chemical bonds. Microwave cannot change molecular structure (Kaufmann et al., 2002; Letellier et al., 1999).

2.4.1.2 Microwave Extraction Mechanism

Microwave causes sudden temperature increase in sample as mentioned above. Moisture in the cell evaporates due to this temperature. As a result of vaporization, high internal pressure is built in the cell. Weak hydrogen bonds are disrupted because of temperature, pressure and molecular rotation. Pressure leads to swell of cell and forces to split, at the end cell wall is ruptured. So, target molecules are extracted (Li et al., 2013; Wang et al., 2006) (Figure 2.12).

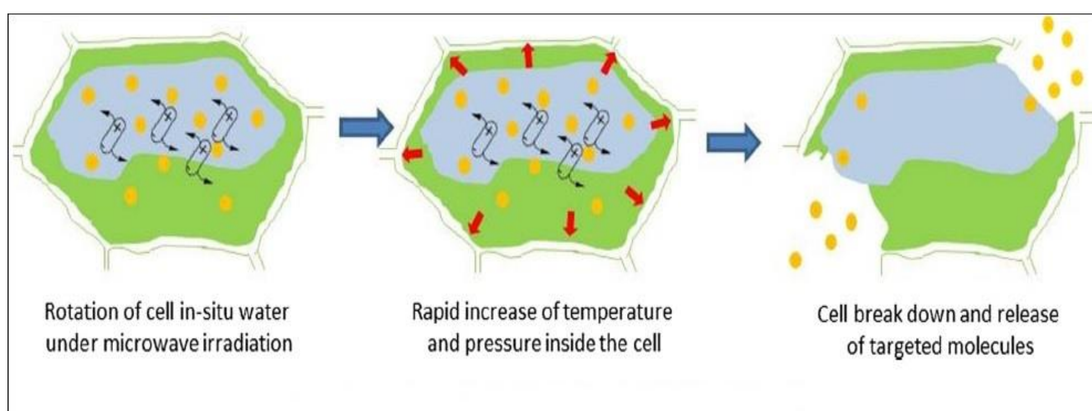


Figure 2.12 Mechanism of solvent-free microwave extraction (Li et al., 2013)

2.4.1.3 Microwave Instrumentation and Extraction Systems

The main components of microwave system are microwave cavity, applicator, magnetron, waveguide and mode stirrer. Microwaves are generated by magnetron which is consist of cylindrical cathode and coaxial anode. Frequency of generated microwaves is not fixed, it can be changed by magnetron. Waveguide, as understood from the name, is a way. Microwaves propagate and reach from magnetron to microwave cavity by using this guide. Microwave cavity is a place where waves are spread and interact to sample (Desai et al., 2009; Kaufmann et al., 2002).

Microwave systems can be monomode or multimode cavities. Monomode cavity can be named as also single, limited or focused mode cavity, because it generates one type of frequency. As shown Figure 2.13.b sample is subjected to direct energy. Thus, temperature of sample increases faster due to high intensity of electric field. Although, heating is homogeneous, monomode systems are useful for small samples. Multimode systems have high volume cavity, so larger samples can be used. As shown Figure 2.13.a multimode systems have a mode stirrer and microwaves are scattered randomly. These waves are reflected by walls and caused non-homogeneous (having different intensities) microwave electric field although every corner of sample is affected (Ameer et al., 2017; Routray et al., 2012).

Microwave extractions can be performed with open systems or closed systems. Open systems are also monomode systems. In open system, extraction is proceed under

atmospheric pressure. It is very simple and safe system, but it is not useful for volatile compounds. The maximum temperature of system can be as much as boiling point of solvent in open system. However, in closed systems temperature can increase above the boiling point. Extractions are carried out under controlled pressure and temperature. Increased pressure in closed system can cause explosion, so these systems require more attention. Volatile compounds can be used in closed systems, but to prevent loss of these compounds temperature must be decreased before opening. This causes increasing of extraction time. Another disadvantage of closed systems to have been multimode reactors. As mentioned above, microwaves disperse within different intensities, so sample is placed on a rotating plate to provide homogeneity (Camel, 2000; Kaufmann et al., 2002; Neas Collins, 1988).

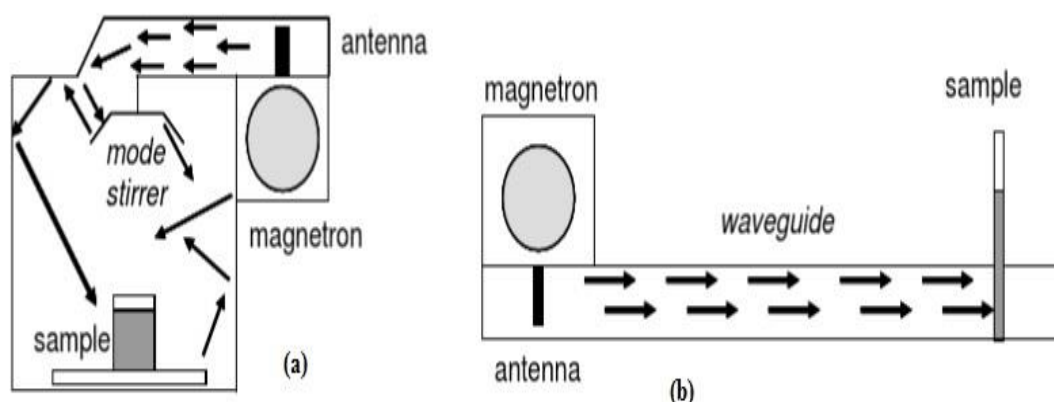


Figure 2.13 (a) Closed System (Multimode) (b) Open System (Monomode)

2.5 Parameters Influence on Microwave Extraction

2.5.1 Solvent Type

Solvent choice is the most important step in microwave extraction. Solvent have interaction with both microwave and sample. Therefore a lot of properties of solvents should be taken into consideration such as polarity, penetration to sample and interaction with analyte. Dielectric constant and dielectric loss factor are important properties for solvents in microwave extraction. Dielectric constant of polar solvents such as methanol, ethanol, water etc. is high and they are mostly used at microwave extractions because of absorbing microwave energy. Nonpolar solvents such as hexane, toluene are not preferred in microwave extraction, because they are

transparent and cannot generate heat. Nonpolar solvents are suitable for thermolabile compounds extraction due to providing rapid cooling. Because of this reason non-polar solvents are used together with a particular amount of polar solvents to increase molecular motion and temperature. In some cases, salt, as a negative and positive ion, is added to solution to increase ionic conduction. Increasing molecular motion and ionic conduction, provides rapid heating and effective extraction (Eskilsson et al., 2000; Letellier et al., 1999; Wang et al., 2006).

2.5.2 Solid Matrix

Physical and chemical properties of solid matrix are among the microwave extraction parameters. Sample shape, size, thickness properties are very important, because microwave heating is volumetric heating. To obtain high yield from microwave extraction, heating must be uniform. If sample size is large, hot spots and cold spots are appeared, heterogeneous heating is observed in microwave systems. Likewise, if sample has sharp edges, corner of sample is heated more or if sample has a uniform shape, in that case center is heated more (Chandrasekaran et al., 2013). Sample size can be decreased, so interaction with microwave increases at the same time extraction yield increases. However, in that point difficulty of separating analytes from solution comes in view as a disadvantage (Wang et al., 2006).

Dielectric constant and dielectric loss factor of sample is as important as that of solvent. Being high dielectric constant and dielectric loss factor of sample allows better microwave penetration and helps to uniform heating (Camel, 2000; Chandrasekaran et al., 2013).

Water inside the sample evaporates due to internal superheating and pressure causes disruption of cells. Porosity of sample increases and solvent penetration become highly. Thus, efficiency of extraction increases with high moisture content (Cheok et al., 2014; Eskilsson et al., 2000).

2.5.3 Solvent to Solid Ratio

Volume of solvent is also important as other solvent properties. Because, optimum ratio of solvent to sample promotes uniform heating and affects efficiency of extraction. If solvent volume is high, more energy is needed for heating. This causes cost and time consumption. Moreover, solute extraction efficiency decreases due to

heterogeneous heating. It can be said that stirring effect or using rotating plate is not sufficient to provide homogeneity. If low amount of solvent is used, in that case interaction of solvent to sample decreases, so analyte cannot be transferred from cell to medium. We cannot say extraction efficiency increases with increasing solvent to sample ratio all the time. In some cases, yield enhances but after a certain time it reaches to steady state. Optimum solvent to sample ratio must be determined specially according to experiment conditions (Eskilsson et al., 2000; Zhang et al., 2011).

2.5.4 Temperature

Temperature is another important factor in microwave extraction as to be in all extraction methods, too. Extraction yield increases with increasing temperature. Water inside the sample evaporates due to high temperature, it creates pressure and finally porosity enhances. Analytes interact with solvent whose solubility increases with elevated temperature. So, more compounds are extracted from sample. However, if temperature continue to increase the higher temperature levels causes degradation of compounds which are sensitive to heat. In closed systems, the temperature can increase the above of boiling point of solvent and the structural deformation of target compounds increases. To prevent this situation, microwave extraction temperature can be adjusted with power. Because, temperature and power affects each other (Camel, 2000; Eskilsson et al., 2000).

2.5.5 Power

Microwave power alone cannot affect extraction efficiency, it is just in a trigger position. If power increases, microwave energy also increases. Elevated energy causes increasing molecular motion, so temperature of system increases. In other words, temperature is affected directly proportional from power changes. Extraction yield increases with power elevation, but at high levels thermolabile compounds are influenced and their structures are destroyed. Additionally, high power level can cause overpressure in closed systems. So, power of system must be selected correctly according to these parameters (Camel, 2000; Routray et al., 2012).

2.5.6 Time

Extraction time of microwave systems is shorter than conventional systems, but exposure time to sample still carries importance on extraction yield. Solvent-sample interaction increases within increasing irradiation time, so efficiency enhances. Sensitive compounds can be damaged because of staying long time at high temperature. To prevent this situation, experiment time is divided into cycles. In that case solution is not exposed to higher temperature, extraction recovery increases without giving damage to analyte (Ameer et al., 2017; Eskilsson et al., 2000).

2.6 Studies on Extraction of Maslinic Acid and Oleanolic Acid

Maslinic and oleanolic acids are extracted from different sources such as olive oil and waste, bark of tree, medicinal plants, fruits, leaves by using different methods. Table 2.3 summaries the studies which performed on extraction of maslinic and oleanolic acid. As shown in Table 2.3 the dried form of plants have been frequently used in extraction of triterpenic acids to extend shelf life of plant during storage and to prevent metabolic activities which can cause degradation of active compounds. However, preparation of plants to extraction, namely drying, takes long times.

Triterpenic acids are distributed in plant matrix and the different parts of plant such as leaves, bark or fruits have different quantity of triterpenic acids. Different extraction methods have also been tried for extraction of them. Polar solvents have been used for extraction of triterpenic acids. In these studies either methanol or ethanol or mixtures of them have studied. Effects of different solvents on extraction yield have been reported in some studies.

Table 2.3 Summary of studies reported in the literature

No	Method	Sample	Compound	Solvent	Quantity	Reference
1	Solid-Liquid Extraction	Dried Leaves of two Ziziphus species	MA-OA and other 7 triterpenic acid types	methanol	MA: 0.44-1.66 mg/g OA: 0.59-1.47 mg/g	Guo et al., 2011
2	Solid-Liquid Extraction	Pomace olive oil	MA-OA	methanol-ethanol mixture	MA : 8000 mg/kg OA : 7000 mg/kg	Garcia et al., 2008
3	Solid-Liquid Extraction	Dried marfil table olive	MA-OA-Erythrodiol	methanol-ethanol mixture	MA :4.8 mg/g dw OA : 3.82 mg/g dw	Gimenez et al., 2015
4	Solid-Liquid Extraction	Dried olive flesh	MA-OA	methanol-ethanol mixture	MA: 287 - 1318 mg/kg olive flesh OA: 169 - 841 mg/kg olive flesh	Romero et al., 2010
5	Solid-Liquid Extraction	The leaves of Eriobotrya japonica	MA-OA and other 5 triterpenic acid types	methanol	MA: 1.88 - 5.39 mg/g crude drug OA: 1.96 - 5.6 mg/g crude drug	Li et al., 2009
6	Solid-Liquid Extraction	Olive leaves	MA-OA and other 3 triterpenoids	ethanol	MA: 0.79 - 1.07 % dw OA:2.16--3.27% dw	Guinda et al., 2015
7	Solid-Liquid Extraction	Dried different fruits peel and flesh	MA-OA and other 4 triterpenoids	ethanol	MA: - OA: 17.2 - 367 mg/kg	Zhang et al., 2014
8	Solid-Liquid Extraction	Dried fermented green olives	MA-OA	methanol-ethanol mixture	MA: 1160 -1420 mg/kg Olive Flesh OA: 501 - 652 mg/kg Olive Flesh	Alexandraki et al., 2014

Table 2.3 Continued

No	Method	Sample	Compound	Solvent	Quantity	Reference
9	Solid-Liquid Extraction	Dried chinese herbs	MA-OA and other 4 triterpenoids	carbon tetrachloride, cloroform, dicloromethane, 1,2-dicloroethane, bromethane	MA: 2.6 - 13.5 mg/kg OA: 28 - 963 mg/kg	Wu et al., 2015
10	Soxhlet, Heat Reflux, Maceration, Ultrasonic, Accelerated solvent, Microwave assissted extraction	Dried Lamii albi flos	OA-Ursolic Acid	acetone	OA: 5.4-22 µg/g dry plant material	Wójciak-Kosior et al., 2013
11	Microwave Assissted Extraction	Dried olive leaves	MA-OA and other 3 triterpenic types	ethanol-water mixture	MA : 2997 - 3353 µg/g OA : 8497 - 13028 µg/g	Sánchez-Ávila et al., 2009
12	Microwave Assissted Extraction	Dried leaves of G. Sylvestre	OA	ethanol	OA: 0.27 - 0.61 % (w/w)	Mandal et al., 2010
13	Microwave Assissted Extraction - Ultrasonic Assissted Extraction (UAE) -Heat Reflux (HR)	defatted residue of Xanthoceras sorbifolia Bunge	Triterpene saponins	ethanol	Triterpene Saponin (MAE): 11.62 % Triterpene Saponin (UAE) : 6.78 % Triterpene Saponin (HR) : 10.82 %	Li et al., 2010
14	Solid-Liquid Extraction	Dried olive ruit	OA - MA	ethyl acetate, methanol, ethanol,methanol-ethanol mixture	OA: 838- 1158 mg/kg Fresh Weight MA: 1905-2524 mg/kg Fresh Weight	Goulas et al., 2011
15	Supercritical fluid extraction	Dried Eucalyptus globulus bark	OA-UA-Betulinic acid, Betulonic acid	ethanol	Total triterpenic acids: 5.1 g/ kg bark	Domingues et al., 2013

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Wet OP kindly obtained from the Güvenal Olive Oil Company located at Gaziantep, Turkey. The wet olive pomace had a moisture content of 52.2 g/100 g. Standards of MA and OA were bought from Sigma- Aldrich. *Staphylococcus aureus* 6538, *Escherichia coli* 25322, *Staphylococcus aureus* 25923, *Klebsiella pneumonia* 700603 and *Pseudomonas aeruginosa* 27853 bacteria were kindly obtained from Gaziantep University, Department of Biology. Methanol, n-Hexane, phosphoric acid, ethyl ether, acetic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), sodium acetate trihydrate (C2H3NaO2•3H2O), concentrated HCl, Iron(III) chloride hexahydrate ($FeCl_3$•$6H_2O$), folin–ciocalteu's phenol reagent, sodium carbonate, gallic acid, trolox and DMSO (Dimethyl sulfoxide) were purchased from Sigma- Aldrich. 2,4,6-tripyridyl-s-triazine (TPTZ) was bought from Fluka. All other reagents and solvents used were of analytical or chromatographic grade.

3.2 Methods

3.2.1 Extraction of Triterpenic Acids (Maslinic Acid and Oleanolic Acid)

3.2.1.1 Centrifuge Extraction

Centrifuge extraction (CE) method which has proceed by Romero, et al., 2010 was used. In CE method methanol/ethanol (1:1, v/v) and hexane were used as an extraction solvent. OP was dried to the constant weight in an oven at 103 ^oC prior to extraction. The dry OP (1 g) was mixed in a centrifuge tube with 4 ml of solvent (methanol/ethanol (1:1, v/v) or hexane) and stirred for 1 min then centrifuged (10000 rpm) for 5 min at 20 ^oC. The liquid phase was separated from the pellet and collected in a separate vessel. This procedure was repeated six times by using fresh solvent for the pellet. The liquid phase collected was vacuum evaporated to get dry MA and OA.

The above procedure has been used for extraction of MA and OA from 2 g wet OP.

3.2.1.2 Soxhlet Extraction

The dry olive pomace (10 g) was weighted into thimble and 220 ml of hexane were transferred into Soxhlet extraction (SE) vessel. After 2 hr extraction, solvent was evaporated by using rotary vacuum evaporator (Heidolph, Hei-VAP Advantage, Heidolph Instruments GmbH & Co. KG, Germany).

3.2.1.3 Preliminary Microwave Assisted Extraction Experiments

The hexane, methanol:ethanol mixture (1:1 v/v) and methanol:hexane mixture (1:1 v/v) have been used as an extraction medium in preliminary experiments in order to decide the type of the solvent for extraction of MA and OA. The dry olive pomace (1 gr) was taken into a Pyrex vial and 10 ml of solvent was added on it. Extraction was done under 250 W for 10 min by using closed vessel pressurized microwave system (CEM Corporation, USA, 3100 Smith Farm Road, Matthews, NC 28105-5044). After extraction liquid phase was decanted and then the solvent was evaporated by using rotary vacuum evaporator. The procedure which has mentioned above was used for 2 gr of wet olive pomace.

3.2.1.4 Microwave Assisted Extraction of Triterpenic acids

Closed vessel pressurized microwave system (CEM Corporation, USA, 3100 Smith Farm Road, Matthews, NC 28105-5044) was used in extraction experiments. This microwave system consisted of an infrared sensor for temperature measurement coupled with an electromagnetic stirring with adjustable speeds and air cooling for simultaneous cooling. The wet olive pomace (2 gr) was taken into a 35 mL Pyrex vials that has a magnet in it and then appropriate amount of hexane was added in to the vial. After that the vial was sealed and placed in to the instrument. In order to determine optimum extraction conditions; microwave power (150-300 W), extraction time (4-20 min) and solvent to sample ratio (5:1-10:1 g/mL for olive pomace) were examined in this microwave system. After microwave irradiation, hexane part that includes the triterpenic acids was transferred to centrifuge tubes and centrifuged (Eppendorf centrifuge 5810 R) at 6000 rpm at 25 ^{o}C for 15 min. Liquid phase was thrown out and the crude pellet (triterpenic acids) was obtained. The weight of the crude pellet was measured after 3 h kept at 25 ^{o}C under the fume hood. The pellet

which obtained at optimum extraction conditions was further washed 6 times by using fresh hexane to remove the remaining fatty acids and derivatives to get the final pure product. Figure 3.1 shows the schematic representation of microwave assisted extraction of MA and OA.

Same experiment was done with dry olive pomace.

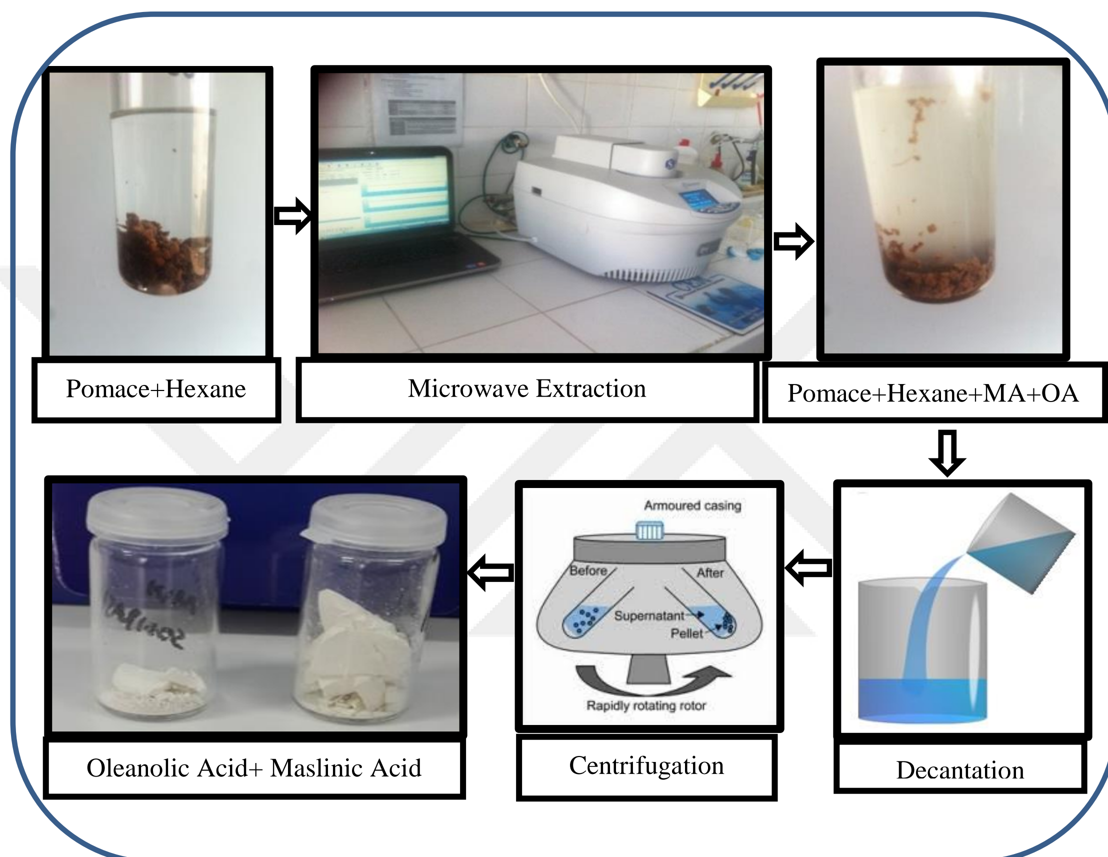


Figure 3.1 Shematic representation of extraction of MA and OA by using microwave assisted extraction

3.2.2 Seperation and Identification of Triterpenic Acids

3.2.2.1 Seperation of Triterpenic Acids by Thin Layer Chromatography

Thin layer chromatography (TLC) was used to find the proper combination of elution solvent before column chromatography and to test whether the components were separated after column chromatography. Different solvent combination have been tried to get the best separation. In the present study, the best separation of MA and OA from the extract on TLC plate has been achieved by using hexane:ethyl

ether:acetic acid mixture in a ratio of 65:34:1. The method modified from Ruiz-Gutierrez et al., (2000) was used in the present study. TLC on silica gel 60 F254 aluminum sheets purchased from Merck (Darmstadt, Germany). Briefly, the TLC plates were cut into correct size and they were activated in oven for 30 min at 110 ^{o}C. A straight line was drawn about 1 cm from the end of plate. Extract was dissolved at methanol and a spot of it is applied to line by using a TLC pipette. The hexane: ethyl ether: acetic acid mixture (65:34:1) has been used as mobile phase for the elution of the sample. Mobile phase was taken into a beaker and then TLC plate was placed into the beaker. The level of the solvent in the beaker was kept under the line that previously signed on the TLC plate. The beaker was closed and allowed that capillary action to draw the solvent up to the plate. After that the plate is placed into a jar containing iodine crystals for a few minutes to make the layers separated visible.

3.2.2.2 Seperation of Triterpenic Acids by Column chromatography

Column chromatography has been used for the separation of the extract into MA and OA (modified from the (Dontha et al., 2015; Lee et al., 2008)) fractions. The separation procedure was completed with 3 steps described below.

Preparation of silica

First of all, the silica gel which was used as a support material for column chromatography was standardized. For standardization, silica gel was put into the oven which was at 150 ^{o}C for 4 hrs and then was cooled to room temperature. The water which was % 5 percent of silica gel was added into the silica gel and mixed by shaking 1 hr.

Packing of the column

Mini column (1.5 cm x 30 cm) was half-filled with this solvent mixture and placed to ring stand. The glass wool was fixed to the bottom of column with the help of a long glass rod. Silica gel (7 gr) was weighed in an Erlenmeyer flask and then enough solvent was added with stirring to obtain slurry. All of the air bubbles were removed from slurry and it was added to the column with the aid of a funnel. As soon as the column began to build, the stopcock was opened and excess solvent was drained.

Column was tapped to remove air bubbles without frustrating the column. When the silica had packed, a final layer of sand (≈ 5 mm) was added to column (Figure 3.2).

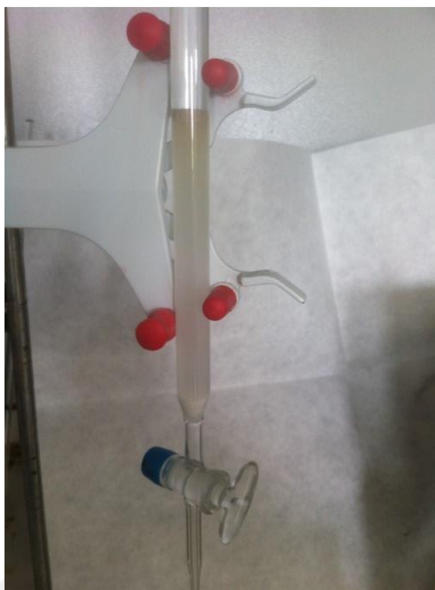


Figure 3.2 Photograph of packed mini column

Elution

Triterpenic acid extract (20 mg) were diluted with 0.55 ml methanol and loaded to column. The hexane: ethyl ether: acetic acid mixture (65:34:1) has been used as mobile phase for the elution of the sample. Mobile phase was added from top of the column and eluent was collected. Elution rate was 15 drops / 10 seconds. While the first 35-40 ml of eluent was collected in a beaker as oleanolic acid, the following eluent was collected as maslinic acid into another beaker. The eluents collected as MA and OA were further analyzed by using high performance liquid chromatography (HPLC) and electrospray ionization mass spectrometry (ESI-MS) as described below to determine the separation performance of the column.

3.2.2.3 Identification of Triterpenic Acids by HPLC Analysis

Chromatographic separation and identification of triterpenic acids was done with HPLC (Shimadzu, Kyoto, Japan) by using ODS-2 column (Sphereclone 5 μ ODS(2), 250 × 4.6 mm; Phenomenex, CA, U.S.A.). Column temperature was set at 35 ^{o}C. Mixture of methanol/water (92:8, v/v), acidified with phosphoric acid at pH 3.0 was used as mobile phase and its velocity was 0.8 mL/min. Eluate was monitored at 210 nm with UV detector (SPD-20A; Shimadzu, Japan). The peak identification has been performed by using standard MA and OA. Sample was diluted with methanol and

injection volume was 20 μL. Total analysis time was 17 min. Different concentrations of MA (1000 ppm, 800 ppm, 600 ppm, 400 ppm, 200 ppm, 100 ppm) and OA (1000 ppm, 750 ppm, 500 ppm, 250 ppm, 100 ppm) were prepared and HPLC analysis were done to obtain calibration curves (Figure 3.3, 3.4).

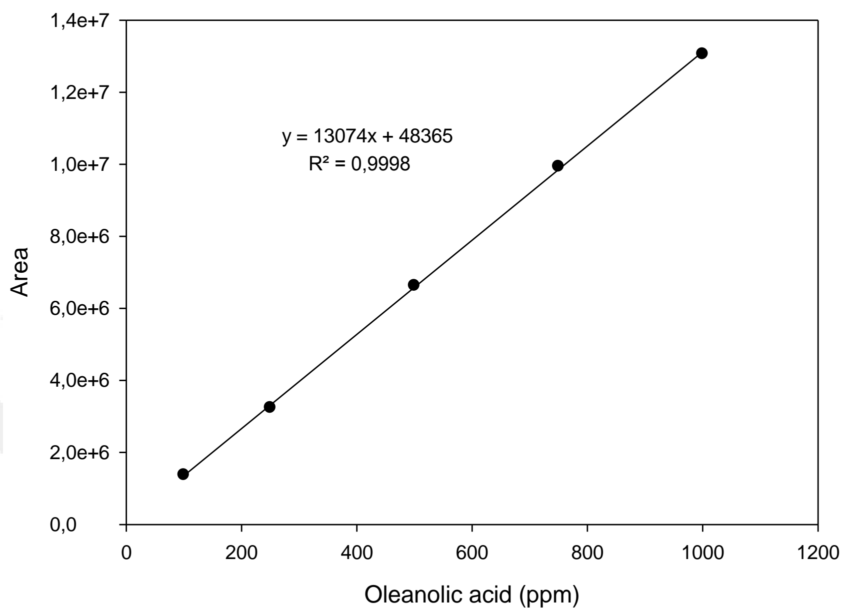


Figure 3.3 Oleanolic acid calibration curve

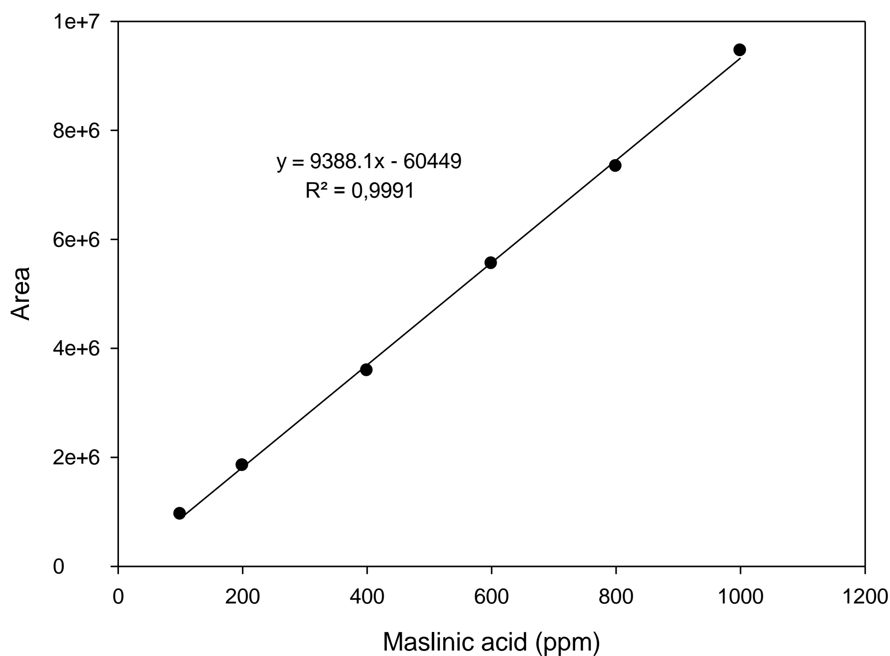


Figure 3.4 Maslinic acid calibration curve

3.2.2.4 ESI-MS Analysis

The maslinic and oleanolic acids were isolated from the extract using column chromatography prior to mass spectra analysis. The mass spectra of fractions were recorded on Ab-SciEx 3200 QTrap MS detector with electrospray ionization probe (Framingham, MA, USA) by direct injection. The method which proposed by Karaman et al., (2016) was used to get the mass spectra of them.

3.2.3 Analysis of Antioxidant Capacity

3.2.3.1 DPPH- Scavenging Activity Method

The DPPH radical scavenging activity of samples (MA, OA and mixture of MA and OA) was analyzed according to the method proposed by Brand-Williams et al. (1995). Methanolic DPPH solution (final absorption adjusted to 0.800 ± 0.010 AU at 517 nm) was prepared by addition of methanol to a 2500 µL of 89.7 µmol/L DPPH. First of all 2 mg of sample was dissolved in 0.4 mL of methanol. Sample (0.1 ml) was withdrawn from this solution and further diluted by addition of 1 mL of methanol. In order to measure the scavenging activity of the final solution 500 µL of solution or blank was added into the 2.5 mL of freshly prepared DPPH solution. The mixture was shaken vigorously and incubated in the dark place for 1 h at room temperature. At the end of the incubation period, the absorbance value of the solution monitored at 517 nm against a blank of pure 95% methanol using a Perkin Elmer Lambda 25 UV/Vis spectrophotometer (Connecticut, USA). The antiradical action of the samples was determined from the difference in absorbance with or without sample (control). The percent inhibition was calculated using the following formula:

$$\% \text{ DPPH-scavenging activity} = (1 - [A_{sample}/A_{control}]) \times 100$$

where A_{sample} is the absorbance of sample with DPPH solution, $A_{control}$ is the absorbance of DPPH solution without sample solution at 517 nm. The all measurements were done in triplicate.

3.2.3.2 Ferric Reducing Antioxidant Power Method (FRAP)

The ferric reducing antioxidant power of samples (MA, OA and mixture of MA and OA) was measured according to the method proceed by Benzie and Strain (1996) with some modifications. The constituents of FRAP solution were sodium acetate buffer (300 mM, pH3.6), 10 ml of 1,3,5-tri (2-pyridyl)-2,4,6-triazine (TPTZ) solution

(10 mM TPTZ in 40 mM HCl) and 20 mM iron (III) chloride solution in 10:1:1 (v/v/v) ratios, respectively. The FRAP solution was prepared and held at 37 ⁰C during experiment. 100 µL of calibration solution (Trolox), blank or sample solution (2 mg sample in 400 µL methanol) were mixed with 3.0 ml of FRAP solution, left in dark for 10 mins. Absorbances of samples were measured at 593 nm, using methanol as blank. Trolox solutions (100µL) in the concentration range of 0-500 µmol/L methanol are prepared to draw calibration curve. FRAP tests were done in triplicate.

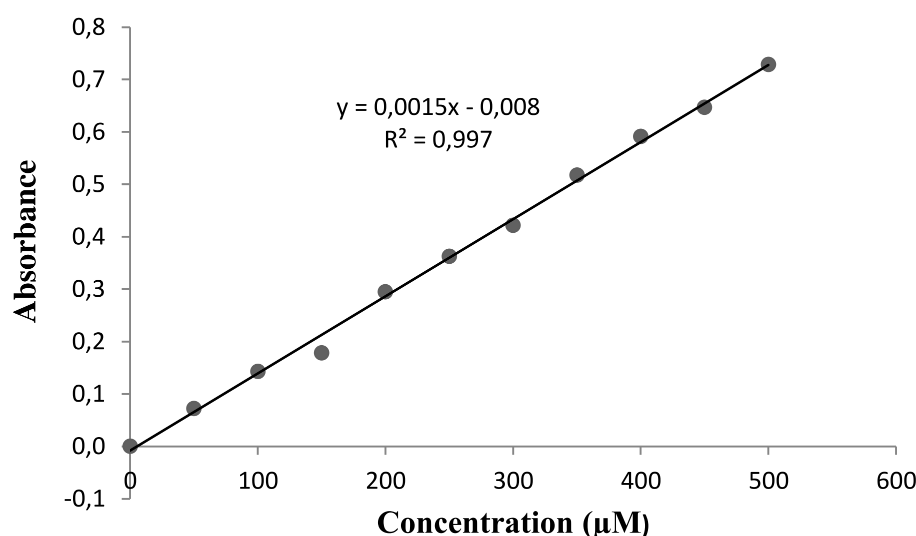


Figure 3.5 Trolox calibration curve

3.2.4 Analysis of Antimicrobial Activity

Antimicrobial analysis was done according to method described by EUCAST (2015). *Staphylococcus aureus* 6538, *Escherichia coli* 25322, *Staphylococcus aureus* 25923, *Klebsiella pneumonia* 700603 and *Pseudomonas aeruginosa* 27853 bacteria were grown in broth culture. Suspension is adjusted to achieve a turbidity equivalent to a 0.5 McFarland turbidity standard by using Mueller Hinton Broth (MHB). EUCAST Standards were taken into consideration in microdilution method. Amoxicillin was used as a control. 512 µg of maslinic acid, oleanolic acid and mixture of them were weighted and dissolved in 1000 µl DMSO and first concentrations were prepared. After incubation for 18–24 h at 37 ˚C, the Minimum inhibitory concentrations (MICs) were determined.

3.2.5 Total Phenolic Content (TPC) by Folin-Ciocalteu's Assay

The Folin-Ciocalteu method was used to determine total phenol levels in samples, which was adapted from Singleton et al. (1999). Briefly, ethanolic extracts (450 µL) was mixed with 2.25 mL of Folin-Ciocalteu reagent, previously diluted with distilled water (1:9, v/v). After 3 minutes of shaking at room temperature, 1.8 mL of sodium carbonate solution (75 g/L) was added to the samples, followed by a reaction time of 2 hours at room temperature. Detection of the phenols was achieved spectrophotometrically (Beckman Coulter, Brea, CA) at a wavelength of 760 nm. Gallic acid solutions (450 µL) at various concentrations (10-100 µg/mL) were used to plot calibration curve (Figure 3.6). TPC values were expressed as gallic acid equivalents (GAE) in mg per g of the dry sample. All measurements were done in triplicate.

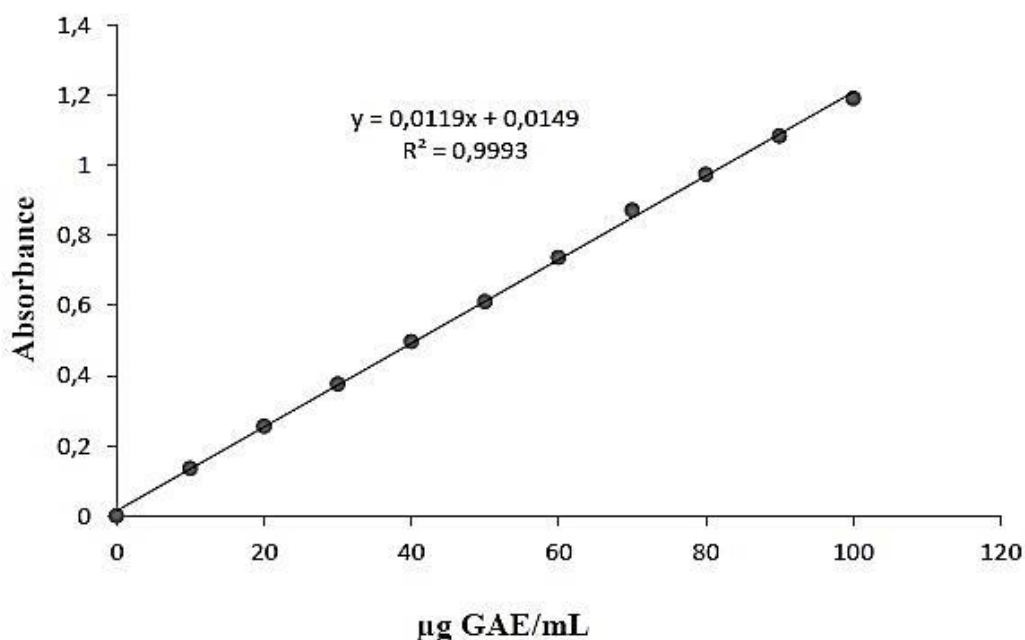


Figure 3.6 Gallic acid calibration curve

3.2.6 Statistical Analysis

The SPSS Statics 15.0, version 2.0 (2006), (SPSS Inc., Chicago) was used for statistical analysis. One-way analyses of variance (ANOVA) were conducted to determine the effect of microwave power, time and solvent to sample ratio on extraction of TTA from olive pomace. Each measurement was triplicated. In order to

determine which means are significantly different from each other, Duncan multiple range test method was used. Trends were considered significant when means of compared parameters differed at $P < 0.05$ significance level.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Results of Preliminary Studies

In preliminary studies extraction of triterpenic acids have been performed by using different extraction methods to get high extraction efficiency and to get high product purity. To this end, microwave assisted extraction, soxhlet and centrifuge extraction methods with different solvent (polar, non-polar, mix) uses have been tried. All of the preliminary extraction studies except soxhlet extraction have been performed on wet and dry olive pomace separately. The results of preliminary studies have been summarized in Table 4.1.

Table 4.1 Results of preliminary studies

No	Extraction Method	Sample	Solvent	Amount (mg TTA extract/ g dry pomace)
1	MAE[1]	Dry olive pomace	Methanol-Ethanol	127.8 ± 2.03
2	MAE[1]	Dry olive pomace	Methanol-Hexane	176.2 ± 1.86
3	MAE[1]	Dry olive pomace	Hexane	9.0 ± 0.9
4	MAE[1]	Wet olive pomace	Methanol-Ethanol	150.3 ± 2.69
5	MAE[1]	Wet olive pomace	Methanol-Hexane	115.7 ± 2.57
6	MAE[1]	Wet olive pomace	Hexane	22.4 ± 0.30
7	Centrifuge[2]	Dry olive pomace	Methanol-Ethanol	32.3 ± 0.53
8	Centrifuge[2]	Dry olive pomace	Hexane	ND
9	Centrifuge[2]	Wet olive pomace	Methanol-Ethanol	86.6 ± 1.43
10	Centrifuge[2]	Wet olive pomace	Hexane	ND
11	Soxhlet[3]	Dry olive pomace	Hexane	28.0 ± 0.75

[1] 250W,10 min, 1:10 solvent to sample ratio; [2] 10000 rpm, 5 min, 20 °C; [3] 2 hr; ND: not detected;
MAE: Microwave assisted extraction

First of all it is seen that the extraction yield is higher in MAE with usage of methanol-ethanol or methanol-hexane than that of in CE and SE. Although extraction yield was high with uses of polar solvent, the brownish coloring agents, phenolic compounds and some other impurities were also extracted together with target acids (MA and OA) which have naturally white in color (Figure 4.1.a- 4.1.b). Same situation was observed in CE and SE methods for polar solvents. It can be discussed

that more phenolic compounds could be extracted together with MA and OA in the use of polar solvent. Because, it is known that the polar solvents (methanol/ethanol) are very effective for the extraction of phenolic compounds. Additionally, it is known that the olive pomace contains huge amounts of phenolic compounds. Moreover, the results of total phenolic content of final products which obtained from polar and nonpolar extractions have confirmed that the total phenolic content of polar extracts were almost 10 times higher than that of nonpolar extracts. For example, while the total phenolic content of the extract after MAE with polar solvent was found as 32.21 mg GAE /g dry TTA extract, it was found as 3.35 mg GAE /g dry TTA extract in the case of hexane usage. Therefore this approach seems reasonable. The HPLC analysis was performed to identify and quantify the extract obtained.

On the other hand, the HPLC analysis showed that there is an extra peak on HPLC-chromatograms (Figure 4.2-4.3). Hence it was supported that some undesired compound was also extracted besides MA and OA in the case of usage of polar solvents. Moreover, in the use of polar solvent the amount of target components (MA and OA) was smaller than that of uses non-polar solvent. For example when the polar solvent was used for the extraction, the amount of desired compounds was almost 35 % of the total yield. However this value was around 95 % in the usage of hexane.

On the other hand, when hexane was used as solvent, there is no yield in centrifuge extraction method. However in MAE with hexane when dry pomace was used, the extraction yield was very low. The usage of wet pomace in MAE with hexane gave higher yield than that of dry pomace**.** In addition to that almost pure (95 %) white colored MA and OA powders were obtained in MAE with hexane (Figure 4.1.c). HPLC results (Figure 4.4) of this extract also showed that only MA (80 %) and OA (20 %) were found as a triterpenic acid in this extract.

When the soxhlet results were examined, there was a little bit higher yield in soxhlet extraction as compared to MAE with hexane. However SE was not chosen as the extraction method for further experiments due to its long extraction time and requirement of dry raw material.

As a result MAE, wet olive pomace, and hexane have been selected as extraction method, raw material and solvent, respectively.

(a) (b) (c)

Figure 4.1 Olive pomace after **(a)** Microwave Assisted Extraction (Polar Solvent) **(b)** Centrifugation (Polar Solvent) **(c)** Microwave Assisted Extraction (Non-polar Solvent)

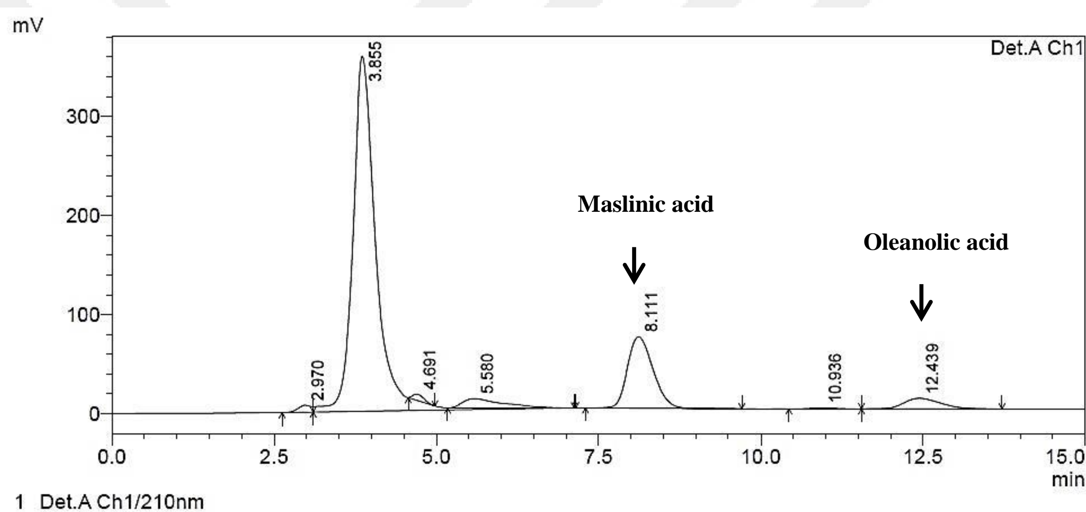


Figure 4.2 HPLC chromatogram at 210 nm of maslinic acid and oleanolic acid after MAE with polar solvent

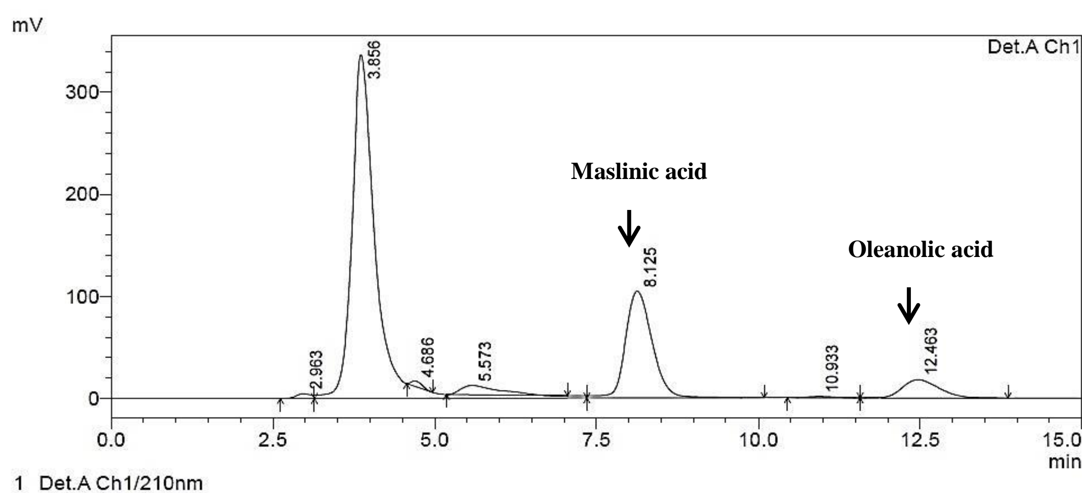


Figure 4.3 HPLC chromatogram at 210 nm of maslinic acid and oleanolic acid after centrifugation with polar solvent

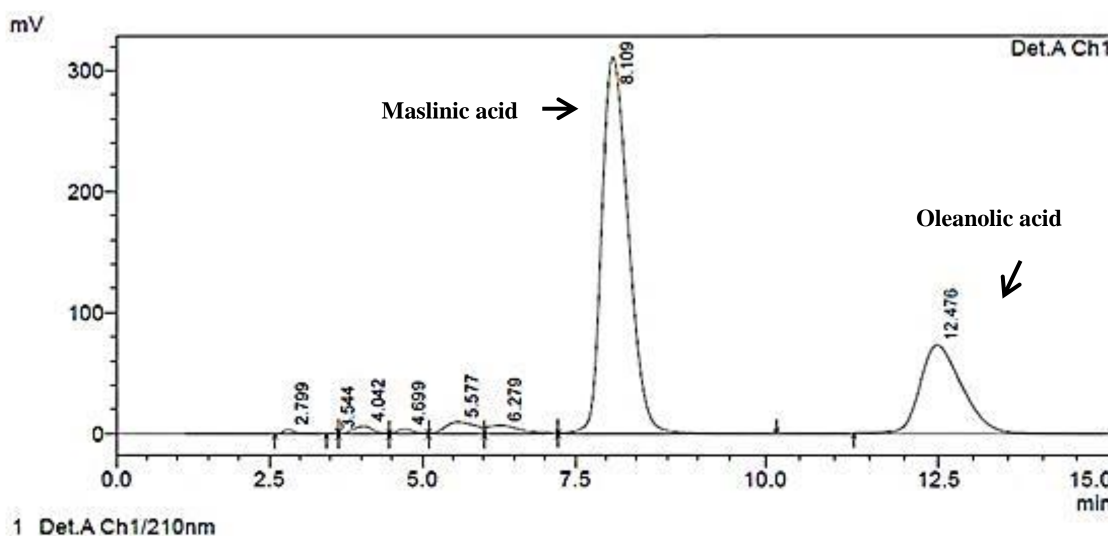


Figure 4.4 HPLC chromatogram at 210 nm of maslinic acid and oleanolic acid after microwave assisted extraction by using hexane

4.2 Effect of Microwave Extraction Parameters on Triterpenic Acids Yield

4.2.1 Microwave Power

The effect of microwave power on extraction efficiency has been shown in Figure 4.5. It has been observed that the extraction yield increases with increasing microwave power and the highest amount of triterpenic acid extract (20.7 mg/g) was obtained at 250 W. The results of statistical analysis also shows that the microwave power is significantly affected ($p<0.05$) on extraction efficiency. Although hexane, microwave transparent solvent, was used as a solvent in this study the temperature of the system was increased due to moisture which present in olive pomace. The increasing microwave power elevates the temperature and the internal pressure. Consequently the cell wall is ruptured and the target compounds release from sample matrix easily (Cheok et al., 2014). Similar explanation was also reported to support the effect of microwave power on microwave extraction of oleanolic acid from *Gymnema sylvestre* (Mandal et al., 2010). Contrary to expectations a significant reduction has been observed on yield after 250 W. The reason of decline would be preventation of pressure. In addition to that extreme temperature increases may have increased the solubility of the analyte in the matrix, thus the migration of analyte from the matrix to the solvent may be prevented.

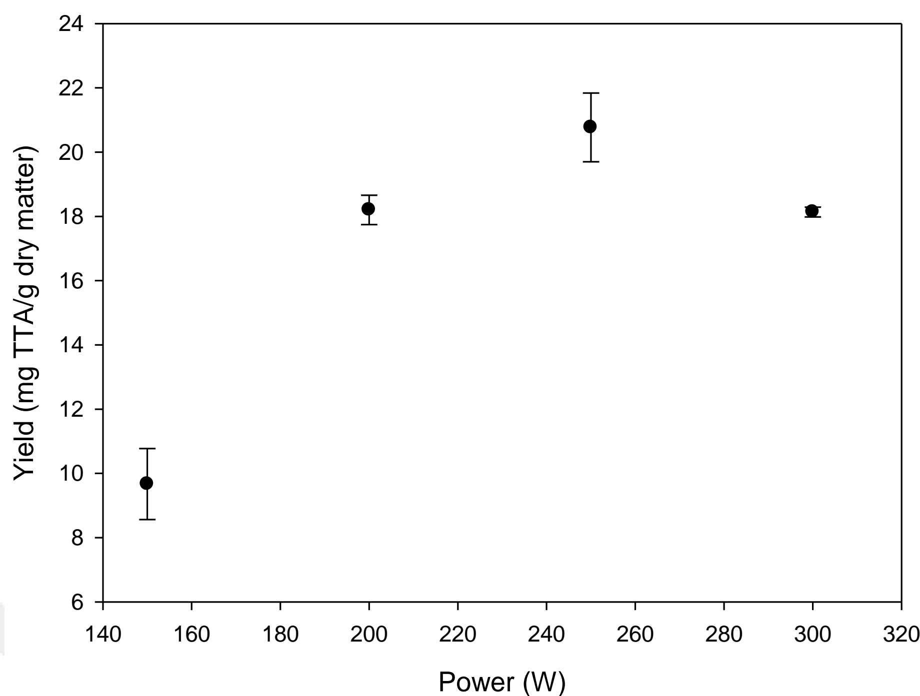


Figure 4.5 The effect of microwave power on yield of triterpenic acid

4.2.2 Time

Figure 4.6 shows the effect of microwave irradiation time on extraction efficiency of triterpenic acid extract from olive pomace. First of all the effect of irradiation time has been found significantly effected ($p<0,005$) on yield. The yield significantly increased from 0 to 12 min irradiation. In theory, if sample is exposed to microwave irradiation for long time extraction yield increases (Ameer et al., 2017; Eskilsson et al., 2000). Similar results were observed in this study for the irradiation time. The highest yield (24.5 mg TTA / g dry matter) was obtained at 12 min irradiation. However after that time (12 min), a decrease was observed. The possible reason is that the material which can be easily taken in the case of free form can stick to the solid matrix over the time and extraction getting harder with time.

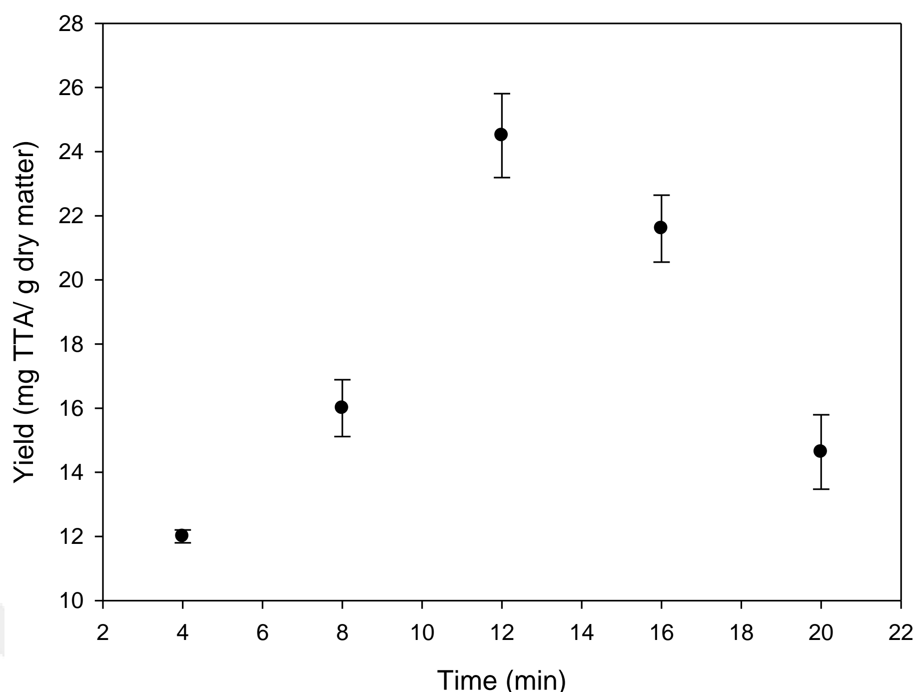


Figure 4.6 The effect of microwave irradiation time on yield of triterpenic acid

4.2.3 Solvent to Sample Ratio

In some MAE, solvent was used as mixture which was hexane with a polar solvent to increase efficiency of yield (Alfaro et al., 2003; Zhou et al., 2006). In this study, hexane was used as an extraction solvent for extraction triterpenic acid.

The amount of solvent which was used in MAE is too low in this study when it compare with conventional methods. The amount of solvent must be enough to immerse sample in MAE (Eskilsson et al., 2000; Wang et al., 2006). In this experiment, 5 ml-10 ml-15 ml and 20 ml solvent was added on to 2 gr wet olive pomace and optimum solvent amount was tried to find. When 5 ml hexane was used, it was sufficient to immerse sample; however microwave application caused to burn. Because of that result of 5 ml was not replaced in Figure 4.7.

In some studies, recovery increases directly proportional with solvent volume (Guo et al., 2001; Xiao et al., 2008). Same results were obtained in this study and it is observed that yield increases with increasing solvent volume.

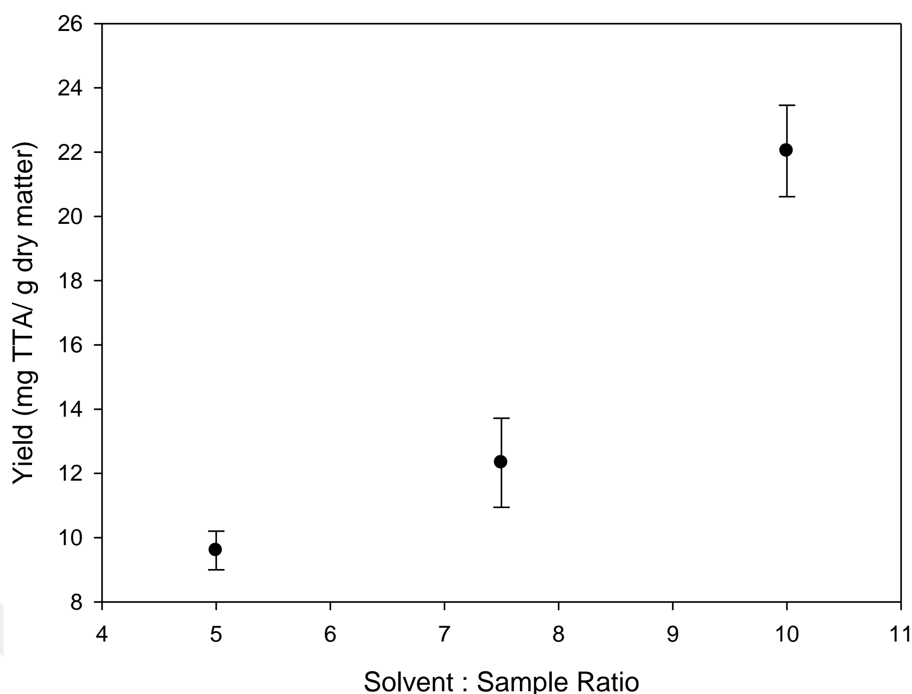


Figure 4.7 The effect of solvent/sample ratio on yield of triterpenic acid

4.3 Characteristics of Triterpenic Acid Extract

The characteristics of the TTA extracted and the individual acid fractions (MA and OA) isolated from the extract were determined in terms of purity, antioxidant activity and antimicrobial activity.

4.3.1. Purity

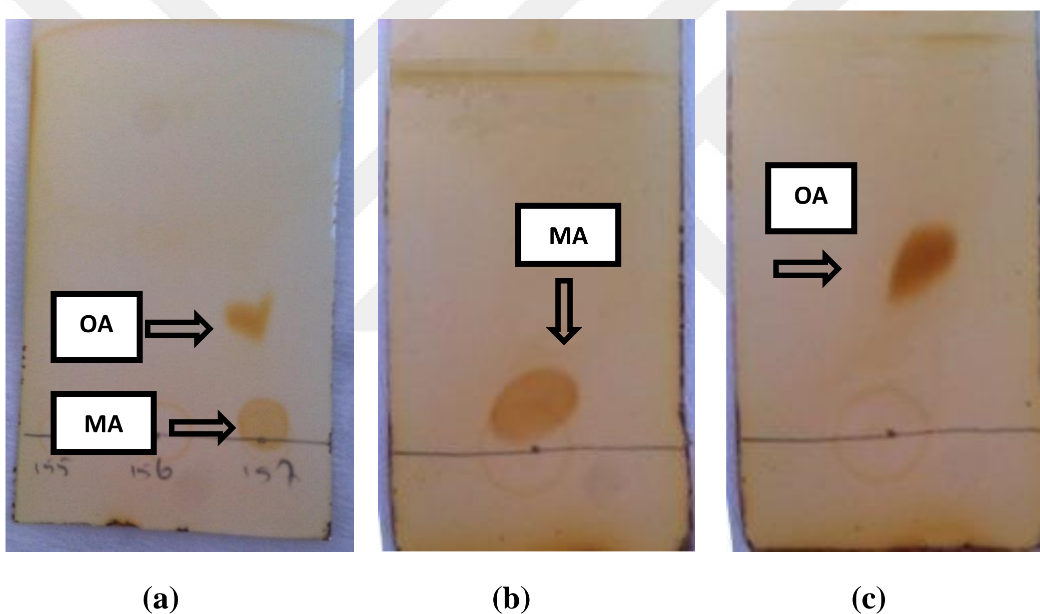
The maslinic and oleanolic acid in extract and the acid fractions isolated by column chromatography were identified and quantified by using HPLC-UV and ESI-MS. The purity of each peak observed in HPLC chromatogram and the purity of fractions collected from column chromatography were confirmed by TLC analysis.

Results of TLC analysis

Firstly, TLC analysis was used to find correct elution solvent combination before the column chromatography. In the present study, the best separation of MA and OA from the extract on TLC plate has been achieved by using hexane:ethyl ether:acetic acid mixture in a ratio of 65:34:1. Figure 4.8. (a) shows the separation of MA and OA on TLC plate. The appearance of two separate points on the TLC plate indicates

the presence and separation of the two fractions. Hence, the hexane: ethyl ether:acetic acid (65:34:1) mixture was used as an elution solvent for column chromatography applications. Ruiz-Gutierrez, et al., (2000) was also used the hexane:ethyl ether:acetic acid mixture in a ratio of 69:30:1 for the separation of triterpenic acids.

Secondly, the TLC analysis was performed to see the seperation performance of the mini column. The TLC results of the first eluent collected from mini column have been given in Figure 4.8. (b). It shows that there is only one fraction. The HPLC analysis result of this fraction confirm that this fraction was MA. The Figure 4.8 (c) shows the TLC result of second fraction. The HPLC results confirm that this layer represents the OA.



(a) (b) (c)

Figure 4.8 TLC pattern of **(a)** MA and OA separation **(b)** MA **(c)** OA

Results of HPLC analysis

The HPLC chromatograms of MA and OA fractions collected from the column chromatography have been given in Figure 4.9. and Figure 4.10, respectively. As it was seen there was only one peak for each fraction. The HPLC results confirm that isolation of acid fractions was succesfully practiced in column chromotography application.

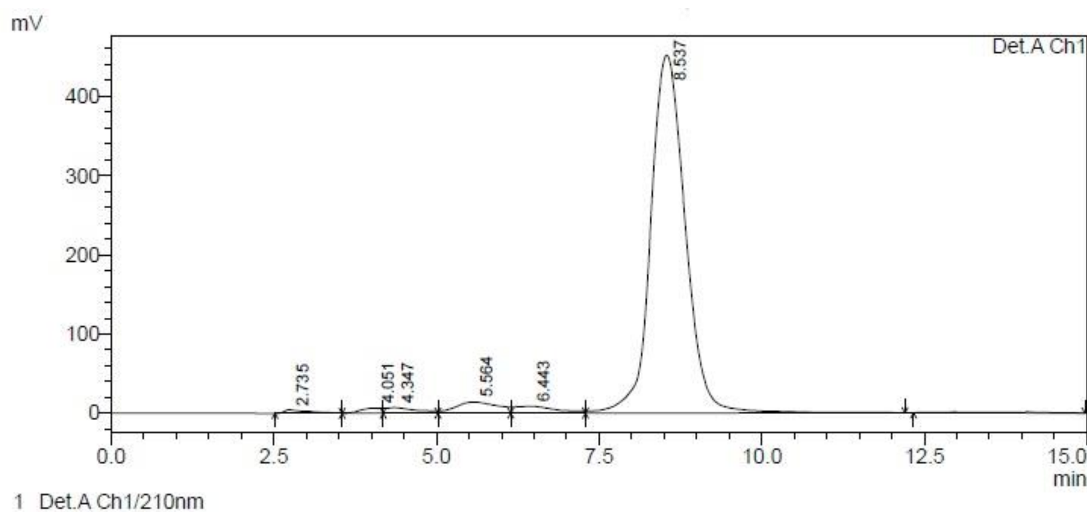


Figure 4.9 HPLC chromatogram of MA fraction after column chromatography

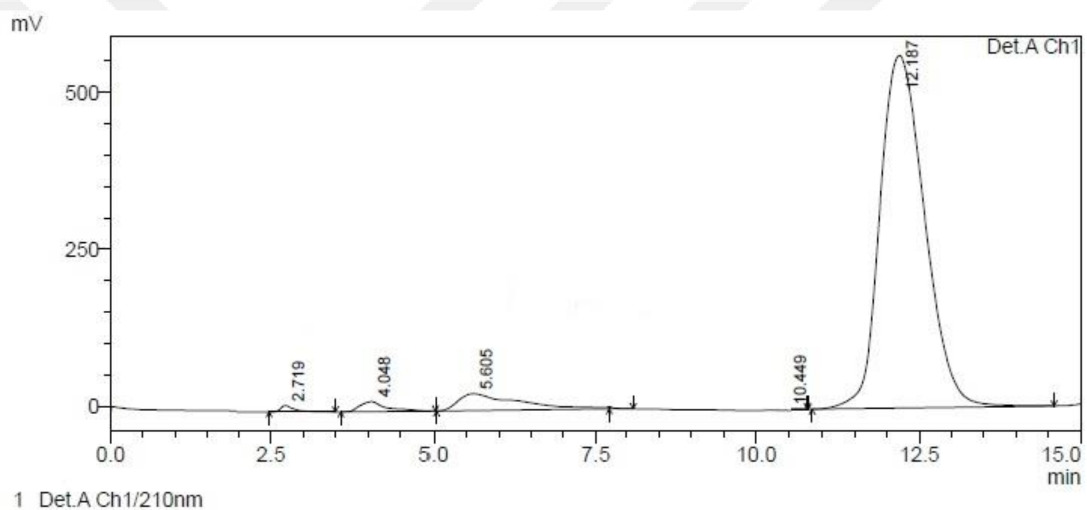


Figure 4.10 HPLC chromatogram of OA fraction after column chromatography

Identification of the TTA by using ESI-MS

In addition to HPLC analysis the structures of the acids isolated from the extract after column chromatography and the structure of each component observed on the HPLC chromatogram were also confirmed by mass spectra. The mass spectra of the MA and OA fractionated using column chromatography is given in Figure 4.11 and Figure 4.12, respectively.

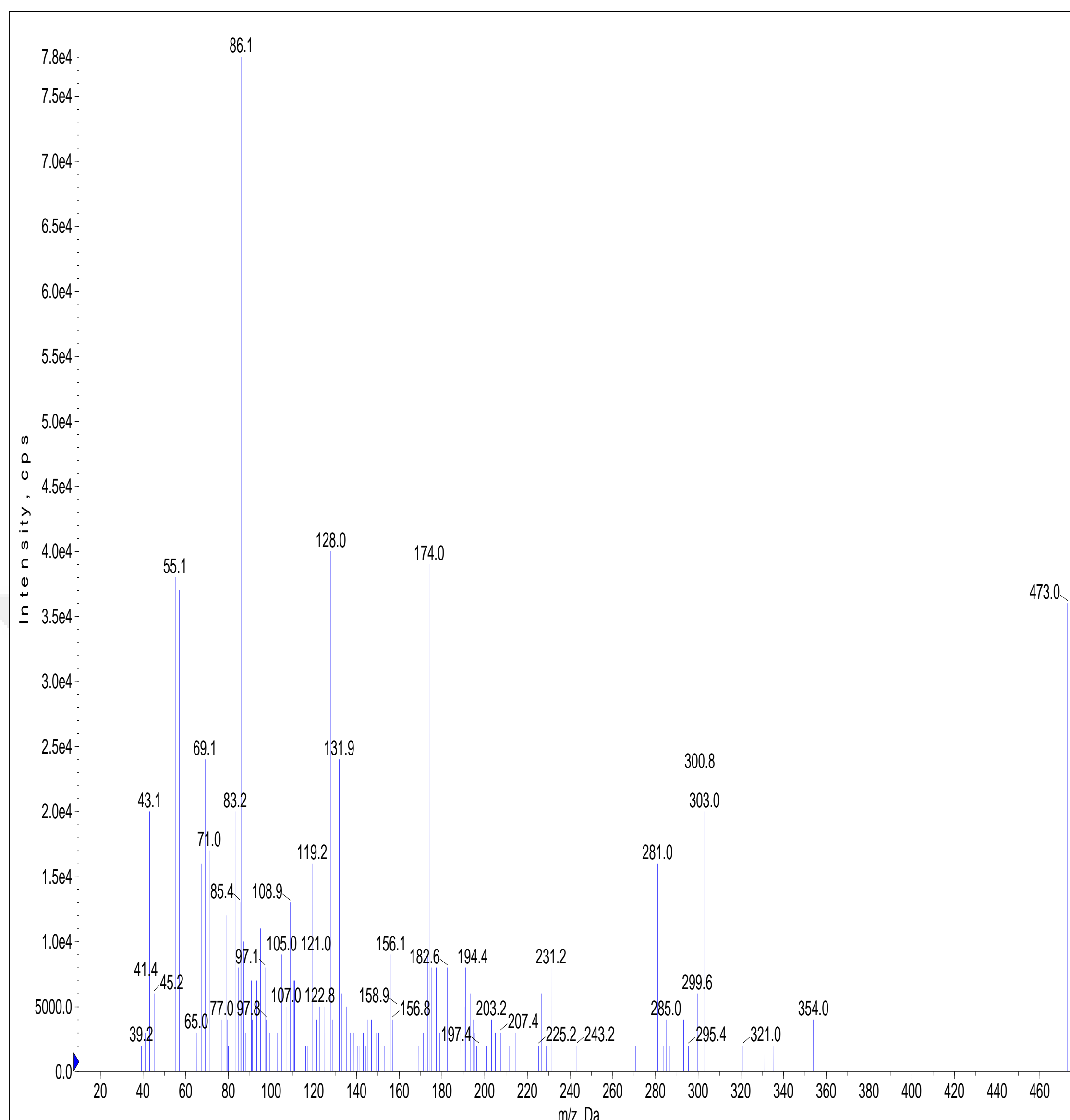


Figure 4.11 MS spectrum of the maslinic acid isolated from the triterpenic acid extract

The molecular mass of MA and OA are 472.7 and 456.7 respectively. The mass spectrum of MA and OA showed peaks at m/z 473 and 457, respectively corresponding with the protonated molecular ion. The major ion peaks in MA spectra showed m/z = 354, 303,300.8, 281, 194.4, 174, 131, 128, 119, 86, 83.2, 69.1, 55.1, 43.1 and 41.4. The m/z values of the major peaks for OA were 269.1, 226.9, 212.9, 195.2, 171.2, 139.1, 97.2, 83.1, 81.1, 67, 57, 55.1, and 39.1. Hence, ESI-MS spectra confirmed that the first eluent collected from column chromatography was OA and the second one was MA.

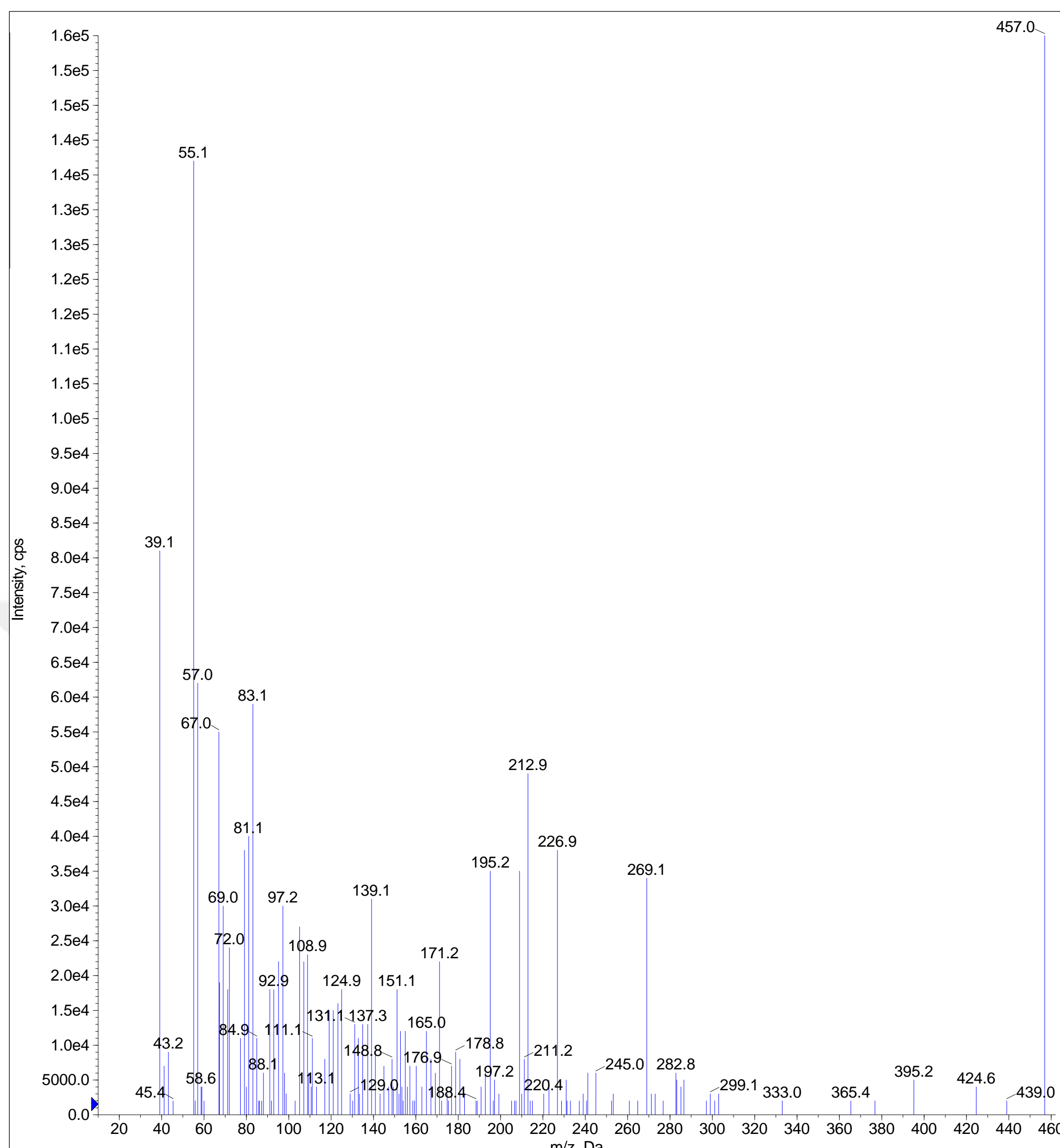


Figure 4.12 MS spectrum of the oleanolic acid isolated from the triterpenic acid extract

4.3.2 Antioxidant Activity

The antioxidant activity of the TTA extract and the antioxidant activities of MA and OA fractions which were isolated from the extract were determined separately and compared with each other. One method is not enough to determine precisely antioxidant activity. Hence, both DPPH and FRAP methods have been used to determine the antioxidant capacity of them in this study. As it is known, DPPH method measures electron-donating activity of compounds. FRAP measures the ability of antioxidant compounds to reduce the ferric ion Fe^{3+} to ferrous Fe^{2+}. DPPH

and FRAP analysis results are given in Table 4.2. As it is known radical scavenging activity of antioxidants are concentration dependent (Jamkhande et al., 2016). Hence the concentrations of the acids used in this study were also given in Table 4.2. According to DPPH results maslinic acid fraction has significantly higher percent inhibition (P<0.01) than both the oleanolic acid fraction and the extact (Table 4.2). Jamkhande et al., (2016) observed that there is a 43. 873 % inhibition for MA in DPPH test at 80 µg/mL. However this value is almost two times higher than that of obtained in this present study for the same concentration. The possible reason of high activity for the MA in the study of Jamkhande et al., (2016) could be related with the usage of alcoholic extract of MA, because the use of alcohol for extraction causes also more phenolic extraction together with MA. In our study, it was thought that the use of nonpolar solvent (hexane) as solvent in the extraction was the main reason of less phenolic in extract and so less radical scavenging activity.

However some other researchers have found no activity in the DPPH test for both triterpenic acids for maslinic acid (Fan et al., 2010) and for oleanolic acid (Çulhaoğlu et al., 2015). In a recently published study, Velasco et al., (2018) have reported that while the pure OA and pure MA did not display activity, the triterpenic acid extract showed slight antioxidant properties in the DPPH test under the same experimental conditions. They explained this difference with the presence of high amounts of phenolic compounds in the triterpenic acid extract obtained in this study. Similarly in the present study, the very slight radical scavenging activity of the extract and the individual acid fractions could be explained by the presence of phenolic compounds.

On the other hand, the results of some other researches indicated that oleanolic acid and maslinic acids are very effective antioxidants. For instance Bai, et al., 2018 have reported that oleanolic acid has strong inhibitory effect on cells in vitro. Additionally Ghafoor (2014) reported that DPPH-scavenging activity of oleanolic acid is enough high to compare with gallic acid, BHT and ascorbic acid. Moreover Jamkhande, et al., (2016) have reported that maslinic acid has strong antioxidant activity. Nur, et al., (2017) have reported that maslinic acid shown high scavenging activity.

When the antioxidant capacity of the extract which is rich in maslinic acid was compared with the antioxidant capacity of individual acids, it is clear that there is no any synergetic effect but rather an adverse effect (Table 4.2).

Table 4.2 The antioxidant activity results for DPPH and FRAP analysis

Assay	Extract (83,3µg/mL) (177.38 µM)	Maslinic acid (83.3 µg/mL) (176 µM)	Oleanolic acid (83.3µg/mL) (182 µM)
DPPH (% Inhibition)	14.39 ± 0.66	22.87 ± 0.78	18.193 ± 0.44
FRAP (µM TE)	224.719 ± 1.34	263.087 ± 3.89	240.989 ± 3.12

The results of FRAP analysis have shown similar trends for extract and individual acids with DPPH results. The ferric ions reduction power was significantly different from each other ($P< 0.01$). Maslinic acid has maximum reduction power followed by oleanolic acid and extract, respectively. In this present study, the results were calculated as µM Trolox equivalent, but other studies in the literature have different units, so it is difficult to compare the results directly. For example the ferric reducing activity of maslinic acid has been found as 1.2 µmol $TE.kg^{-1}$ and 827.44 µM in the studies performed by Bai, et al., (2018) and Nur, et al., (2017), respectively. In another study the reducing power of oleanolic acid is slightly higher than that of gallic acid (Ghafoor, 2014).

The antioxidant activity results for both maslinic and oleanolic acids appear to be inconsistent in the literature, the possible reasons could be undefined dosage and purity of the MA and OA. Also, different units have been used to state the activity in literature. Moreover, the results of the antioxidant tests seem to be dependent on the experimental conditions so the discrepancies between different studies could be justified.

4.3.3 Antimicrobial Activity

In this study, two different strains of *S. aureus* were used as gram positive bacteria and *E. coli*, *K. pneumonia* and *P. aeruginosa* were used as gram-negative bacteria to investigate antimicrobial activity. The antimicrobial activities of the samples have

been compared with the activity of amoxicillin which is widely used antibiotic as control. MIC results of extract and individual acids have been summarized in Table 4.3. It is seen that extract is almost twice as effective as MA and OA in deactivating *S. aureus* 6538. Moreover, its effect was as high as amoxicillin. Inhibitory effect of triterpenic acids on *S. aureus* (ATCC 6538) was found lower than *S. aureus* (ATCC 25923). *S. aureus* (ATCC 25923) strain is one of the widely used in the studies and the results show that maslinic acid and oleanolic acid have inhibition activity on these gram-positive bacteria (Acebey-Castellon et al., 2011; Fontanay et al., 2008; Horiuchi et al., 2007). On the other hand, the effect of extract and acids on *S. aureus* 25923 and *P. aeruginosa* 27853 was found almost same. Additionally MA and OA show similar effect against used bacteria strains except *E. coli*. When the effect of acids and extract on *E. coli* has been compared, MA was the most effective. Similar results are obtained at other studies (Chouaïb, et al., 2015; Wang, et al., 2015). This difference could be explained as to have maslinic acid has two hydroxyl group. According to the Table 4.3, the inhibition effect of both extract and individual acids on *P. aeruginosa* 27853 was more powerful than that of amoxicillin. However, Acebey-Castellon, et al., (2011) have reported that maslinic acid has no inhibitory effect on *P. aeruginosa*. Some other studies indicate that antimicrobial inhibition of oleanolic acid is very poor or none (Fontanay, et al., 2008; Horiuchi, et al., 2007). Another striking result is that there is an adverse effect of combination of MA and OA in extract on *K. pneumonia*.

According to the results of some previous studies triterpenic acids were reported as more effective on gram-positive bacteria than gram-negative bacteria (Fontanay, et al., 2008; Horiuchi, et al., 2007). However, for gram negative and gram positive bacteria strains used in this study, the extract, MA and OA did not show such a difference.

Table 4.3 Antimicrobial activities of compounds

	MIC (µg/ml)				
Compounds	*S. aureus* (ATCC 6538)	*E. coli* (ATCC 25322)	*S. aureus* (ATCC 25923)	*K. pneumonia* (ATCC 700603)	*P. aeruginosa* (ATCC 27853)
OA+MA	64	128	64	256	64
MA	128	64	64	128	64
OA	128	128	64	128	64
Amoxicillin	64	64	64	128	128
DMSO	512	512	512	512	512

CHAPTER 5

CONCLUSION

In this study, the extraction of triterpenic acids from wet olive pomace was successfully achieved by microwave irradiation in a closed system with a high yield.

Optimum conditions which result in high quantity of triterpenic acid extract were determined as 250 W, 12 min, and 10:1 for microwave power, process time and solvent to solid ratio, respectively.

Triterpenic acids, OA and MA, were successfully separated from each other's by using the mixture of hexane: ethyl ether: acetic acid (65:34:1) as an eluent solvent in a mini column chromatography.

The antioxidant capacities of the triterpenic acid extract and the antioxidant capacity of maslinic acid and oleanolic acid fractions isolated were weak. It can be concluded that the observed antioxidant activity could be related with the presence of phenolic compounds in extract and acid fractions.

The triterpenic acid extract and the maslinic acid and oleanolic acid fractions isolated were shown antimicrobial action in any extent on all studied microorganisms. In addition, the effect of MA fraction was at least as effective as amoxicillin on all microorganisms except *S. aureus* (ATCC 6538).

In conclusion, the findings of the present study have shown that microwave assisted extraction is one of the efficient and promising method to extract triterpenic acids from wet olive pomace within the short extraction time.

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APPENDIX

Table A.1 ANOVA result for microwave power & yield

Test of Homogeneity of Variances

Levene Statistic	df1	df2	Sig.
2,222	3	8	,163

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	210,929	3	70,310	108,169	,000
Within Groups	5,200	8	,650		
Total	216,129	11			

Post Hoc Tests

Homogeneous Subsets

Duncan[a]

		Subset for alpha = 0.05		
VAR00003	N	1	2	3
150,00	3	9,6667		
300,00	3		18,1333	
200,00	3		18,2000	
250,00	3			20,7667
Sig.		1,000	,922	1,000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3,000.

Table A.2 ANOVA result for microwave irradiation time & yield

Test of Homogeneity of Variances

Levene Statistic	df1	df2	Sig.
2,028	4	10	,166

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	318,671	4	79,668	80,095	,000
Within Groups	9,947	10	,995		
Total	328,617	14			

Post Hoc Tests

Homogeneous Subsets

Duncan[a]

VAR00005	N	Subset for alpha = 0.05			
		1	2	3	4
4,00	3	12,0000			
20,00	3		14,6333		
8,00	3		16,0000		
16,00	3			21,6000	
12,00	3				24,5000
Sig.		1,000	,124	1,000	1,000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3,000.

Table A.3 ANOVA result for solvent/sample ratio & yield

Test of Homogeneity of Variances

Levene Statistic	df1	df2	Sig.
1,803	2	6	,244

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	256,149	2	128,074	89,216	,000
Within Groups	8,613	6	1,436		
Total	264,762	8			

Post Hoc Tests

Homogeneous Subsets

Duncan[a]

VAR00007	N	Subset for alpha = 0.05		
		1	2	3
5,00	3	9,6000		
7,50	3		12,3333	
10,00	3			22,0333
Sig.		1,000	1,000	1,000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3,000.

Table A.4 ANOVA result for DPPH

Test of Homogeneity of Variances

DPPH

Levene Statistic	df1	df2	Sig.
,755	2	6	,510

ANOVA

DPPH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	93,594	2	46,797	113,243	,000
Within Groups	2,479	6	,413		
Total	96,074	8			

Post Hoc Tests

Homogeneous Subsets

DPPH

Duncan[a]

Sample	N	Subset for alpha = 0.05		
		1	2	3
3,00	3	14,3900		
2,00	3		18,1933	
1,00	3			22,2873
Sig.		1,000	1,000	1,000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3,000.

Table A.5 ANOVA result for FRAP

Test of Homogeneity of Variances

FRAP

Levene Statistic	df1	df2	Sig.
1,600	2	6	,277

ANOVA

FRAP

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2225,174	2	1112,587	125,140	,000
Within Groups	53,345	6	8,891		
Total	2278,519	8			

Post Hoc Tests

Homogeneous Subsets

FRAP

Duncan[a]

Sample	N	Subset for alpha = 0.05		
		1	2	3
3,00	3	224,7187		
2,00	3		240,9890	
1,00	3			263,0870
Sig.		1,000	1,000	1,000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3,000.