USAGE OF MICROWAVE AND ULTRASOUND IN THE EXTRACTION OF ESSENTIAL OILS AND PHENOLIC COMPOUNDS

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USAGE OF MICROWAVE AND ULTRASOUND IN THE EXTRACTION OF ESSENTIAL OILS AND PHENOLIC COMPOUNDS

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ABSTRACT

USAGE OF MICROWAVE AND ULTRASOUND IN THE EXTRACTION OF ESSENTIAL OILS AND PHENOLIC COMPOUNDS

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The objective of this study is to extract phenolic compounds from nettle and melissa by using microwave and ultrasound and to compare these methods with conventional extraction and maceration, respectively. Extraction of melissa essential oil was also studied.

In extraction of phenolics, effects of extraction time (5-20 min for microwave; 5-30 min for ultrasound) and solid to solvent ratio (1:10, 1:20, 1:30 g/ml) on total phenolic content (TPC) were investigated for microwave and ultrasound extractions. Different powers were also studied for ultrasound extraction. In addition, effect of solvent type (water, ethanol, ethanol-water mixture at 50:50 v/v) was analyzed and water was found as better solvent.

Optimum conditions for microwave extraction of phenolics were determined as 10 min and 1:30 solid to solvent ratio for nettle, and as 5 min and 1:30 solid to

solvent ratio for melissa. TPC at these conditions for nettle and melissa extracts were 24.6 and 145.8 mg GAE/g dry material, respectively. Optimum conditions for ultrasound extraction was 30 min, 1:30 solid to solvent ratio, and 80% power for nettle and 20 min, 1:30 solid to solvent ratio, and 50% power for melissa. TPC at these conditions for nettle and melissa were 23.9 and 105.5 mg GAE/g dry material, respectively. Major phenolic acids were determined as naringenin in nettle and rosmarinic acid in melissa.

Major aromatic compounds in melissa essential oil were found as limonene, citral, and caryophyllene oxide. Yields of essential oil obtained by microwave and hydrodistillation were 4.1 and 1.8 mg oil/g dry sample, respectively.

Keywords: Extraction, leaching, melissa, nettle, phenolic

ÖZ

UÇUCU YAĞLARIN VE FENOLİK BİLEŞİKLERİN ÖZÜTLENMESİNDE MİKRODALGA VE ULTRASON KULLANIMI

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Bu çalışmanın amacı, mikrodalga ve ultrason kullanarak ısırgan otu ve melisadan fenolik bileşenleri özütlemek ve bu metotları sırasıyla klasik özütleme ve maserasyonla karşılaştırmaktır. Ayrıca, melisa uçucu yağının özütlenmesi de çalışılmıştır.

Fenoliklerin özütlenmesinde, mikrodalga ve ultrason özütlemeleri için özütleme zamanının (mikrodalga için 5-20 dak; ultrason için 5-30 dak) ve katı maddenin çözücü miktarına oranının (1:10, 1:20, 1:30 g/ml) toplam fenolik madde (TFM) miktarına etkileri araştırılmıştır. Ayrıca ultrason özütlemesi için değişik ultrason güçleri çalışılmıştır. Çözücü türünün (su, etanol ve etanol-su karışımı 50:50 v/v) etkileri de analiz edilmiş ve suyun daha iyi bir çözücü olduğu bulunmuştur.

Fenoliklerin mikrodalga özütlemesinde, ısırgan otu için 10 dak, 1:30 katı madde:çözücü oranı ve melisa için 5 dak ve 1:30 katı madde:çözücü oranı en

uygun koşullar olarak belirlenmiştir. Bu koşullarda, ısırgan otu ve melisa özütlerinin TFM miktarları, sırasıyla 24.6 ve 145.8 mg GAE/g kuru maddedir. Ultrason özütlemesinde en uygun koşullar, ısırgan otu için 30 dak, 1:30 katı madde:çözücü oranı ve %80 ultrason gücü, melisa için ise 20 dak, 1:30 katı madde:çözücü oranı ve %50 ultrason gücüdür. Bu koşullardaki TFM miktarları ısırgan otu ve melisa için sırasıyla 23.9 ve 105.5 mg GAE/g kuru maddedir. Başlıca fenolik bileşenler ısırgan otunda naringenin, melisada ise rozmarinik asit olarak belirlenmiştir.

Melisa uçucu yağındaki başlıca aromatik bileşenler limonen, sitral ve karyofilenoksit olarak bulunmuştur. Mikrodalga ve hidrodistilasyon ile elde edilen uçucu yağ verimleri sırasıyla 4.1 ve 1.8 mg yağ/g kuru numunedir.

Anahtar sözcükler: Fenolik, ısırgan otu, katıdan özütleme, melisa, özütleme

To my family

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TABLE OF CONTENTS

LIST OF TABLES

TABLE

LIST OF FIGURES

FIGURE

CHAPTER 1

INTRODUCTION

1.1. Phenolic compounds

Phenolic substances are found in herbs, fruits, and vegetables in a wide range. They, which are produced as secondary metabolites in plants, have inhibitory and protective characteristics against pests, pathogens, or parasites (Rosa et al., 2010; Singleton and Esau, 1969).

Phenolic compounds have an important effect on taste and odor of food materials, such as bitterness, astringency, sourness, or sweetness. Especially, the flavor of tea is thought to be mostly coming from polyphenols and catechins, and these compounds give tea sweet and juicy taste (Singleton and Esau, 1969). In addition, phenolic substances, in the form of flavonoids, are responsible for color formation in plants (Jaganath and Crozier, 2010).

Polyphenols are divided into two main groups as flavonoids and non-flavonoids, according to their structures.

1.1.1. Flavonoids

Flavonoids have the basic structure of C6-C3-C6, and there are seven different subgroups with respect to the functional groups. Their structures are shown in Appendix A.

1.1.1.1. Flavonols

Flavonols are mostly found in vegetables such as onions, capers, lettuce; and some herbs and spices like parsley, fennel, and oregano. Also, cocoa, red wine, and brewed tea contain flavonols in abundance.

Quercetin, which presents in onions, asparagus, berries, and lettuce, is the most common polyphenol in flavonols. Kaempferol is the second largest group in flavonols. Kale, spinach and berries are highly kaempferol containing foods. Myricetin is another flavonol, found in parsley, fennel, oregano, grape, red cabbage, brewed tea, and red wine. Onions and pears contain isorhamnetin, which is one of the least common flavonols (Rosa et al., 2010).

1.1.1.2. Flavones

Flavones in plants are found in smaller quantities compared to flavonols. Apigenin and luteolin are the most common flavones especially present in parsley, celery, thyme, basil, artichoke, and other herbs. Tangeretin and chrysin are the other flavones, which are found in citrus peel and passion flower, respectively (Hurst, 2007). Also, lemongrass contains orientin and isoorientin flavones (Jaganath and Crozier, 2010).

1.1.1.3. Flavanones

Hesperetin, naringenin, and eriodictyol are the most abundant flavanones, especially in citrus fruits such as lime, lemon, grapefruit, and orange. Sakuranetin and isosakuranetin are the two uncommon ones (Jaganath and Crozier, 2010). Other than citrus fruits, prunes and kiwi also have flavanones. Flavanones are usually found in fruits in glycosylated forms like naringin (naringenin-7-*O*-neohesperidoside), hesperidin (hesperetin-7-*O*-rutinoside), narirutin (naringenin-

7-*O*-rutinoside), or neohesperetin (hesperetin-7-*O*-neohesperidoside). These compounds are responsible for the bitter taste in fruits (Hurst, 2007).

1.1.1.4. Isoflavones

Isoflavones, which are polar and water soluble compounds, are structurally different from other flavonoids. They are mostly found in legumes and are not detected in fruits and vegetables (Rosa et al. 2010). Isoflavones are mostly found in soybeans and nuts and grains have smaller quantities (Hurst, 2007). Daidzein, genistein, and glycitin are the most common isoflavones (Jaganath and Crozier, 2010).

1.1.1.5. Anthocyanidins/Anthocyanins

Anthocyanins, which are glycosidic derivatives of anthocyanidins, are water soluble and color determinative pigments in plants. As they are responsible for color formation from red to purple, they have a protective effect on plants against high intensity light. The concentration of anthocyanidin increases during ripening and it accumulates mostly in the peel of fruits (Rosa et al., 2010). Grapes, berries, plums, peaches, cherries, eggplant, and red cabbage have generally cyanidin, delphinidin, petunidin, peonidin, pelargonidin, malvidin and their glycosylated derivatives as anthocyanidins (Jaganath and Crozier, 2010).

1.1.1.6. Flavan-3-ols

Catechin and epicatechin are the most common representatives of flavan-3-ols. They are responsible for different tastes such as bitterness, astringency, sourness, or sweetness. Flavan-3-ols are found in tea and chocolate in higher amounts than they are in fruits, such as apricots, apples, cherries, and grapes; red wine, beer, and nuts. Epigallocatechin is included in cocoa beans (Pan et al., 2003; Othman

et al., 2007) and tea. Tea leaves also have theaflavin and its derivatives in large quantities (265-530 mg theaflavin/100 ml fresh weight) (Rosa et al., 2010; Jaganath and Crozier, 2010).

1.1.1.7. Proanthocyanidins

Proanthocyanidins (condensed tannins) are oligomeric or polymeric flavan-3-ols. They are condensation products of flavanols. Procyanidins (including catechin or epicatechin units), prodelphinidins (including (epi)gallocatechin), and propelargonidins (formed by (epi)afzelechin) are the main proanthocyanidins, and the rich food sources of them are cinnamon, sorghum, beans, nuts, chocolate, berries, and plums. Apples and grapes also contain proanthocyanidins in small concentrations (Rosa et al., 2010; Hurst, 2007).

1.1.2. Nonflavonoids

Nonflavonoid phenolic substances have three main groups; namely phenolic acids, hydroxycinnamic acids, and stilbenes.

1.1.2.1. Phenolic acids

Phenolic acids mainly include gallic, p-hydroxybenzoic, protocatechuic, ellagic, vanillic, and syringic acids. The basic structure of phenolic acids is C6-C1, and can be seen in Appendix A. There is a considerable amount of ellagic acid in berries, such as raspberry, strawberry, and blackberry. Phenolic acids can be present in bound form or in isomer form as in the pomegranate. Pomegranate juice contains a significant amount of ellagitannins, which are hydrolysable tannins. Also there are gallagic acid and punicalagin isomers in pomegranate juice. White wine includes protocatechuic acid in high concentration. It is also found in dates, raspberry, cinnamon bark, and clove buds. Gallic acid is rich in grapes, and is

constituted gallotannins, which are another type of hydrolysable tannins (Rosa et al., 2010; Jaganath and Crozier, 2010).

1.1.2.2. Hydroxycinnamic acids

The most known hydroxycinnamic acids are p-coumaric, caffeic, ferulic, sinapic acids and their tartrate esters like coutaric, caftaric acids. Chlorogenic acid, that is a compound formed by quinic and caffeic acid, is also an important hydroxycinnamic acid. These are found mostly in the skin of the fruits or vegetables, and also in coffee beans and some herbs. For example, green coffee beans contain high amount of chlorogenic acid (1158-2741 mg/100 g dry material). Although this amount is reduced by nearly 90% (96 mg chlorogenic acid/100 g fresh weight), after roasting antioxidant activity still remains in a coffee drink (Rosa et al., 2010). Some chlorogenic acid types in green coffee beans are 3-*O*-caffeoylquinic acid, 4-*O*-feruloylquinic acid, and 3-5-*O*-dicaffeoylquinic acid (Jaganath and Crozier, 2010).

There are curcuminoids, another group in hydroxycinammates, in ginger, cardamon, and turmeric. Curcumin is a diferuloylmethane, which is found highly in turmeric together with demethoxycurcumin and bisdemethoxycurcumin (Jaganath and Crozier, 2010).

Coumarins, including p-coumaric acid and m-coumaric acid, are present mostly in essential oils of cinnamon bark, cassia leaf, and lavender. They are also found in some fruits and green tea (Rosa et al., 2010).

1.1.2.3. Stilbenes

Stilbenes are included in foods in very restricted concentrations. Resveratrol and its glycoside piceid are the main representatives and they are present in grape and

grape products, such as red wine. Resveratrol can be found in cis and trans forms in berries, cabbage, spinach, and some herbs (Jaganath and Crozier, 2010; Rosa et al., 2010).

1.2. Antioxidants

Phenolics have free-radical scavenging effect, and this supplies a shielding against oxidative injury in various parts of human body, such as skin, blood, or tissue (Rosa et al., 2010). It is reported that there is a strong correlation between antioxidant activity and phenolic compounds in plants. Antioxidants have three main mechanisms that are prevention of free radical formation, inhibition of initiation of chain reactions (free radical scavenging), and transition-metal chelating activity (Shan et al., 2005). Free radicals can be in the form of reactive oxygen species or reactive nitrogen species. These two radicals can be formed in living systems via endogenous or exogenous sources. Even normal aerobic respiration is an endogenous source. Smoking, ionizing radiations, and some chemicals constitute the exogenous sources. Free radical scavenging mechanism of antioxidants can be explained by the following reactions:

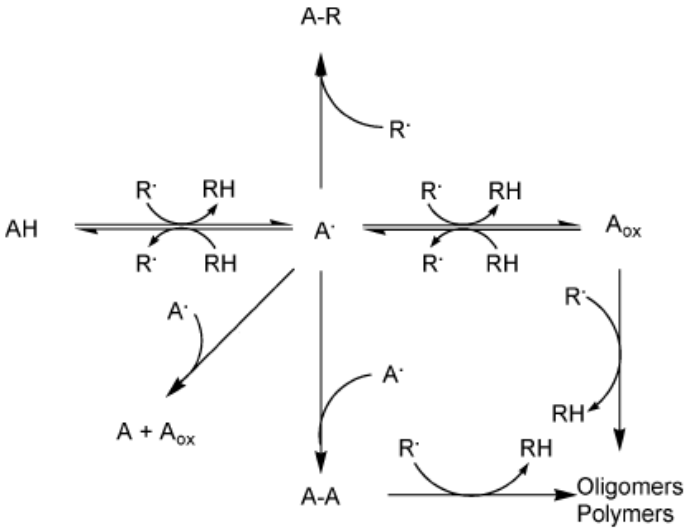


Figure 1.1. Reaction mechanism of antioxidants
AH – antioxidant; $R^{\bullet}$ – free radical (Adapted from Eklund et al., 2005)

Synthetic or natural antioxidants are required to overcome some problems such as DNA damage, tissue injury, or lipid oxidation to a certain extent. Antioxidants have protective or reparative characteristics against free radicals (Mavi et al., 2004). Although synthetic antioxidants, such as BHT and BHA, are used in food materials, there is an increasing demand for natural antioxidants in recent years. Common sources of antioxidants are whole grain, fruits, vegetables (Prakash, 2001), cocoa (Othman et al., 2007), tea, and coffee (Wang and Ho, 2009). Antioxidants can prevent heart diseases, cancer (Ghasemzadeh et al., 2010), and even they can be used for therapies in traumatic brain injuries (Hall et al., 2010). Besides these positive health effects in human body, there are different prevention mechanisms from free radicals in food materials, which may prolong the shelf life of food. Some of the natural antioxidants in plants are flavonoids, cinnamic acids, benzoic acids, ascorbic acid, and rutin (Ghasemzadeh et al., 2010). Specifically, catechins are found in green tea, and hydroxycinnamic acids (caffeic acid and chlorogenic acid) are in coffee (Wang and Ho, 2009).

There are different methods to determine the antioxidant activity. 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), oxygen radical absorbance capacity (ORAC), and trolox equivalent antioxidant capacity (TEAC) are the most common methods (Othman et al., 2007; Prakash, 2001).

In this thesis, DPPH method was used due to its simplicity and practicability. Its reactive mechanism can be explained by the reactions:

$$DPPH^{\bullet} + AH \rightarrow DPPH\text{-}H + A^{\bullet}$$
$$DPPH^{\bullet} + R^{\bullet} \rightarrow DPPH\text{-}R$$

AH is antioxidant and R is radical species. The color change of $DPPH^{\bullet}$ radical was measured at 515 nm and antioxidant activity was determined (Brand-Williams et al., 1995).

1.3. Extraction (Leaching) of phenolic compounds

There are different extraction types such as maceration, Soxhlet, microwave, and ultrasound extractions.

Different extraction solvents can be used, like ethanol, methanol, acetone, water, or their mixtures for different polarities; however it needs a special attention for food processing. Solvent should be chosen as non-toxic or it should not leave residues after application in food industry regarding health and safety considerations (Hasbay Adil et al., 2008).

1.3.1. Maceration

Maceration is the extraction of analytes from a solid material into the solvent by immersion. Solid and liquid are in contact to reach the equilibrium of analytes (Self, 2005). Maceration was used for the extraction of phenolic compounds in different studies. For example, phenolic compounds from propolis (Trusheva et al., 2007), melissa (Marques and Farah, 2009) and citrus (Ma et al., 2009) were obtained by maceration. Extraction media, including solid particles and solvent, is kept at constant temperature for a definite time period with no mechanical effect such as agitation or stirring.

1.3.2. Soxhlet extraction

Conventional solid-liquid extraction (leaching) is mostly conducted with a Soxhlet apparatus, shown in Figure 1.2. Leaching is the principle mechanism of solid-liquid extraction. Solid material is put in the refluxed extraction tube, and solvent is in the extraction flask. Boiled and evaporated solvent goes up inside the system and when it reaches the condenser it condenses and falls back into the refluxed extraction tube, where the solid material is placed. Solvent contacts with

the solid in this place, and it comes back into the extraction flask when it completes the siphon. During solid and liquid contact, soluble components pass through the solvent until the equilibrium is reached. Mass transfer continues from solid to solvent until the equilibrium of each component is completed (Self, 2005).

Conventional system is usually a long-time procedure, such as 2-4 hours (Richter et al., 1996). Continuous heating and high temperatures may degrade the bioactive components including phenolics in food materials for long times.

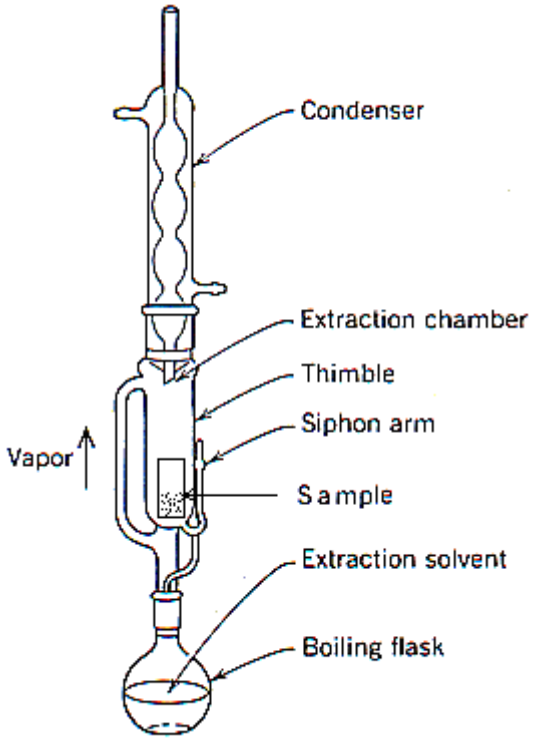


Figure 1.2. Basic parts of Soxhlet extraction apparatus

1.3.3. Microwave extraction

Microwaves are electromagnetic waves that have frequencies between 300 MHz and 300 GHz. They are non-ionizing radiations (Schubert and Reiger, 2005).

Heating is supplied by electromagnetic radiations within the extraction matrix in microwave extraction, instead of conventional heating. Microwaves heat the media very rapidly and uniformly due to its volumetric heating characteristic, thus undesirable results of thermal damage would be minimized (Rosa et al., 2010). Ionic conduction and dipole rotation are the two mechanisms that occur in microwave heating. The movement of ions in aqueous solutions plays an important role in dielectric heating especially at lower frequencies. In addition, polar liquids, such as water or alcohol, induce the rotational movement in molecular level in external electric field, and dominate the heating mechanism of microwave (Schubert and Reiger, 2005). In extraction systems, where a polar solvent like water is used, dipolar polarization is the major heating mechanism (Schubert and Reiger, 2005).

Since microwaves are composed of high frequency electromagnetic waves, heating mechanism is different than conduction and convection. Dielectric properties of heated material and solvent are very important. One of them is dielectric constant (ε') which is defined as the ability of a material to store energy in an electromagnetic field, and the other one is dielectric loss factor (ε'') which is the ability of a material to dissipate energy (Schubert and Reiger, 2005). The ratio of dielectric loss factor of a material to its dielectric constant is defined as dissipation factor (tan δ). Dissipation factor is dependent on the heat generation in the material. From this theoretical knowledge, it can be inferred that nature of solvent and the solid material in microwave extraction are very important. Dielectric properties of some solvents are given in Table 1.1. Polar compounds have principally high dielectric loss factors. Solvents that have high dielectric

properties and their mixtures such as ethanol-water are generally chosen in extraction studies (Sanchez-Prado et al., 2010). Although for the extraction of most organic analytes from herbs, polar solvents are usually selected, sometimes for different purposes non-polar solvents can also be used. For example in a study, conducted with fresh mint leaves for its essential oil, non polar solvents were preferred (Schubert and Reiger, 2005). The reason was here that the solvent was transparent to microwaves and microwaves directly reach the solid sample without causing any heating of solvent. It affects the water molecules inside the cellular structures. That causes the disruption of cell membrane and the easy passage of target compounds into the solvent (Schubert and Reiger, 2005).

Shorter extraction time (Xiao et al., 2008), less solvent consumption (Proestos and Komaitis, 2008), and higher extraction efficiency (Chen et al., 2008) are the advantages of microwave assisted extraction found in literature. Temperature and time are critical parameters in extraction processes. Higher temperatures enhances the diffusion rate and thus the extraction rate, however heat sensitive components decompose at high temperatures for long extraction times. Since shorter times are enough for microwave heating compared to conventional one, microwave extraction can be recommended for heat sensitive components (Schubert and Reiger, 2005).

Microwave extraction of phenolic compounds with different solvents such as acetone, methanol, water, and ethyl acetate-water mixture was performed by Proestos and Komaitis (2008). They have used conventional reflux system as control. Different aromatic plants including *Rosmarinus officinalis*, *Origanum dictamnum*, and *Vitex agnus*-cactus, were used. As a result of their study, it was stated that microwave assisted extraction reduced the extraction time significantly, decreased the required solvent amount, and increased the extraction yield of phenolic compounds.

Table 1.1. Dielectric properties of some solvents (Adapted from Sanchez-Prado et al., 2010)

Solvent	ε'	ε''	tan δ ($x10^4$)
Acetone	21.1	11.5	5555
Acetonitrile	37.5	2.3	620
Ethanol	24.3	6.1	2500
Ethyl acetate	6.02	3.2	5312
Hexane	1.89	0.00019	1
Methanol	23.9	15.3	6400
Water	76.7	12.0	1570

In another study, phenolic compounds and antioxidants from buckwheat were obtained with water, ethanol, and aqueous ethanol by microwave extraction (Inglett et al., 2010). Control experiments were done in water bath with different temperatures in the range 23°C to 150°C. Highest total phenolic content was achieved with 50% aqueous ethanol by microwave extraction at 150°C and highest antioxidant activity was obtained with pure ethanol at 100°C and 150°C for both microwave and water bath extraction (Inglett et al., 2010).

Stability of different phenolic compounds under microwave extraction conditions were also studied (Liazid et al., 2007). Investigated temperature range for 22 different phenolic compounds was between 50°C and 175°C. It was found that phenolics were stable up to 100°C; and epicatechin, resveratrol, and myricetin were degraded at 125°C. As a conclusion, Liazid et al. (2007) stated that phenolics that have higher number of hydroxyl-type groups degrade more easily under microwave extraction conditions. Cerretani et al. (2009) studied on microwave heating of olives and changes on phenolic compounds and they found that phenolic compounds decreased with increasing microwave heating time.

They have specifically stated that elenolic acid decreased rapidly and highly, o-diphenols slightly decreased, and lignans remained constant under microwave conditions, with increasing heating time. In another study, Brenes et al. (2002) stated that yield of phenolic compounds increased with microwave heating time, polyphenols slightly changed, however α-tocopherol decreased rapidly upon microwave heating of olive oils. Both Cerretani et al. (2009) and Brenes et al. (2002) expressed that microwave heating was less degradative to olive oils than conventional heating.

1.3.4. Ultrasound extraction

Ultrasound extraction is a kind of non-thermal application. It includes sound waves in the range of 18 kHz and 500 MHz (Self, 2005). There are two occurrences during extraction procedure; one of them is the phenomenon of cavitation that is specific for ultrasound application, and the other one is mechanical effect that is simply the stirring. Ultrasonic waves produce bubbles in the solvent and ultrasonic vibrations compress these bubbles. Temperature and pressure of the compressed bubbles increase and finally the bubbles collapse. Collision produces a shock wave in the solvent and enhances the passage of solvent through the solid material. This is known as phenomenon of cavitation. Sound waves also have a mechanical effect related to the mixing. Mixing increases the contact surface area of the solid and solvent and another enhancement is obtained other than acoustic cavitation (Rostagno et al., 2003; Ghafoor et al., 2009).

Ultrasound extraction has several advantages such as reduced extraction time, lower operating temperature, and increased extraction yields (Ma et al., 2008). Lower temperature avoids thermal decomposition of bioactive compounds and mixing effect supplies an additional efficiency to the extraction yield (Shalmashi, 2009).

There are various studies in literature on ultrasound assisted extraction. Extraction of phenolic compounds and antioxidants from citrus (Ma et al., 2009; Londono et al., 2010), grape seeds (Ghafoor et al., 2009), pomegranate seed (Abbasi et al., 2008), strawberry (Herrera and Castro, 2004); vanillin from vanilla pods (Jadhav et al., 2009); isoflavones from soy beans (Rostagno et al., 2003); and oil from tea seeds (Shalmashi, 2009) were studied by using ultrasound extraction. In all these studies, solvent selection and temperature were the important issues in order not to degrade the target compound or in order to increase the yield compared to the control method. Pan et al. (2003) used ultrasound as a control method for comparison of microwave-assisted extraction of tea polyphenols.

Ultrasound application can be done either with a probe system or with a bath set up. Although there are different application areas, both of them were used for extraction purposes (Rostagno et al., 2003). However, it is defended by Chowdhury and Viraraghavan (2009) that probe system is more efficient considering the transferring of ultrasonic energy. Probe has a direct contact with the solvent media, and there will be no external interference that causes the loss of energy. However, in ultrasonic bath system, since the solid and solvent are put in a beaker or another cup, they are not in direct contact with given energy. Since some of the ultrasonic energy can be absorbed by the beaker, there will be an energy interruption.

1.4. Essential oils

Essential oils are volatile and aromatic substances that are obtained from different parts of plants. Essential oils are commonly used for their flavor and fragrance characteristics in food industry, and in addition antimicrobial (Bassole et al., 2003), antiviral, antifungal (Pandey et al., 2000), and antioxidant properties are

also present (Burt, 2004; Kulisic et al., 2004). Besides food industry, essential oils are used in perfumery and pharmaceuticals (Burt, 2004).

In obtaining the essential oils, spices and some herbs have great importance. The volatile components of spices and herbs are used in food industry as preservative ingredient or flavoring agents (Güllüce et al., 2003). Individual essential oil components do not have the same preservative characteristics as a food additive (Burt, 2004). Natural additives are more preferable than synthetically manufactured ones and there is an increasing demand for naturally obtained essential oils, and also the studies on properties of individual components are ongoing (Kulisic et al., 2004).

Hydrodistillation is the most common method to extract the essential oils (Dang et al., 2001; Jerkovic et al., 2001). It is performed with a heater under the extraction flask, a Clevenger apparatus, and a condenser on top of Clevenger apparatus. However, there are different methods that were experienced such as microwave hydrodiffusion and gravity (Vian et al., 2008), microwave assisted solvent extraction, microwave hydrodiffusion (Sahraoui et al., 2008), solvent free microwave extraction (Bayramoglu et al., 2009; Lucchesi et al., 2004), and ultrasound assisted extraction (Hashemi et al., 2009). With these novel methods, it is aimed to use less solvent, less energy, and to consume less time; in addition other aims are to decrease the degradation and losses of some volatile compounds and increase the extraction efficiency. Lucchesi et al. (2004) stated that solvent free microwave extraction is a green technique which is an alternative for the extraction of essential oils, that requires less solvent, less energy, and shorter time compared to the conventional hydrodistillation method.

Vian et al. (2008) used a different set up including microwave oven in solvent free extraction process. They compared this new method, which is named as microwave hydrodiffusion and gravity, with hydrodistillation in terms of

extraction time, extraction yield, and rejected carbon dioxide amount; and found that for the same extraction yield, time and rejected carbon dioxide amount were less in microwave setup. In another study, Sahraoui et al. (2008) studied different extraction methods for the extraction of essential oil of dry lavender flowers. They also stated that microwave assisted extraction techniques were better than the conventional ones in terms of required time, energy, and cleanliness of the process.

1.5. Nettle (*Urtica diocia*)

Nettle is a member of Urticaceae family which has sharp leaves (Pinelli et al., 2008) and stinging hairs containing different kinds of acids (Yarnell, 1998) in its fresh form. It is used as a folk medicine, especially in Turkey due to its polyphenol content and their antioxidant characteristics (Yıldız et al., 2008; Gülçin et al., 2004). Aerial parts and roots of nettle can be used as tea or cooked in meals. According to many studies, nettle has been used as diuretic, hypoglycemic, anti-inflammatory (Yarnell, 1998), anti-rheumatic, and hypotensive (Ozyurt et al., 2007; Yıldız et al., 2008; Riehemann et al., 1999; Sezik et al., 2001). The anti-inflammatory characteristics, which come from mainly caffeic acid, malic acid, and chlorogenic acid (Yarnell, 1998), are used for treatments of some allergic rhinitis (Pinelli et al., 2008). Nettle leaves and seeds have become important traditional medicine for preventing and curing the cancer disease (Ozyurt et al., 2007; Pinelli et al., 2008) by their antioxidant characteristics. It is also an important source of vitamin C (Ozyurt et al., 2007; Gülçin et al., 2004; Al-Ismail et al., 2007). Antioxidant characteristics are related with the phenolic content of nettle. Extraction of phenolic compounds from different parts of nettle was studied before by Pinelli et al. (2008) and Hudec et al. (2007). Also, there are various studies on water extraction of nettle in order to investigate antioxidant activity, total phenolic contents, and specific phenolic acids (Gülçin et al., 2004; Matsingou et al., 2001; El, 2008).

1.6. Melissa (*Melissa officinalis*)

Melissa is another traditional medicine in the culture of Asia and Europe (Dastmalchi et al., 2008; Sarı and Ceylan, 2002; Allahverdiyev et al., 2004; Carnat et al., 1998). It is cultivated all around Mediterranean region, including coasts of Turkey (Adinee et al., 2008). The word "Melissa" comes from "melitos" meaning honey in Greek, an affinity to bees is intended. The term "officinalis" comes from the French word "officine" meaning "laboratory" (Herodez et al., 2003).

Melissa is rich in phenolic compounds (Karasová and Lehotay, 2006; Caniova and Brandsteterova, 2001). It has antioxidative characteristics due to its rich phenolic contents such as caffeic acid and rosmarinic acid (Caniova and Brandsteterova, 2001; Carnat et al., 1998). These phenols are produced in plant as a protective mechanism against microorganisms (Karasová and Lehotay, 2006).

Ziakova and Brandsteterova (2002) investigated the phenolic antioxidants in melissa, that was extracted with different methods such as solid phase extraction, pressurized solvent extraction, and supercritical fluid extraction; and they found that solid phase extraction was the best method for their target phenolics. In another study, air dried melissa leaves were extracted with aqueous ethanol; total phenol content and also antioxidant activity were investigated by different assays (Dastmalchi et al., 2008).

Melissa is also rich in essential oils (Tran-Thi et al., 2006; Rozzi et al., 2002). There are many different essential oils in *Melissa officinalis*, however most abundant ones are citronellal and citral (neral and geranial) (Topal et al., 2008; Carnat et al., 1998; Allahverdiyev et al., 2004). Obtainable essential oil amount from melissa is very low, thus it is quite valuable and expensive (Sarı and Ceylan, 2002). Its essential oil content was found as 0.010-0.097% (v/w) by Sarı and

Ceylan (2002). In addition, the value of the oil is also due to the fact that melissa is an officinal medicinal plant for long times (Tran-Thi et al., 2006). It is used for treatment of headaches, toothaches, gastrointestinal disorders, fever, influenza, migraine, rheumatism, insomnia, and depression (Sarı and Ceylan, 2002; Herodez et al., 2003; Mimica-Dukic et al., 2004; Allahverdiyev et al., 2004; Topal et al., 2008). Leaf essential oils have sedative, antiviral, antibacterial, antispasmodic characteristics (Sarı and Ceylan, 2002; Toth et al., 2003). Mimica-Dukic et al. (2004) studied the antioxidant and antimicrobial characteristics of essential oil of melissa.

Sarı and Ceylan (2002) and Adinee et al. (2008) studied composition of essential oil of melissa, which was conventionally extracted with a Clevenger apparatus. In a different study, lemon scented herbs including melissa were compared with respect to their citral compounds. Citral and citronellal content of essential oil of melissa were also studied by Tran-Thi et al. (2006); and it was found that lemon balm from different places was containing 5.7-22.9% neral and 4.8-31.7% geranial.

Most common extraction method for essential oils is hydrodistillation, similar with the other herbs (Dang et al., 2001; Jerkovic et al., 2001). In the extraction of essential oils from herbs, generally water is used; however Mimica-Dukic et al. (2004) used n-hexane to obtain essential oils from melissa. They also investigated the antimicrobial activity of essential oil of melissa besides the composition and antioxidant activity; and they obtained a yield of 0.2% (v/w). In another study, Carnat et al. (1998) investigated some aromatic compounds and their properties by infusion of melissa like the preparation of tea. They stated that melissa tea had digestive and antispasmodic characteristics. Supercritical fluid extraction was also used and compared to the hydrodistillation of lemon-scented botanicals including melissa by Rozzi et al. (2002), and it was found that geranial, neral acetate, and caryophyllene; which are some essential oils in melissa; was higher in

concentration in supercritical fluid extraction and citronellal, neral, and caryophyllene oxide was higher in hydrodistillation.

1.7. Objectives of the study

Nettle is a traditional medicine that is commonly used as blood purifier and diuretic. Besides, as an herbal tea, it is used for diabetes, rheumatism, eczema, and anemia (Akbay et al., 2003). Phenolic compounds in nettle are known as a cure for hair loss. Melissa is another traditional medicine in especially coastal regions of Turkey. Melissa is not only rich in phenolic compounds but also has a quite valuable essential oil. Its sedative property is commonly known; in addition it can be used as a flavoring agent in food, medicine, and preservative for food due to its essential oil content (Adinee et al., 2008). Essential oil of melissa is valuable due to its low obtainability and its specific volatile compounds (Tran-Thi et al, 2006). Both plants are important for their phenolic contents, in addition aromatic substances in melissa are worth to be searched. Phenolic compounds are associated with their antiulcer, antimutagenic, and anticarciogenic properties (Vilkhu et al., 2008) and natural ones are more preferable than synthetically produced ones due to health and safety aspects.

In literature, there are different studies on phenolic compounds of nettle and melissa, however there is no study on microwave and ultrasound extractions of these plants. It was aimed to reduce extraction time and increase extraction yield in microwave and ultrasound extractions. Microwave heating is very fast thermal process compared to the conduction heating. In addition, ultrasound can be considered as a non-thermal process, because extraction media is kept at very low temperatures. The disadvantages of conventional methods, such as long extraction times and exposure to high temperatures, were tried to be eliminated in this study by using different extraction methods. These drawbacks cause degradation of phenolic compounds and decrease the extraction yield. Microwave and ultrasound

extraction parameters were tried to be optimized in this study. Antioxidant activity and phenolic compounds of nettle and melissa extracted by different methods were also compared.

Another objective of this study was to obtain essential oil of melissa with microwave, and compare it with the essential oil obtained by conventional hydrodistillation in terms of yield and composition. Microwave extraction of aromatic compounds in melissa has not been studied before. Thus, this study would be a former one in this respect.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Aerial parts of dry nettle and melissa that was obtained from local markets was used (Istanbul Baharatları, Ankara, Turkey) in this study. They were used as in their original dried conditions without doing any crushing or grinding preapplication.

Standards of gallic acid, catechin hydrate, caffeic acid, chlorogenic acid, p-coumaric acid, naringenin, naringin, vanillic acid, syringic acid, trans-3-hydroxycinnamic acid, rosmarinic acid, hydrocinnamic acid, and hesperetin were purchased from Sigma-Aldrich. Standards for essential oils (α-pinene, limonene, cineol, γ-terpinene, linalool, α-terpineol, citronellol, citral (neral and geranial), geraniol, β-caryophyllene, and caryophyllene oxide were also obtained from Sigma-Aldrich and Fluka.

DPPH, ethanol, methanol (HPLC grade), alkane standard mixture (C_{10}-C_{40}), and n-hexane were also purchased from Sigma-Aldrich.

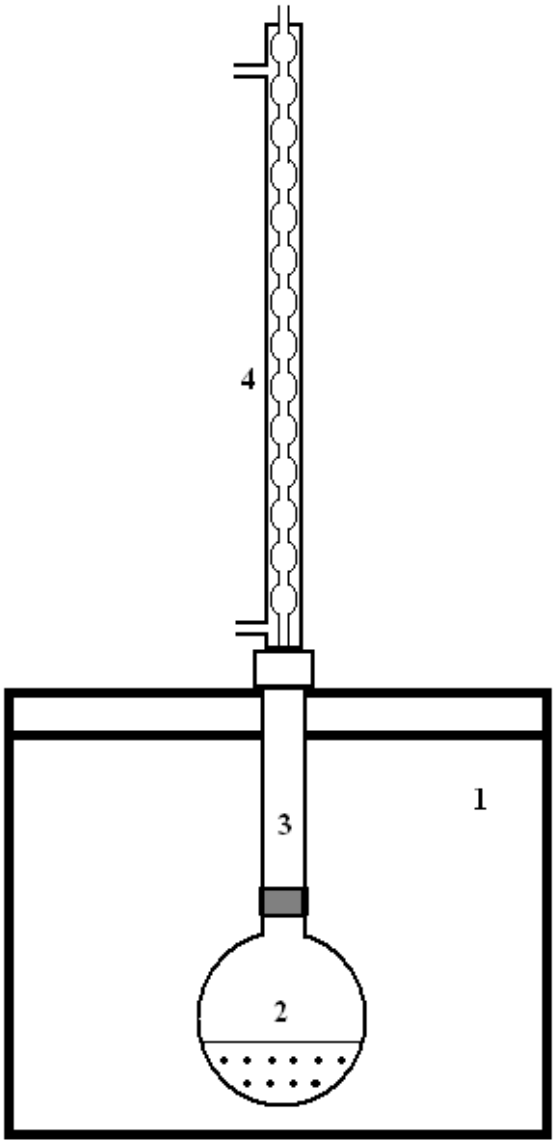
2.2. Extraction of phenolic compounds

2.2.1. Microwave extraction

Laboratory scale microwave oven (Milestone Ethos D, Sorisole, Italy) was used for the heating purpose in extraction. It has approximately 45 L inner volume with 35x35x35 cm dimensions. Experimental set up consists of heating unit, extraction flask (1 L), condenser, and an adaptor that links the extraction flask and condenser. Microwave oven set up is shown in Figure 2.1.

Samples (5 g) were placed into flask and solvent was poured on them. Then, flask was placed into the chamber of microwave oven. Power was chosen as constant (407 W, which was measured by IMPI-2L test) and different times of extractions (5, 10, 15, and 20 min) were done. Three different solid to solvent ratio, which were 1:10, 1:20, and 1:30 g/ml, were experienced. Water, ethanol, and 50:50 (v/v) ethanol:water combination were used as the solvent. After the extraction was completed, sample was allowed to cool down, and then extract was filtered, weighed, and its volume was recorded. All extractions were done in two replicates.

During extraction, temperature within the flask was measured by a fiber optic probe system (FISO-UMI4, FTI-10 Signal Conditioner; Quebec, Canada) and it was recorded as 97°C for water.



1 microwave oven cavity
2 extraction flask
3 adapter
4 condenser

Figure 2.1. Basic parts of microwave extraction apparatus for extraction of phenolic compounds

After each extraction process, extracts were roughly filtered through a piece of cloth and were centrifuged at 10000 rpm (8720 *g*) for 10 minutes (Sigma 2-16PK Centrifuge; Buckinghamshire, England). Extracts to be analyzed were kept in 20 ml dark colored bottles in refrigerator at most for 2 days. Amber colored and tight capped bottles were preferred because degradation reactions are known to be enhanced with air, light, and temperature (Ghasemzadeh et al., 2010; Liazid et al., 2007).

2.2.2. Ultrasound extraction

Sonic Ruptor400 Ultrasonic Homogenizer (Omni Sonic Ruptor400 Ultrasonic Homogenizer, Kennesaw, USA) with a standard probe (1" Processing Tip for Ultrasonic Homogenizer) was used. Experimental set up consists of a water bath, a beaker, and the ultrasonic probe.

Water was the only solvent used in ultrasound extractions. Ten grams of samples were placed into a 200 ml beaker with the appropriate amount of distilled water. Ultrasound power, pulser, and time were adjusted on the panel. It has a maximum power of 300 W and 20 kHz frequency. Two power levels were chosen, which were 50% and 80%. Ultrasound was operated at 50% pulser mode. Samples were put into the cooler water bath in the beaker and ultrasonic probe was dipped at most 1.5 cm depth into the extraction media. Extractions were performed at different times (5, 10, 20, and 30 min). 1:10, 1:20, and 1:30 g/ml solid to solvent ratios were tried, and extraction temperature was 40°C. After extraction, extract was immediately filtered and centrifuged as explained above.

2.2.3. Conventional extraction

Conventional extraction was used only for comparison with microwave extraction. The similar experimental set up used in microwave extraction was used. The only difference was that heating was achieved with conventional hot plate from Şimşek Laborteknik (PI-404, 4x1000 W; Ankara, Turkey) instead of microwave. Ethanol and water were tested as solvents. Solid and solvent mixture was placed into the flask together with solvent. Extraction was performed for different times up to 10 h and solid to solvent ratio of 1:10, 1:20, and 1:30 g/ml were tried.

During extraction, temperature within the flask was measured by the same fiber optic probe system in microwave extraction and it was recorded as 97°C for water.

Extracts were filtered and centrifuged after the extraction was completed, and were kept in dark and cold until being analyzed.

2.2.4. Maceration

Maceration was done at 40±1°C for comparison with ultrasound extraction. An incubator (NÜVE EN 400; Ankara, Turkey) was used. Only water was used as solvent. Samples (10 g) were placed into the beakers with an appropriate amount of distilled water at 40°C. They were mixed for a few seconds in order to soak all the solid particles. Top of beakers were capped with aluminum foil and they were left to stand at 40°C for 24 hours.

Extracts were filtered and centrifuged after the extraction was completed, and were kept in dark and cold until being analyzed.

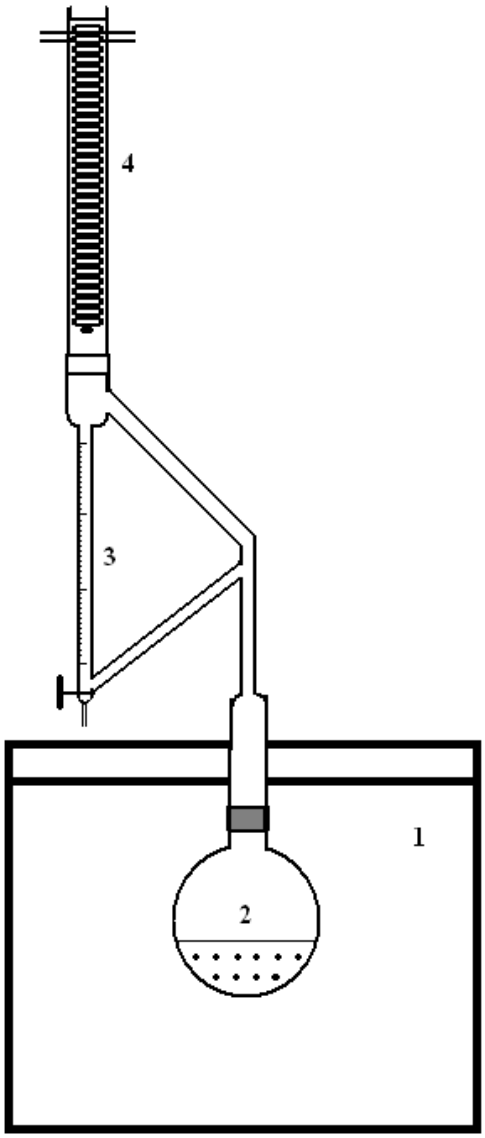
2.3. Extraction of essential oils of melissa

2.3.1. Microwave extraction

Experimental set up consists of heating unit, extraction flask (2 L), Clevenger apparatus, and condenser. Extraction set up is shown in the Figure 2.2. The same microwave oven used for phenolic extraction was used for extraction of essential oils from melissa.

Microwave oven power was set to 407 W which was measured by IMPI-2L test (Buffler, 1993), and it was operated for 1 h. 50 g dry melissa leaves were put into extraction flask (2 L) and 1 L distilled water was poured on leaves. This was placed into the microwave oven and Clevenger apparatus and condenser were connected to the flask. Extraction was continued until no more essential oil was obtained.

After extraction, essential oil was kept in an amber colored and tight capped vial in order to prevent from light and air. Attention was paid to enter no water into vial, while taking the essential oil. Obtained essential oil was weighed and measured volumetrically, and yield was calculated in terms of mg essential oil / g dry sample. Then, gas chromatography analysis was performed.



1 microwave oven cavity
2 extraction flask
3 Clevenger apparatus
4 spiral condenser

Figure 2.2. Basic parts of microwave extraction apparatus for extraction of essential oil

2.3.2. Hydrodistillation

Hydrodistillation was done as the control method for extraction of essential oil. It was performed with a heater, an extraction flask containing 50 g dry melissa leaves and 1 L distilled water, a Clevenger apparatus, and a spiral condenser. Heater was operated at 500 W power level, and extraction was continued until no more essential oil was obtained.

After extraction, essential oil was kept in an amber colored and tight capped vial in order to prevent from light and air. Attention was paid to enter no water into vial, while taking the essential oil. Obtained essential oil was weighed and yield was calculated in terms of mg essential oil / g dry sample. Then, gas chromatography analysis was performed.

2.4. Analysis of extracts

2.4.1. Determination of total phenolic content

Folin-Ciocalteu method was used (Singleton and Rossi, 1965; Singleton et al., 1999) for the determination of total phenolic content (TPC). In the presence of phenolic substances, Folin-Ciocalteu reagent is reduced by sodium carbonate, and this is observed by a color change.

200 µl diluted sample and 1 ml 0.2 N Folin-Ciocalteu (2N, SIGMA-ALDRICH F9252) reagent was put into a tube. They were vortexed and left to stand in dark for 5 min. After 5 minutes, 800 µl 75 g/L sodium carbonate (SIGMA-ALDRICH S7795) solution was added and vortexed again. Tube was kept in dark at room temperature (25°C) for 1 hour and then the absorbance was measured at 760 nm.

Using a spreadsheet program (MS Office Excel 2003), calibration curve was prepared with different gallic acid concentrations (30, 40, 50, 60, 70, 80, 90, 100, 110, and 120 ppm) in distilled water, so total phenolic content was expressed as mg gallic acid equivalent. Ethanol calibration curve was prepared in the same manner with different concentrations of gallic acid (30, 40, 50, 60, 70, 80, 90, 100, and 110 ppm). Also, another calibration curve for 50:50 (v/v) ethanol:water mixture was prepared with 20, 30, 40, 50, 60, and 70 ppm gallic acid concentrations.

Calibration curves are given in Appendix B (Figure B.1-B.3).

2.4.2. Determination of antioxidant activity

$DPPH^{\bullet}$ method was used for the determination of antioxidant activity (Brand-Williams et al., 1995). Method is based on color change of $DPPH^{\bullet}$ in the presence of substances that have antioxidant characteristics. $DPPH^{\bullet}$ solution is quickly degraded with light and due to its sensitivity; it is always kept in dark.

For this determination, 0.025 g $DPPH^{\bullet}$ / L methanol was prepared and 1.95 ml from this solution was added on 0.05 ml extract in cuvette. Cuvettes were totally covered with aluminum foil in order to prevent the light. Absorbances were measured at 515 nm immediately after $DPPH^{\bullet}$ solution was put (at t=0) and after 2 h of waiting in dark (at t=2h).

Time was determined according to the constant absorbance reading of gallic acid. $DPPH^{\bullet}$ was added to different concentrations of gallic acid and absorbances were recorded in every 15 minutes. After 1 hour, gallic acids gave constant absorbance measurements. For the samples, it was found that 2 hour waiting period was necessary for the reaction to be completed.

Calibration curve was prepared with different concentrations of $DPPH^{\bullet}$ in methanol, with the highest concentration point of 0.025 g $DPPH^{\bullet}$ / L methanol. The equation for the calibration curve was shown in Appendix B (Figure B.4). The antioxidant activity was determined according to the following formula:

$$\text{mg DPPH / g dry material} = (C_{t=0} - C_{t=2h}) * DF * V_{extract} / m_{extract} ; \quad (2.1)$$

where $C_{t=0}$ is the concentration of $DPPH^{\bullet}$ calculated immediately after the sample and $DPPH^{\bullet}$ solution was mixed, and $C_{t=2h}$ is the concentration of $DPPH^{\bullet}$ calculated after 2h the sample and $DPPH^{\bullet}$ solution was mixed, DF is the dilution factor, $V_{extract}$ is the volume of extract in ml, and $m_{extract}$ is the amount of dry sample in g.

2.4.3. Determination of ascorbic acid content

Ascorbic acid analysis was done to check if there was a significant contribution to the determination of total phenolic content. Ascorbic acid content of extracts was determined by 2,6-dichlorophenol indophenol titration method (AOAC, 2006). For this method, metaphosphoric acid (HPO_3) solution (3% w/v) and indophenol standard solution were prepared with distilled water. Indophenol standard solution was prepared by mixing the 50 mg 2,6-dichlorophenol indophenol with 50 ml distilled water and adding 42 g $NaHCO_3$ to this solution. Then this solution was completed to 200 ml with distilled water. In addition, ascorbic acid standard solution (1 mg/ml) was also prepared for the titration. For titrations, extracts were diluted with the ratio of 1/5 with distilled water. Two ml of this dilute extract was mixed with 5 ml 3% (w/v) HPO_3 solution and the mixture was completed to 30 ml with distilled water. Then, this was titrated with 2,6-dichlorophenol indophenol solution and used volume was recorded. Blank and ascorbic acid standard solution were titrated with the same procedure, and ascorbic acid content was determined by the following formula:

mg ascorbic acid / ml sample = 2 * [(V_s – V_b) / (V_{st} – V_b)] ; (2.2)

where V_s, V_b, and V_{st} were the volumes of 2,6-dichlorophenol indophenol solution used for the sample, blank, and ascorbic acid standard solution, respectively.

2.4.4. Determination of phenolic compounds by HPLC

In this study, for the determination of phenolic compounds in nettle and melissa, a modification of the method proposed by Yıldız et al. (2008) was used. Agilent Zorbax SB-C18 (Santa Clara, USA) reversed phase column (250 x 4.6 mm, 5 µm particle size) was used in Shimadzu UFLC equipment. The model of degasser was GDU-$20A_5$, pump was LC-20AD, autosampler was SIL-20A HT, column oven was CTO-20A, and the diode array detector was SPD-M20A.

Two mobile phases were used which were 0.2%-CH_3COOH distilled water (A) and 90% aqueous methanol solution (B). Standards were prepared in 90% methanol solution. Calibration curves were obtained for each phenolic acid and had R^2 values greater than 0.98. All standards, samples, and mobile phases were filtered through 0.45 µm filter before injection. Standards were scanned in the range of 190 and 800 nm and the peak values were obtained. The wavelength that gave the peak value was chosen specifically for each standard.

2.4.4.1. HPLC conditions for nettle extract samples

For nettle samples; gallic acid, naringenin, and naringin were analyzed at 280 nm; p-coumaric acid was analyzed at 310 nm; and caffeic acid and chlorogenic acids were analyzed at 330 nm. Nettle extracts were diluted in the ratio of 1/2 with 90% aqueous methanol.

Table 2.1. HPLC conditions for nettle extract

Column: Agilent Zorbax SB-C18 (250 x 4.6 mm, 5 μm particle size)

Temperature: 40°C

Detector: Shimadzu DAD SPD-M20A

Wavelength: 280 nm, 310 nm, and 330 nm

Mobile Phases: A - 0.2%-CH_3COOH distilled water

B - 90% aqueous methanol solution

Elution rate: 1ml/min

Gradient program:

t (min)	% A	% B
0	100	0
2	100	0
4	98	2
6	98	2
8	97	3
10	97	3
12	96	4
14	96	4
16	94	6
19	94	6
20	92	8
24	92	8
25	90	10
30	90	10
34	80	20
36	75	25
38	70	30
40	65	35

(Table 2.1. cont.'d)

41	65	35
45	60	40
46	60	40
50	50	50
52	50	50
53	80	20
55	100	0

2.4.4.2. HPLC conditions for melissa extract samples

For melissa samples; vanillic and hydrocinnamic acids were analyzed at 260 nm; gallic acid, catechin hydrate, syringic acid, naringenin, trans-3-hydroxycinnamic acid, naringin, and hesperetin were analyzed at 280 nm; p-coumaric acid was analyzed at 310 nm; and caffeic and rosmarinic acids were analyzed at 330 nm. Melissa extracts were diluted in the ratio of 1/5 with 90% aqueous methanol.

Chromatographic conditions were adapted from the paper related with melissa and rosmarinic acid (Toth et al., 2003).

Table 2.2. HPLC conditions for melissa extract

Column: Agilent Zorbax SB-C18 (250 x 4.6 mm, 5 μm particle size)
Temperature: 40°C
Detector: Shimadzu DAD SPD-M20A
Wavelength: 260nm, 280 nm, 310 nm, and 330 nm
Mobile Phases: A - 0.2%-CH_3COOH distilled water
B - 90% aqueous methanol solution
Elution rate: 0.5 ml/min

(Table 2.2. cont.'d)

Gradient program:

t (min)	% A	% B
0	100	0
2	100	0
3	99	1
4	99	1
5	95	5
6	95	5
7	90	10
8	90	10
10	80	20
13	70	30
15	70	30
18	69.6	30.4
20	69.6	30.4
23	69.3	30.7
25	69.3	30.7
28	69	31
30	69	31
32	68	32
33	68	32
35	66	34
36	66	34
38	64	36
39	64	36
41	62	38
42	62	38
44	60	40
45	60	40

(Table 2.2. cont.'d)

47	57	43
48	57	43
50	55	45
51	55	45
53	54	46
54	54	46
56	50	50
57	50	50
59	80	20
60	100	0

2.5. Determination of volatile compounds of melissa essential oil by GC-MS

Gas chromatography/Mass spectrometry (GC-MS) analysis of essential oils was done with a GC-MS system from Agilent Technologies, which had autosampler (7683B Series Injector), 6890N Network GC System, and 5973Network Mass Selective Detector. There were two columns in GC-MS system, which had basically the same characteristics, however outlet of one column was mass selective detector and the other was front detector (flame ionization detector – FID). GC columns were Agilent 19091s-433 HP-5MS 5% Phenyl Methyl siloxane, which were 30 m in length, 250 μm inner diameter, and 0.25 μm film thickness (temperature limits were from -60°C to 325°C). The carrier gas was helium.

GC-MS conditions

Injection volume: 1 µl (syringe size: 10 µl)

Front Detector (FID)

Temperature: 250°C
Hydrogen flow: 40 ml/min
Air flow: 450 ml/min
Mode: Constant makeup flow
Makeup flow: 45.0 mL/min
Makeup Gas Type: Helium

Oven

Initial temperature: 60°C
Initial time: 2 min
Final temperature: 260°C
Rate: 5°C/min
Run time: 47 min

Before injection, 5 µl sample was mixed with 5 µl alkane standard mixture (C_{10}-C_{40} 1% v/v diluted with n-hexane) and diluted with 980 µl n-hexane.

Calibration curves were prepared with five concentrations of standards either in ml/ml (1/25, 1/50, 1/75, 1/100, and 1/125 ml/ml) if the standard was liquid or in ppm (mg/L) (100, 200, 300, 400, and 500 mg/L) if the standard was solid. Standards were prepared with n-hexane. R^2 values of all calibration curves were obtained as greater than 0.98.

2.6. Statistical analysis

Statistical Analysis Software (SAS 9.1) was used. One-way analysis of variance (ANOVA) was applied to determine the optimum conditions of conventional extraction and maceration. For comparison of extraction methods, one-way ANOVA was used. If significant difference was found ($p \leq 0.05$), means were compared using Duncan's multiple comparison method. Two-way ANOVA was performed for microwave extraction, while three way ANOVA was used for ultrasound extraction.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Extraction of phenolic compounds from nettle

Moisture content of dry nettle was determined as 9%. Microwave and ultrasound techniques were used for the extraction of phenolic compounds as novel methods, and they were compared to conventional extraction and maceration, respectively, in terms of total phenolic contents. The extraction conditions giving the highest total phenolic compounds were chosen as the optimum for each method. The antioxidant activity and also the concentration of phenolic compounds of the extract obtained at the optimum conditions were determined.

Three different solvents were used in extractions, which were water, ethanol, and ethanol-water (50:50 v/v) mixture. Figure 3.1 shows the concentrations of total phenolics in nettle extracts obtained using microwave for different solvent types. Although ethanol is known as a good solvent for phenolic compounds (Lapornik et al., 2005), it gave considerably lower concentrations in this study. The efficiency of heating of solvent under microwave is dependent on the dissipation factor (tan δ) which represents the measure of the ability of the solvent to absorb microwave energy and transfer it as heat to the surrounding molecules (Mandal et al., 2007). Higher efficiency was expected when ethanol was used in microwave extraction, since ethanol has higher dissipation factor than water. However, the results were different than expected. This may be due to higher solubility of nettle phenolic compounds in water. Also, the state of solid phase might have effect on solubility. Dried nettle leaves were not grounded for extraction, so the cellular

structure was not disrupted. Although leaves were very thin and had very low resistance to mass transfer, water channels in cellular structure might have been effective in allowing the passage of water by increasing the contact surface area (Chen et al., 2008; Trabelsi et al., 2010). There are some studies in which water was used as solvent for the extraction of phenolics from nettle (Gülçin et al., 2004).

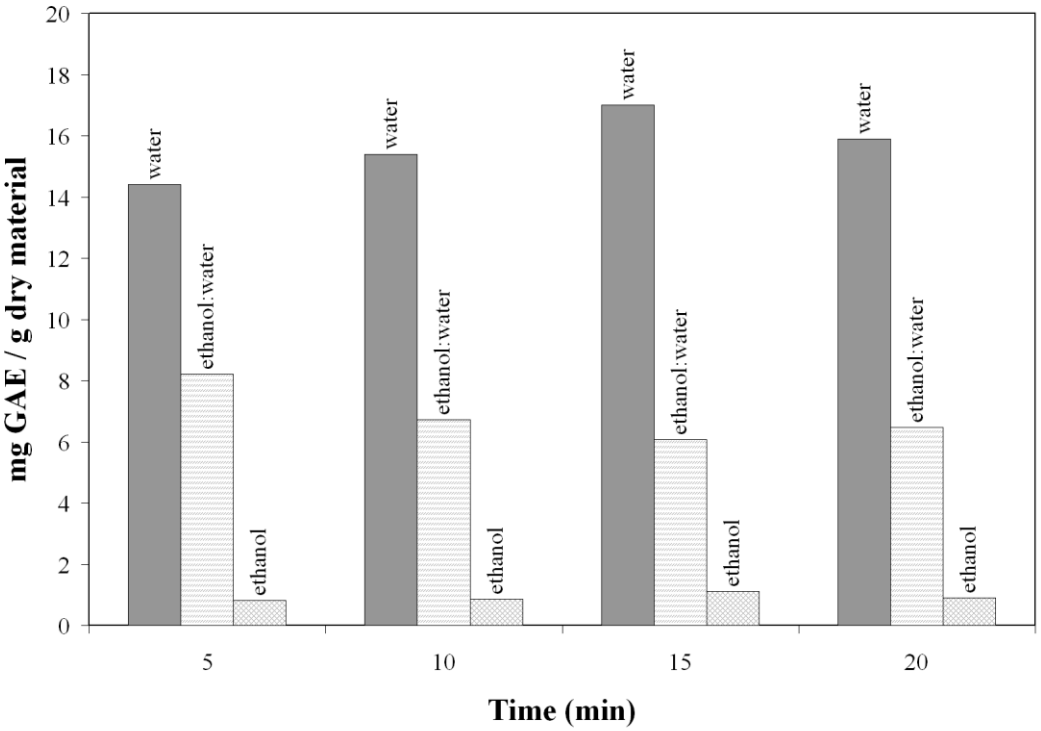


Figure 3.1. Total phenolic contents of nettle extract obtained by microwave extraction with 1:10 solid to solvent ratio for different solvent types

Ethanol was not a good solvent for the extraction of phenolics in nettle using conventional methods. Conventional extraction of nettle with ethanol was done for 30 minutes with 1:20 solid to solvent ratio and total phenolic content was found as 1.7 mg GAE / g dry material. This value was 21.5 mg GAE / g dry

material when water was used as the solvent. Therefore, the experiments were continued using water as solvent.

3.1.1. Effect of microwave and conventional extractions on total phenolic content of nettle extract

Time and solid to solvent ratio are the two parameters used in microwave extraction. Four different times (5, 10, 15, and 20 min) and three different solid to solvent ratios (1:10, 1:20, and 1:30) were experienced and total phenolic contents of the extracts were determined. Microwave power of 407 W was used in the experiments. No other power level was tested because different power levels showed no significant difference (Yan et al., 2010). Experimental data can be seen in Appendix C (Table C.1). Total phenolic content of nettle extracts changed in the range of 14.4 and 26.3 mg GAE / g dry material.

As can be seen from Figure 3.2, concentrations of nettle extracts remained almost constant with respect to extraction time. There are two main reasons for this situation. One of them is the very fast heating mechanism of microwave (Schubert and Regier, 2005), and the other one is the very thin structure of nettle leaves. Microwaves can provide the required energy for extraction even in a very short time. In addition, thin structure of nettle leaves allows phenolic substances to diffuse into water very quickly (Anonymous, 2005).

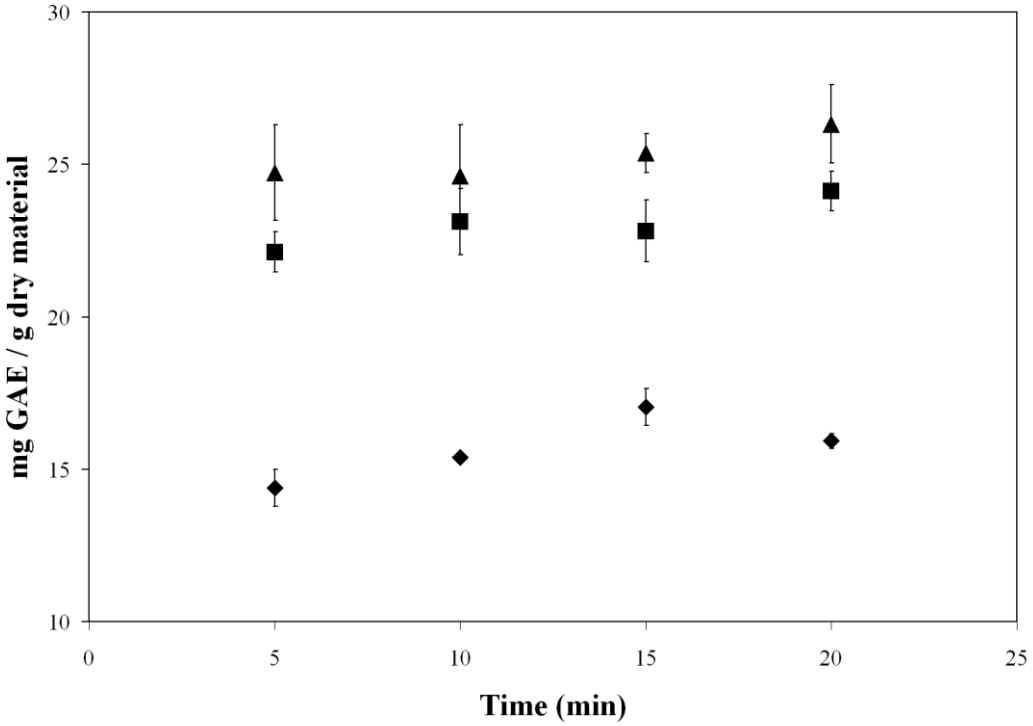


Figure 3.2. Total phenolic contents of nettle extract obtained by microwave extraction with water; solid to solvent ratios ♦ 1:10, ■ 1:20, ▲ 1:30.

Solid to solvent ratio plays an important role for the extraction process. As various studies stated, concentration of total phenolic substances increased with solid to solvent ratio (Sayyar et al., 2009; Xiao et al., 2008; Bi et al., 2010; Cacace and Mazza, 2003; Richter et al., 1996). The reason for this situation is the increase in concentration gradient with increase in solvent amount. Concentration of total soluble phenolic substances extracted increases with the amount of the solvent (Figure 3.2).

According to the statistical analysis, there was a significant difference between solid to solvent ratio. The best result in terms of total phenolic contents was obtained at 10^{th} minute for 1:30 solid to solvent ratio for microwave extractions of nettle (Table E.1).

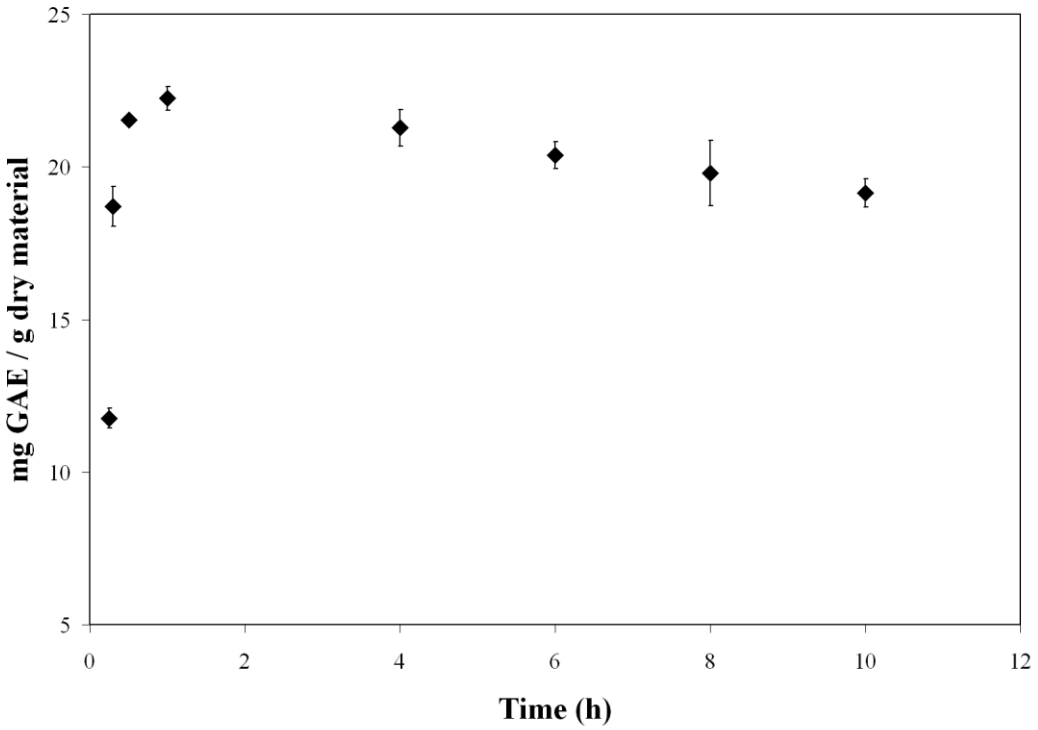


Figure 3.3. Total phenolic contents of nettle extract obtained conventionally with water and 1:20 solid to solvent ratio for different times

For comparison of microwave extraction, extraction was also performed with the same apparatus but with a conventional heater. To determine the optimum time in conventional extraction, in terms of total phenolic compounds, 1:20 solid to solvent ratio was used. Figure 3.3 shows that extraction of phenolic substances in nettle was nearly completed at 30th minute. For 15 and 20 minutes extractions, it is clearly seen that time is not sufficient to get all the phenolic substances. After 30 minutes, it can be regarded as concentration of total phenolic content remained almost constant.

According to one-way ANOVA results (Table E.2); there is no significant difference between extraction times 30 min, 1 h, 4 h, and 6 h. After 6 hour, total

phenolic content decreased due to degradation of phenolic substances as a result of high heat exposure (Schubert and Regier, 2005). These results are also consistent with the study of Xiao et al (2005). Thus, extraction time of 30 min was chosen as the optimum time.

Figure 3.4 shows the effects of solid to solvent ratios on total phenolic content of conventionally extracted nettle for 30 min.

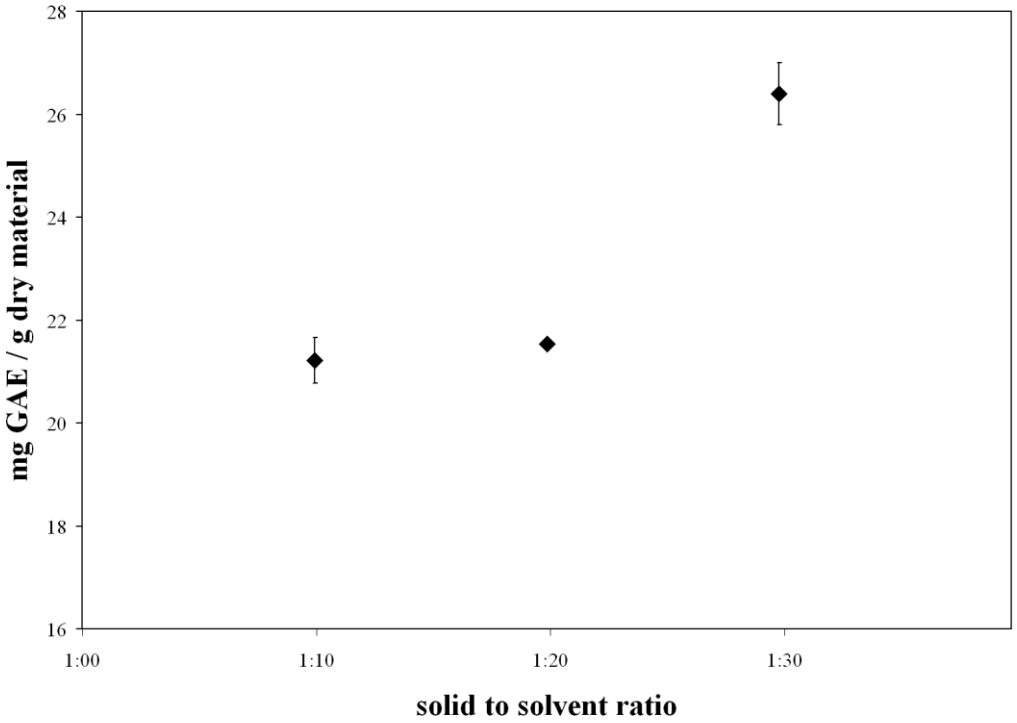


Figure 3.4. Total phenolic contents for 30 min conventional extractions of nettle with water in different solid to solvent ratios

Increasing solid to solvent ratio increases the concentration of total phenolic content. Similar results were found by other researchers too (Alekovski et al., 1998; Sayyar et al., 2009; Bi et al., 2010; Cacace and Mazza, 2003). Large

volumes of solvent enhances extraction yield, because amount of phenolic compounds that can be solved in extraction solvent increases due to the increase of concentration gradient. So, the optimum total phenolic content was obtained at 1:30 solid to solvent ratio.

According to the statistical analysis 1:30 solid to solvent ratio was found to be significantly different than the other solid to solvent ratios (Table E.3).

3.1.2. Effect of ultrasound extraction and maceration on total phenolic content of nettle extract

In ultrasound extraction; effects of ultrasonic power (50% and 80%), extraction time (5, 10, 20, and 30 min), and solid to solvent ratio (1:10, 1:20, and 1:30) on extraction of phenolic compounds at constant temperature (40°C) were investigated. Many people suggested that phenolic thermodegradation occurs at temperatures higher than 40°C (Ma et al., 2009; Chowdhury and Viraraghavan, 2009; Shalmashi, 2009; Xia et al., 2006), so the possible maximum temperature was selected as 40°C.

Ultrasonic power had a significant effect on the extraction efficiency of nettle (Figure 3.5). Increasing power from 50% to 80% increased total phenolic content of nettle (Table E.4). Ultrasound extraction has mainly two effects which are acoustic cavitations and mechanical effects. Cavitation phenomenon is related to the cycles of compression and expansion of cavitation bubbles in extraction media that generates high pressure and temperature spots (Ohlsson and Bengtsson, 2002). Compression increases the pressure and temperature and thus leads to the implosion of bubbles. These compression and expansion cycles disrupt the cell walls, so extraction is enhanced by both the passage of phenolic compounds from the cell to the extraction solvent and the passage of solvent into the cell (Shalmashi, 2009). Implosion of bubbles causes a mixing effect, which is the

mechanical effect of ultrasound in extraction process (Chemat et al, 2004a). Increasing power level of ultrasound supplies a faster and stronger mixing effect that reduces external resistance and enhances the mass transfer, so increasing power enhances the extraction and increases the efficiency (Shalmashi, 2009; Chemat et al., 2004b; Ma et al., 2009). By means of ultrasound mixing occurs in the solid-liquid interface. As a result, the thickness of the boundary layer decreases.

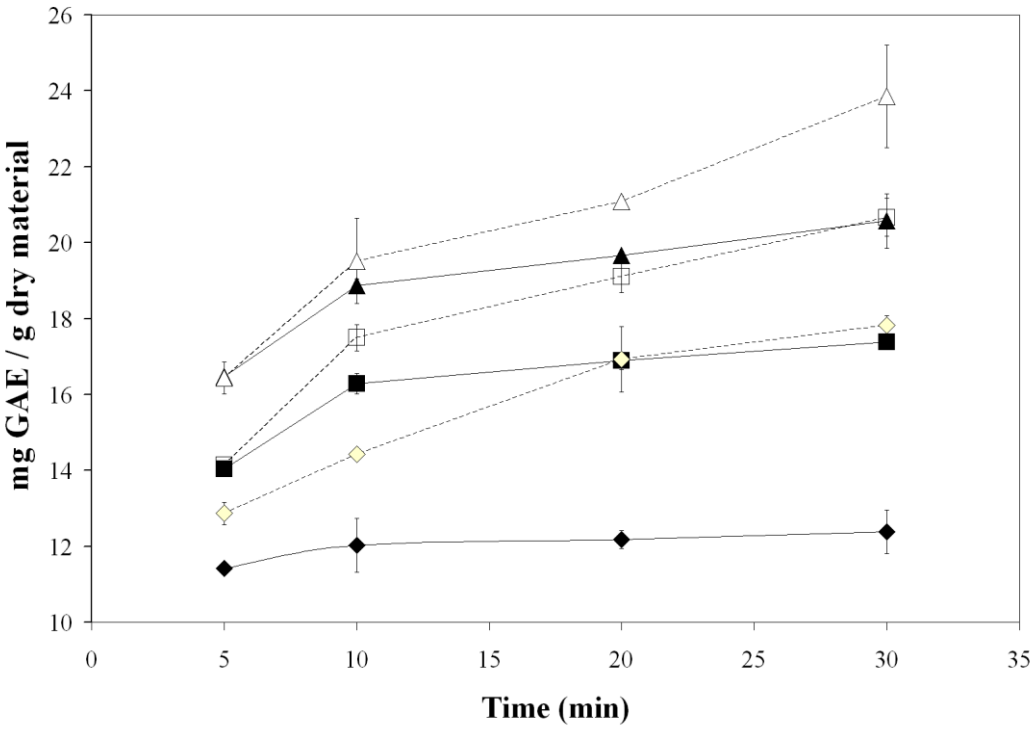


Figure 3.5. Total phenolic contents of nettle extracts obtained by ultrasound extraction with water; ♦ 50% power and 1:10, ■ 50% power and 1:20, ▲ 50% power and 1:30, ◊ 80% power and 1:10, □ 80% power and 1:20, Δ 80% power and 1:30

Time was also a significant parameter in ultrasound extraction of phenolic compounds. As the extraction time increased, total phenolic content also increased (Figure 3.5). Similar results had been obtained by Rostagno et al. (2003). The local heating of the tip of the probe gave an excess energy to extraction matrix (Chemat et al., 2004b). Although this increasing temperature enhances extracting of some of the phenolic substances, it might also cause degradation for the others. Continuous mixing of the media or a cooling jacket system may prevent local heating for the probe ultrasound system. In this experiment, cooling system was preferred due to its uniformity and simpler set up. By this way, temperature could be kept at 40 ± 5°C, and temperature effect was eliminated for longer times. Extraction time of 30 min was convenient for this experiment, which is also in accordance with the extraction of bioactive compounds from herbs with the help of ultrasound in the study of Vinatoru (2001).

In addition, solid to solvent ratio, also, showed a significant effect on ultrasonic extraction of phenolic compounds from nettle (Table E.4). It can also be clearly seen from Figure 3.5. The reason is the same as explained before, that is, more extractable phenolic substances can pass to larger volumes of solvent (Alekovski et al., 1998; Sayyar et al., 2009; Bi et al., 2010; Cacace and Mazza, 2003).

According to 3-way ANOVA results, the optimum conditions in terms of total phenolic substances were 80% power, 30 minute, and 1:30 solid to solvent ratio (Table E.4).

Maceration was done at 40 ± 1°C for 24 hour in an incubator for the comparison of ultrasound extraction (Shalmashi, 2009; Chung et al., 2010; Trusheva et al., 2007). Since ultrasound extraction can be considered as non-thermal, control method should also be similar, that is should be at the same temperature.

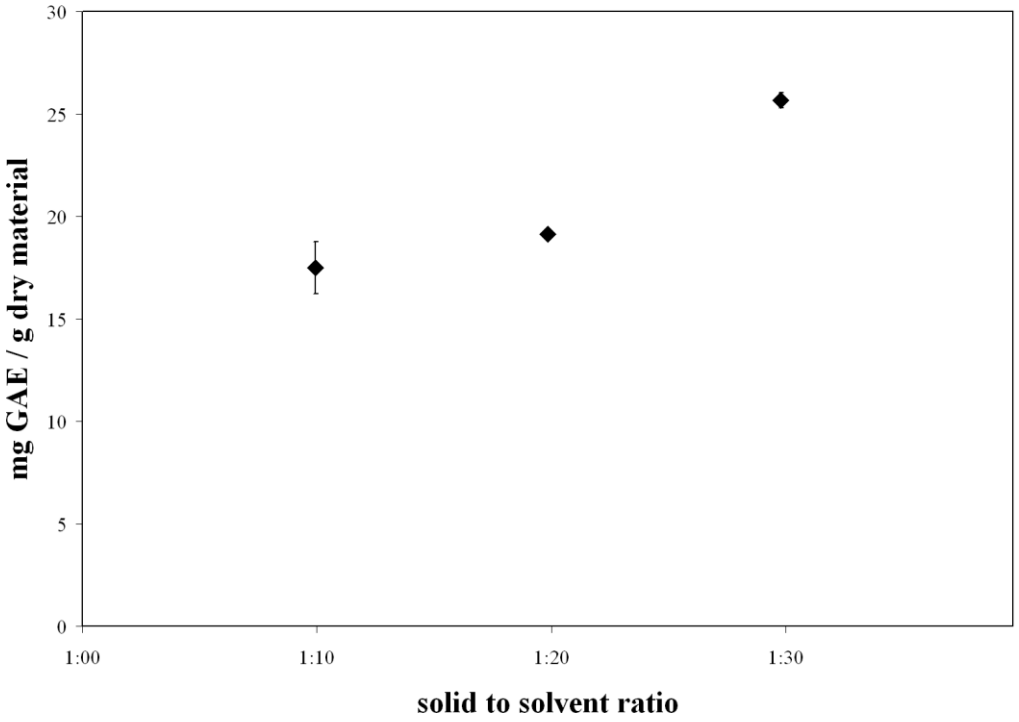


Figure 3.6. Total phenolic content of nettle extract obtained by maceration with water and with different solid to solvent ratios

Total phenolic contents of nettle extract obtained by maceration are shown in Figure 3.6. Solid to solvent ratio of 1:30 was found to be significantly different from the other solid to solvent ratios, thus this ratio was chosen as the optimum (Table E.5).

3.1.3. Comparison of different extraction methods according to total phenolic content

Table 3.1 shows total phenolic contents of nettle extracts obtained at the optimum conditions of different extraction methods. There is no significant difference between microwave extraction and conventional extraction. However, extraction

time was reduced by 67% by microwave extraction. This result is in accordance with the studies of Chen et al. (2008) with *Herba Epidemii*; Trusheva et al. (2007) with propolis; and Abbasi et al. (2008) with pomegranate.

The comparison of ultrasound and maceration extractions in terms of total phenolic contents also shows no significant difference statistically (Table E.6). However, total phenolic content of extract obtained by ultrasound was slightly lower. Khan et al. (2010) stated that at higher frequencies than the required in ultrasonic extraction, hydroxyl radical formation is favored, and hydroxyl radicals disrupt the phenolic substances.

Table 3.1. Experimental data for total phenolic content (TPC) and antioxidant activity (AA) of nettle for different extraction methods

Extraction method	Extraction time	TPC (mg GAE / g dry material)	AA (mg DPPH / g dry material)
MW[1]	10 min	24.6 [a]	4.2 [b]
Conv[2]	30 min	26.4 [a]	3.9 [c]
US[3]	30 min	23.9 [a]	3.0 [d]
Mac[4]	24 h	25.7 [a]	4.5 [a]

[1] MW, microwave extraction (1:30 solid to solvent ratio); [2] Conv, conventional extraction (1:30 solid to solvent ratio); [3] US, ultrasound extraction (80% power, 1:30 solid to solvent ratio); [4] Mac, maceration (1:30 solid to solvent ratio, at 40°C)

When all methods were compared, there was no statistically significant difference determined among them in terms of total phenolic contents. However, microwave extraction significantly reduced the time compared to the other three methods (Table 3.1). This is due to the rapid heating mechanism and fast diffusion of phenolic compounds into water. Since water within the plant material absorbs

microwave energy, cell disruption is promoted by internal superheating which facilitates diffusion of phenolic compounds from the matrix. Since ultrasound extraction temperature was lower and the total phenolic content of nettle extract obtained by ultrasound was not higher, it can be concluded that there was no thermal degradation of phenolics in microwave extraction of nettle.

Folin-Ciocalteu assay has the principle of reduction of reagent in the presence of phenolic substance and this reduction is accompanied by an observable color change (Singleton and Rossi, 1965; Singleton et al., 1999). This assay can be affected by non-phenolic substances including sugar, sulfur dioxide, ascorbic acid, organic acids, and Fe (II) (Roura et al., 2006; Singleton et al., 1999). Among these interferences to the assay, ascorbic acid might be critical in nettle extract (Ozyurt et al., 2007; Gülçin et al., 2004; Al-Ismail et al., 2007). Ascorbic acid content of extracts was determined titrimetrically and only negligible amount was detected. Since ascorbic acid is very heat sensitive, at 40°C and above, ascorbic acid in extracts may be damaged. In the study of Nunes et al. (1998); it was shown that even at 20°C maceration, 70% of initial ascorbic acid content was lost in samples. In addition, Capecka et al. (2005) demonstrated that drying decreases ascorbic acid content significantly, and in this study dried leaves were used. If considerable amount of ascorbic acid had been detected, then a correction for the Folin-Ciocalteu assay results would have been required (Asami et al., 2003). However, extractions at 40°C and boiling conditions do not constitute interference to the assay, so there is no need for a correction factor.

3.1.4. Comparison of different extraction methods according to antioxidant activity

Antioxidant activities of extracts obtained at the optimum extraction conditions were determined. There was a significant difference between the extracts of different methods in terms of antioxidant activity (Table E.6). In Table 3.1, total

phenolic content and antioxidant activities of different methods are shown. Maceration extract of nettle gave the higher antioxidant activity, and microwave extract was the second highest one, while the ultrasound extract had the lowest.

There was no correlation between antioxidant activity and total phenolic content of extracts obtained by microwave and conventional heating. There was no significant difference between total phenolic contents of extracts obtained by both methods; however antioxidant activity was significantly higher in microwave extract of nettle.

Total phenolic content and antioxidant activity in ultrasound extract was lower (Table 3.1) compared to maceration extract and this might be due to the degradation of some phenolic substances, which have hydroxyl type substituents such as naringin. That is, ultrasonic waves damaged the structure of some high hydroxyl substituent containing phenolics (Ma et al., 2009).

Although it is stated that there is a strong correlation between antioxidant values and total phenolic content in different papers (Shan et al., 2005; Gallo et al., 2010, Chrpova et al., 2010; Pirbalouti et al., 2010), antioxidant activities of individual phenolic compounds may be different (Londono et al., 2010). Londono et al. (2010) have shown in their study that aglycones and polymethoxyflavones, such as hesperetin and tangeritin, are more efficient than glycosides, such as hesperidin and neohesperidin. Definitely, it might be related with the assay that they have used for the antioxidant activity determination; however as a general statement, it can be said that aglycones show more antioxidant activity than their glycosides (Londono et al., 2010). Different phenolic substances can be extracted with different methods (Chung et al., 2010). DPPH test and Folin-Ciocalteu assay may also detect different type of phenolic substances, so it is not surprising if a strict correlation between total phenolic content and antioxidant activity was not observed (Matsingou et al., 2001). Therefore, the difference of total phenolic

content and antioxidant activity of microwave and conventional extractions can be explained with the reaction of different phenolic compounds for the two assays (Folin-Ciocalteu and DPPH). Thus, generally expected positive correlation between total phenolic content and antioxidant activity could not be seen in this study.

3.1.5. Comparison of different extraction methods according to concentration of phenolic compounds

Table 3.2 shows the concentration of some specific phenolic compounds found in nettle. Among a large variety of phenolic compounds; the selected ones (gallic acid, caffeic acid, chlorogenic acid, p-coumaric acid, naringenin, and naringin) have lower concentrations in microwave extract of nettle. There are unstable phenolics in this group, which means microwave radiation easily degrades them due to their structural properties. Greater number of hydroxyl group containing phenolic compounds are unstable and can easily be degraded under microwave radiation (Ma et al., 2009). This may be due to the severe conditions of microwave regarding sudden and excessive heating (Gallo et al., 2010). Therefore microwave extraction under vacuum may be recommended. There are different studies on vacuum microwave extraction proving the increasing yield and efficiency of extracts (Xiao et al., 2009; Wang et al., 2008).

Table 3.2. Concentrations of main phenolic acids in nettle detected by HPLC (mg / g dry material)

Extraction method	Gallic acid	Caffeic acid	Chlorogenic acid	p-Coumaric acid	Naringenin	Naringin
MW	1.125	1.223	4.798	1.157	5.582	0.665
Conv	1.256	1.327	5.108	1.255	6.034	0.865
US	1.209	1.289	4.453	1.100	5.735	0.784
Mac	1.185	1.343	5.009	1.180	5.881	0.779

In general, concentrations of phenolic compounds obtained by ultrasound extraction were less than those obtained by maceration. It was stated above that higher stability of phenolics are related to the more hydroxylic type and less methoxylic type groups, although there are exceptions. Some of these phenolics are suffering from not only microwave radiation but also ultrasound treatment. For example, caffeic and p-coumaric acids have only hydroxylic type groups, but they are unstable phenolics (Ma et al., 2009). The expected slight decrease can be seen from Table 3.2 for microwave and ultrasound extractions compared to their control methods. There is a possibility of formation of oxidative radicals during ultrasound extraction of nettle in water (Proestos and Komaitis, 2006). Proestos and Komaitis (2006) have studied with aromatic plants, and investigated the effect of solvent type under ultrasonic treatment. They have found lower phenolic acid concentration in water extracts, and this was explained by the formation of hydrogen peroxide because of the aqueous solution subjected to the ultrasonic energy (Paniwnyk et al., 2001). Although gallic acid, caffeic acid, p-coumaric acid, and naringenin gave similar results with the study of Proestos and Komaitis (2006); the difference between the ultrasonically assisted and conventional extractions in that study was very much higher. Most probably, phenolic compounds have suffered from formation of oxidative radicals, such as peroxides; but aqueous extractions of nettle did not show such a great degradation; thus application of ultrasound to an aqueous solvent may be convenient. In the presence of ultrasonic treatment, higher concentration of naringin was obtained as compared to maceration (Table 3.2). This is consistent with the results of Khan et al. (2010) in which polyphenols were investigated in orange peel.

For gallic acid and naringin, ultrasound selectivity can also be seen (Trusheva et al., 2007). They showed higher stability due to their structures (Ma et al., 2009).

HPLC chromatograms for different wavelengths are shown in Appendix D.

3.2. Extraction of phenolic compounds from melissa

Moisture content of melissa was determined as 8.6%. Microwave and ultrasound extractions at different conditions were done for obtaining the phenolic compounds of melissa, and conventional extraction and maceration were performed for comparison, respectively, in terms of total phenolic contents. The extraction conditions giving the highest total phenolic compounds were chosen as the optimum for each method. The antioxidant activity and the concentration of phenolic compounds of the extract obtained at these conditions were also determined.

Ethanol and water were tested as extraction solvents for melissa, as in the case of nettle extraction. As it is seen in Figure 3.7, water gave significantly better efficiency in terms of total phenolic content for melissa, like in the case of nettle extracts. The reason may be due to the solubility. Phenolics in melissa might have better solubility in water. Hong and Kim (2010) showed that water was better than ethanol for extraction of rosmarinic acid, which is the most abundant phenolic compound in melissa (Table 3.4). The swelling of water by the leaf tissue would be effective (Chen et al., 2008; Hemwimol et al., 2006). It was stated that water was the best solvent for the extraction of catechins, which constitute an important part of phenolic compounds (Trabelsi et al., 2010). Since outcomings were very similar to nettle extracts, ethanol-water mixture was not tried. Extractions were carried out by using water only.

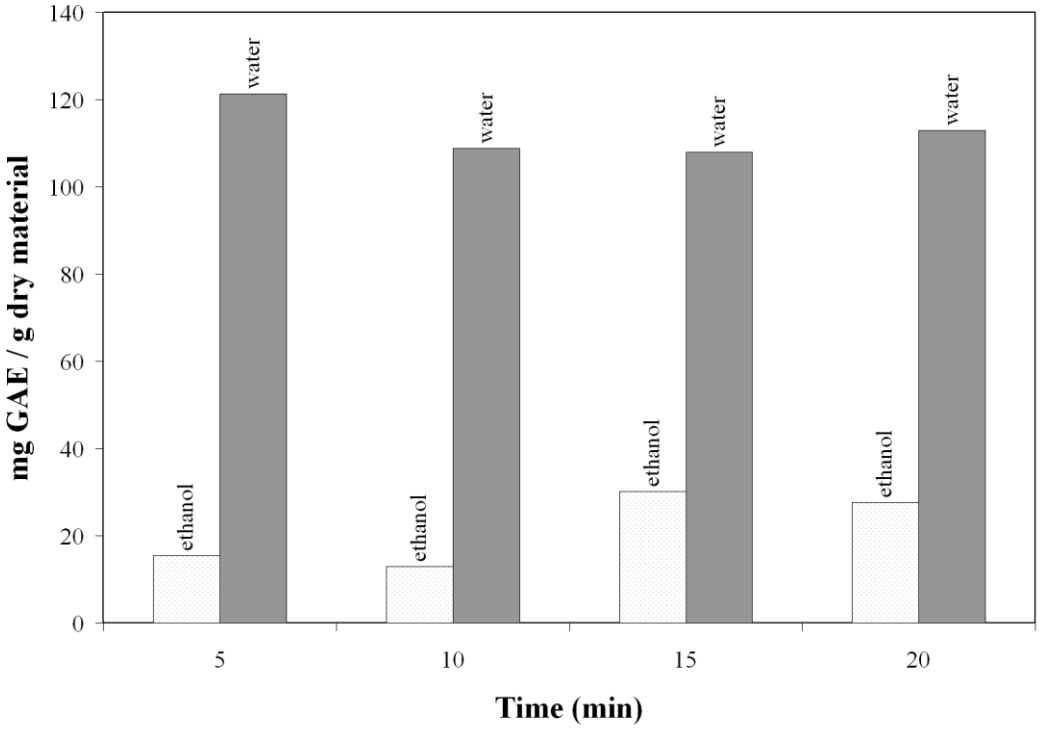


Figure 3.7. Total phenolic contents of melissa extract obtained by microwave extraction with 1:10 solid to solvent ratio for two different solvent types

3.2.1. Effect of microwave and conventional extractions on total phenolic content of melissa extract

As in the case of nettle extracts, time and solid to solvent ratio are the two parameters for microwave extraction. Four different times (5, 10, 15, and 20 min) and three different solid to solvent ratios (1:10, 1:20, and 1:30) were tested in terms of total phenolic contents. Related data can be seen in Table C.7 in Appendix. Total phenolic content of melissa extracts changed in the range of 107.9 and 145.8 mg GAE / g dry material, which are about six times that of nettle.

Both statistically and graphically (Figure 3.8), it can be seen that solid to solvent ratio had an important effect on total phenolic content. Solid to solvent ratio of

1:30 was significantly different from the other ratios and gave higher total phenolic content results. There was no significant difference between 1:20 and 1:10 ratios in terms of total phenolic content. These results are in accordance with the literature for optimization of solid to solvent ratio in microwave extractions (Yan et al., 2010).

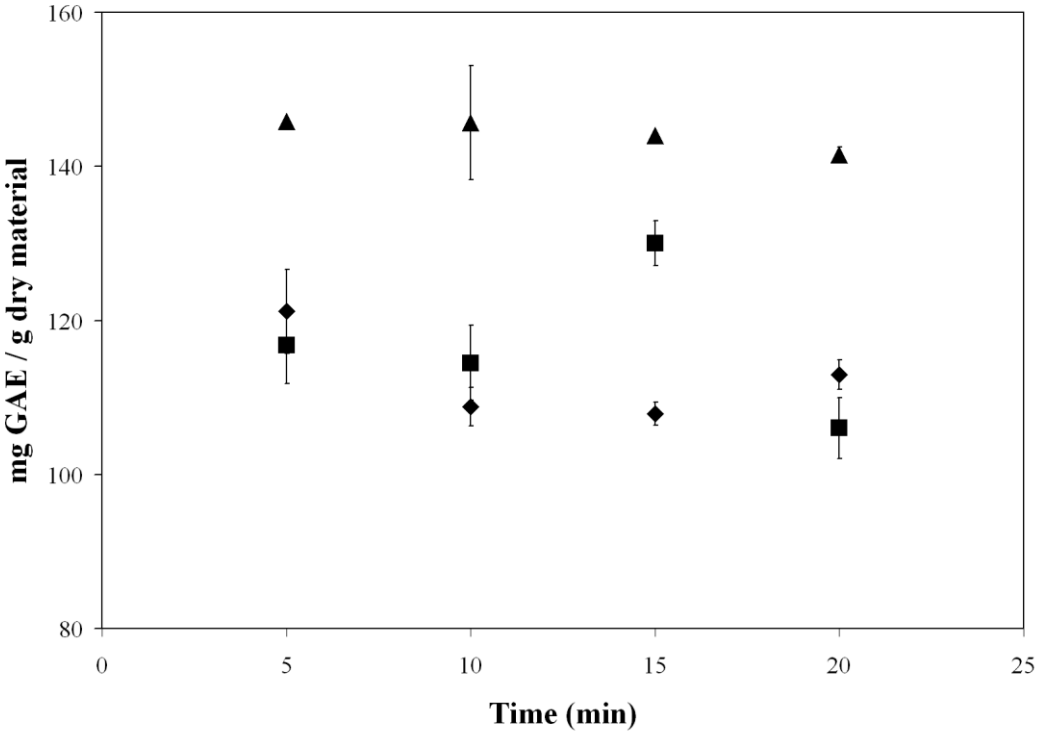


Figure 3.8. Total phenolic contents of melissa extract obtained by microwave extraction with water; solid to solvent ratios ♦ 1:10, ■ 1:20, ▲ 1:30.

There was no significant difference between 5 and 10 min. So, 5 min extraction time can be selected as the optimum time. This showed that all the extractable phenolics readily diffused to the solvent in microwave extraction. Microwave power and temperature were sufficient for obtaining the maximum results for total phenolic content in a very short time. Aerial parts of melissa were very thin, and

very fast diffusion occurred from solid material into the solvent after boiling of water.

Extraction time for conventional method was also optimized. Four different time values, which are 30 min, 1 h, 2 h, and 4 h, were tried and none of them showed a difference, statistically (Table E.8). So, 30 minute was chosen for the time efficiency and economy.

From Figure 3.9, it can be inferred that melissa has readily soluble phenolic compounds in water, and also the phenolic characteristics are very heat stable under these conditions. Even for 4 h extraction, there was not a significant difference in terms of total phenolic contents.

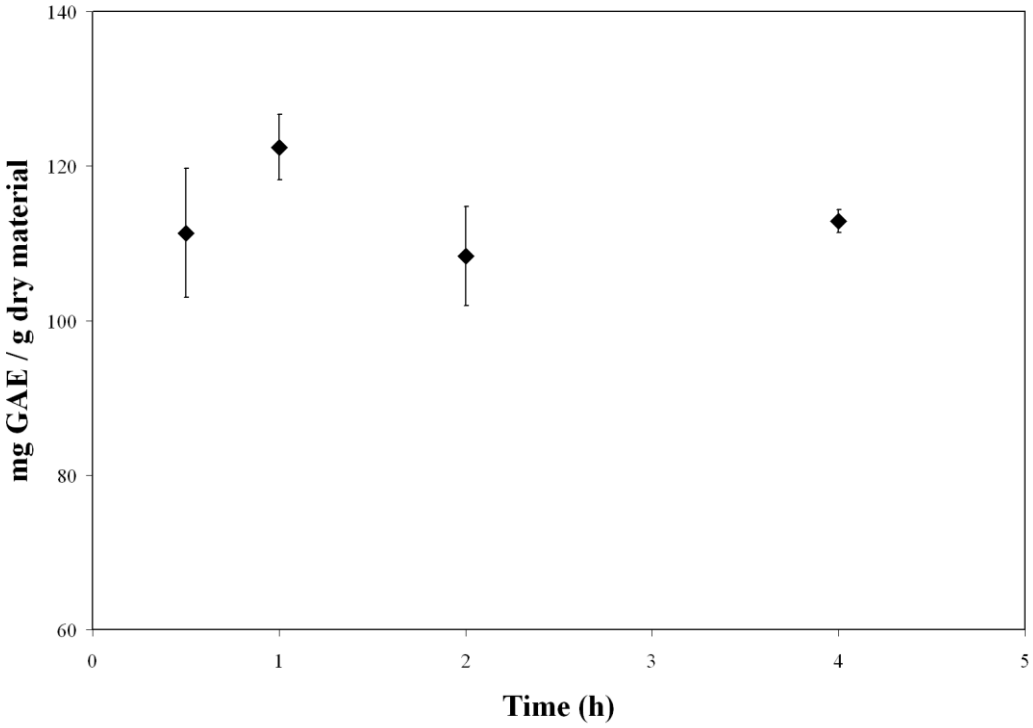


Figure 3.9. Total phenolic contents of melissa extract obtained conventionally with water and 1:20 solid to solvent ratio for different times

There is an expected increase in terms of total phenolic contents of melissa with the increasing solid to solvent ratio according to the Figure 3.10 (Yan et al., 2010; Xiao et al., 2008; Richter et al., 1996; Bi et al., 2010; Cacace and Mazza, 2003). Although 1:30 solid to solvent ratio seems to provide the highest total phenolic content, there was no significant difference between 1:20 and 1:30 solid to solvent ratio statistically. This means 1:20 solid to solvent ratio was sufficient in order to extract almost all phenolic substances from melissa.

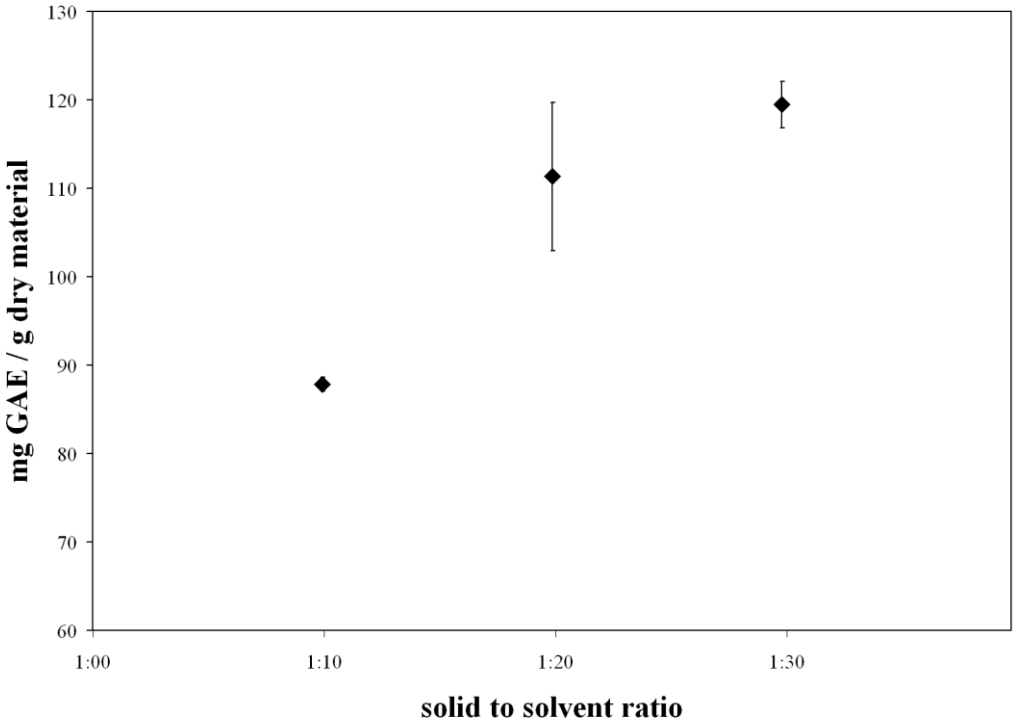


Figure 3.10. Total phenolic contents for 30 min conventional extractions of melissa with water in different solid to solvent ratios

3.2.2. Effect of ultrasound extraction and maceration on total phenolic content of melissa extract

Power had a significant effect on total phenolic content of the extracts. In the presence of 50% power, extracts had higher total phenolic content than the ones obtained with in presence of 80% power level (Figure 3.11). Positive correlation of power of ultrasound and total phenolic content of extracts has been stated in literature (Alupului et al.c, 2009; Ma et al., 2008; Khan et al., 2010). However, it was also mentioned that some phenolic substances survived degradation under ultrasound extraction conditions (Ma et al., 2009; Chemat et al., 2004b; Chowdhury and Viraraghavan, 2009; Gogate et al., 2004). Although the overall temperature was kept at 40°C, hot spots at the tip of probe may have caused the degradation of phenolic compounds at 80% power level.

Time was found to affect total phenolic content significantly (Table E.10, Figure 3.11). Extraction time of 20 and 30 minute showed statistically no difference. In terms of economy, 20 min was chosen as the optimum extraction time. General trend of extraction time was in accordance with the literature (Shalmashi, 2009; Rostagno et al., 2003; Chemat et al., 2004b). Increasing solvent amount increases obtainable total phenolic content to a certain extent (Bi et al., 2010).

For ultrasound extraction of melissa; the effects of power, time, and solid to solvent ratio were all significantly effective (Table E.10). According to the statistical analysis 50% power of ultrasound, 20 minutes, and 1:30 solid to solvent ratio were determined to be the optimum extraction conditions.

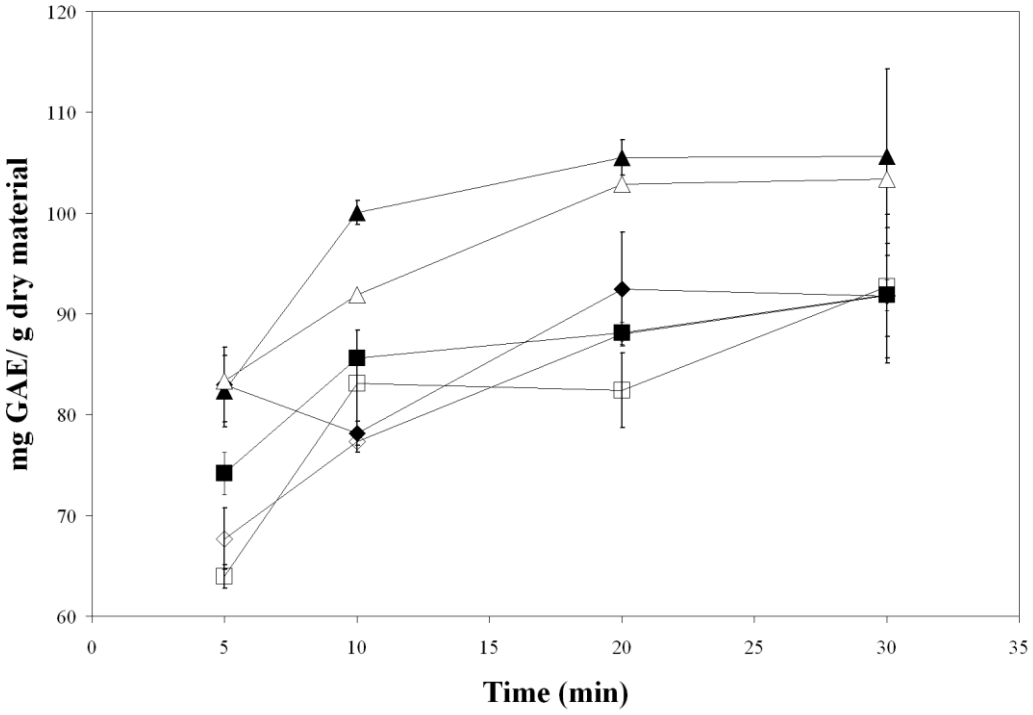


Figure 3.11. Total phenolic contents of melissa extract obtained by ultrasound extractions with water; ♦ 50% power and 1:10, ■ 50% power and 1:20, ▲ 50% power and 1:30, ◊ 80% power and 1:10, □ 80% power and 1:20, Δ 80% power and 1:30

Comparison was done with 1:30 solid to solvent ratio and total phenolic content value for maceration was 90.2 ± 1.3 mg GAE / g dry material.

3.2.3. Comparison of different extraction methods according to total phenolic content

The possible effect of ascorbic acid to Folin-Ciocalteu method (Roura et al., 2006) was investigated. Although there is not a published study showing that dried melissa leaves have ascorbic acid, its analysis were done titrimetrically. The result

showed that processed melissa extract at 40°C did not contain ascorbic acid. So, it did not taken into account for total phenolic content values.

According to one-way ANOVA results (Table E.12) for four different extraction techniques, microwave extraction was significantly better than the others, and other three methods showed no significant difference from each other in terms of total phenolic content (Table 3.3). This may be due to the rapid generation of heat inside the melissa leaves and the subsequent formation of higher pressure gradient which enhances mass transfer.

Table 3.3. Experimental data for TPC and AA of melissa for different extraction methods (1:30 solid to solvent ratio)

Extraction method	Extraction time	TPC (mg GAE / g dry material)	AA (mg DPPH / g dry material)
MW	5 min	145.8 [a]	30.64 [a]
Conv	30 min	119.5 [b]	30.58 [a]
US	20 min	105.5 [b]	22.51 [c]
Mac	24 h	90.1 [b]	25.21 [b]

Microwave extraction (1:30 solid to solvent ratio), conventional extraction (1:30 solid to solvent ratio), ultrasonic extraction (50% power, 1:30 solid to solvent ratio), and maceration (1:30 solid to solvent ratio, at 40°C)

In addition, microwave extraction, reduced the extraction time by 83%. In literature, it is stated that microwave assisted extraction not only reduces the time, but also can enhance the extract quality in terms of phenolic compounds (Schubert and Regier, 2005; Gallo et al., 2010; Rosa et al., 2010). Although it is known that specifically type and structure of phenolic compounds play important role, long

time exposures to heat denature the phenolics (Schubert and Regier, 2005; Shouqin et al., 2004). However, no heat denaturation was seen in microwave extraction.

As it can be seen from Table 3.3, ultrasound extraction is slightly better than maceration in terms of total phenolic content however, it was not statistically significant. Ultrasonic extraction can be preferred to maceration in terms of time efficiency.

3.2.4. Comparison of different extraction methods according to antioxidant activity

Table 3.3 shows the total phenolic contents and antioxidant activities of melissa for different extraction methods. Total phenolic content of microwave extract was higher than its control experiment; however antioxidant activity showed no significant difference.

Total phenolic content of ultrasound and maceration extracts were not found to be different statistically, however antioxidant activity of maceration was higher.

Total phenolic contents do not also show correlation with antioxidant activities (Chung et al., 2010). This may be due to the effective phenolic types with respect to the applied method, like Folin-Ciocalteu and DPPH, as in the case of nettle extracts.

3.2.5. Comparison of different extraction methods according to concentration of phenolic compounds

In Table 3.4, concentrations of eleven different phenolic compounds (catechin, caffeic acid, vanillic acid, syringic acid, p-coumaric acid, naringenin, trans-3-

hydroxycinnamic acid, naringin, rosmarinic acid, hydrocinnamic acid, and hesperetin) from melissa extracts were shown. All of the acids gave very similar concentration values for microwave and conventional extractions, except trans-3-hydroxycinnamic acid and rosmarinic acid. Gallic acid could not be detected in the melissa extracts. As stated before; phenolic compounds gave different responses to different extraction methods with respect to their degradation levels. Hydroxyl type substituents show less stability under microwave extraction conditions (Ma et al., 2009; Liazid et al., 2007). This phenomenon is valid for catechin, with five hydroxyl groups; naringenin, with three hydroxyl groups; hesperetin, with three hydroxylic groups and one methoxyl group; caffeic acid, p-coumaric acid, and hydrocinnamic acid, with one hydroxyl group in their structures. Vanillic acid with its one methoxyl group, is known as a stable phenolic compound (Ma et al., 2009); and it is convenient with the result of this experiment. Its concentration was higher in microwave extraction that in conventional extraction. Trans-3-hydroxycinnamic acid, which is a derivative of trans-cinnamic acid in the group of chlorogenic acids, is known to be present in melissa (Marques and Farah, 2009). Marques and Farah (2009) showed that cinnamic acids can be obtained by methanolic extracts or infusion in water. This is supported with obtained results of this study, and enhanced by showing the convenience of microwave extraction for this compound. It may also be thermally unresistant, because it could not be detected in conventional extraction (Nkhili et al., 2009).

Two abundant phenolic acids that could be detected in melissa extract, were rosmarinic acid and hydrocinnamic acid. Of these two, rosmarinic acid could have been obtained by microwave extraction in higher concentrations. This shows that rosmarinic acid is heat sensitive. Extraction time of 30 min may cause degradation in the structure and effectiveness of rosmarinic acid (Ma et al., 2009; Schubert and Regier, 2005; Shouqin et al., 2004).

Catechin (Proestos and Komaitis, 2006), syringic acid, p-coumaric acid (Ma et al., 2009), narigenin (Proestos and Komaitis, 2006), trans-3-hydroxycinnamic acid, and rosmarinic acid were seemed to suffer from ultrasound extraction. Among them, naringenin and trans-3-hydroxycinnamic acid were not detected in ultrasonic melissa extract. On the contrary, naringin could not be obtained from maceration, but the recovery of it with ultrasound was quite high.

Rosmarinic acid, naringenin, and trans-3-hydroxycinnamic acid have suffered from ultrasound application, because they have only hydroxyl type substituents in their structures (Ma et al., 2009). Syringic acid has two methoxylic and one hydroxylic type groups, and this structure made it relatively unstable (Ma et al., 2009), so it showed a slight decrease in concentration for ultrasound extraction. Caffeic acid, vanillic acid, and hesperetin gave higher concentrations for the tested method in accordance with literature (Ma et al., 2009; Ma et al., 2008).

Table 3.4 shows that individual phenolic compounds had different responses to the applied extraction method. For example; caffeic acid, vanillic acid, syringic acid, p-coumaric acid, and hesperetin generally gave similar concentration values for different methods. Vanillic acid and catechin seemed to be heat sensitive (Liazid et al., 2007), since under boiling conditions of microwave and conventional extractions, their concentrations decreased. In addition, catechin survived degradation under microwave and ultrasound applications. Naringenin gave very similar concentration values in all methods, except ultrasound extraction. In ultrasound extraction, it could not be detected (Ma et al., 2009). Interestingly, trans-3-hydroxycinnamic acid suffered from both conventional and ultrasound extraction. Trans-3-hydroxycinnamic acid might have been degraded with high temperature for long times (Ma et al., 2008; Ma et al., 2009), and also ultrasound mechanism might not have been convenient for its obtaining. Naringin was not detected in maceration. Short time extractions gave similar results, but 24 hour waiting period may have caused degradation of naringin. Rosmarinic acid

and hydrocinnamic acid exhibited similar trends; they seemed to be extractable under high temperatures (Gallo et al., 2010). Their concentrations were high for microwave and conventional extractions; and low for ultrasound extraction and maceration. In addition to that, rosmarinic acid was mostly suffered from ultrasound among the four methods.

Table 3.4. Concentrations of main phenolic acids in melissa detected by HPLC (mg / g dry material)

Extraction method	Catechin	Caffeic acid	Vanillic acid	Syringic acid	p-Coumaric acid	Naringenin	trans-3-hydroxycinnamic acid	Naringin	Rosmarinic acid	Hydro-cinnamic acid	Hesperetin
MW	1.353	2.345	0.219	3.718	2.590	15.269	3.012	6.210	39.804	21.442	13.171
Conv	1.729	2.510	0.211	3.603	2.878	15.793	nd	6.097	34.193	23.962	13.345
US	2.008	2.459	0.480	3.267	2.469	nd*	nd	5.787	16.902	7.744	13.067
Mac	3.426	2.445	0.450	3.654	2.716	15.749	2.966	nd	23.318	6.030	12.829

* nd, not detected

3.3. Extraction of essential oil from melissa

Microwave extraction of essential oil from melissa was also studied and it was compared with the hydrodistillation. Quantitative analyses were done for both methods. Essential oil yield was higher in microwave extraction (4.1 mg essential oil / g dry sample) than in conventional hydrodistillation (1.8 mg essential oil / g dry sample). These results were in accordance with literature. Higher yields of essential oil from *Origanum husnucanbaseri* (Uysal et al., 2010) and from oregano (Bayramoglu et al., 2008) by solvent free microwave extraction than by hydrodistillation. In addition, Wang et al. (2010) compared the microwave assisted hydrodistillation and hydrodistillation in the extraction of essential oil of mango, and found that a yield of 0.16% and 75 min extraction time for microwave assisted hydrodistillation and a yield of 0.11% and 4 h extraction time for hydrodistillation. In another study, it was stated that melissa leaves contain at least 0.05% (v/w) essential oil on dry basis (Allahverdiyev et al., 2004); which was also in accordance with the findings of this study.

Individual components and amounts are shown in Table 3.5 comparatively with control method. It can be seen that concentrations of α-pinene, cineol, γ-terpinene, linalool, α-terpineol, and β-caryophyllene were close to each other for both methods; limonene, neral, geranial, and caryophyllene oxide gave higher concentrations in microwave extraction. The most abundant four compounds (limonene, neral, geranial, and caryophyllene oxide) in melissa (Topal et al., 2008; Rozzi et al., 2002) were successfully extracted with microwave within 1 h. Extraction time was 6 h for conventional extraction. That is, extraction time was decreased by 83% by using microwave.

Table 3.5. Concentrations of some essential oils in melissa detected by GC-MS (μl oil / g dry material)

Essential oil	Conventional	Microwave
α-pinene	0.0005	0.0005
Limonene	0.0027	0.0035
Cineol	0.0003	0.0003
γ-terpinene	0.0003	0.0003
Linalool	0.0015	0.0016
α –terpineol	0.0003	0.0003
Citronellol	0.0008	nd
Neral	0.0020	0.0029
Geraniol	nd	0.0001
Geranial	0.0027	0.0036
β-caryophyllene	0.0003	0.0004
caryophyllene oxide*	72.6593	103.9363

* μg oil / g dry material

Citronellol could be obtained in hydrodistillation only; this might have been due to the degradation of this compound under microwave conditions. In contrast, geraniol could be obtained by microwave extraction, although concentration of detected geraniol was very small. Hydrodistillation with 6 h period might have been detrimental for geraniol or a smaller concentration than 0.0001 μl oil / g dry material might have been extracted by conventional method which was considered as trace.

GC chromatograms of essential oil of melissa obtained by hydrodistillation and microwave extraction can be seen in Appendix F.

CHAPTER 4

CONCLUSION AND RECOMMENDATIONS

In this study, nettle and melissa extracts obtained by microwave and ultrasound were compared to the conventional and maceration extractions, respectively, in terms of total phenolic contents, antioxidant activity, and concentrations of individual phenolic compounds.

Among three different solvent types, water was determined as the best solvent. In microwave extraction for phenolic compounds from nettle and melissa, time and solid to solvent ratio were found to have significant effect. As a common trend, decreasing solid to solvent ratio increases the concentration of total phenolic compounds for both plants.

In ultrasound extractions; power, time, and solid to solvent ratio had significant effect on concentration of total phenolic compounds for nettle and melissa. Increasing extraction time and decreasing solid to solvent ratio increased the total phenolic content. Power had different effects for both plants. Increasing power level increased the total phenolic content for nettle, however for melissa it was the reverse, that is increasing power level decreased the total phenolic content.

When microwave extraction, ultrasound extraction, conventional extraction, and maceration were compared, no significant difference in terms of total phenolic content was determined among the methods. In general, microwave extraction can be recommended in extraction of phenolic compounds from nettle and melissa, since there was no thermal degradation and extraction time was significantly reduced.

Quantitative analysis was done for individual phenolic compounds by HPLC. The most abundant phenolic compounds were chlorogenic acid and naringenin, in nettle; and rosmarinic acid, in melissa. The highest antioxidant activity was obtained by maceration for nettle. On the other hand, melissa extracts obtained by microwave and conventional extractions were similar and the highest in terms of antioxidant activity.

Microwave extraction of essential oils of melissa was also done in this study, and compared to conventional hydrodistillation. Individual essential oil compounds were compared quantitatively by GC-MS. Microwave extraction of essential oil of melissa gave higher concentrations as compared to conventional extraction.

As a recommendation, microwave-vacuum extraction can be studied in order to investigate the stability or degradation degree of phenolic compounds. Further research on essential oil of melissa can be related to the antimicrobial characteristics.

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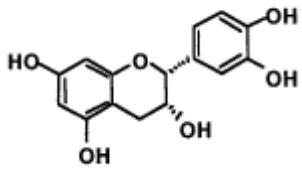
APPENDICES

APPENDIX A

STRUCTURES OF SOME PHENOLIC COMPOUNDS

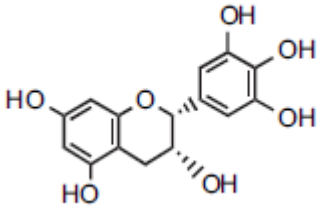
Flavonoids

Flavan-3-ol



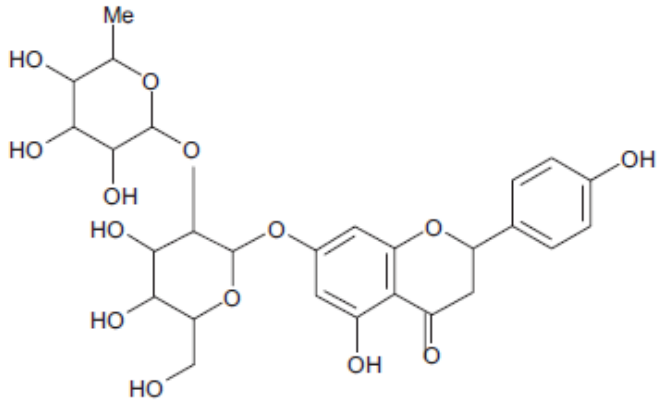
Catechin[a]

Proanthocyanidin

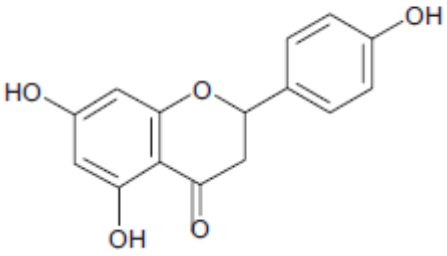


(-)-Epigallocatechin[b]

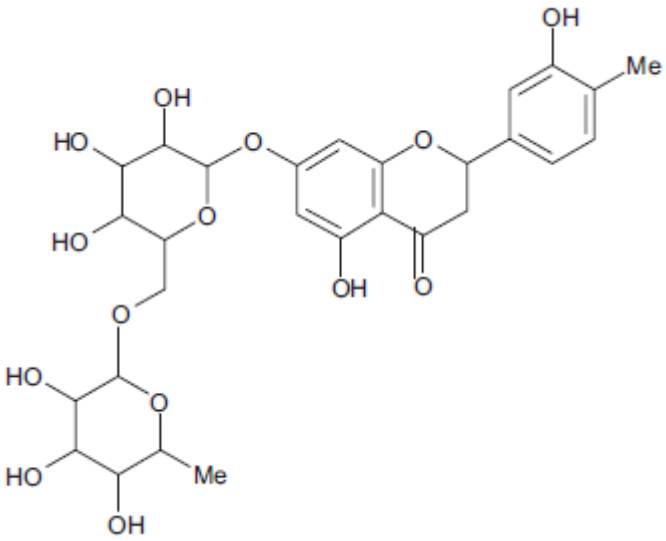
Flavanones



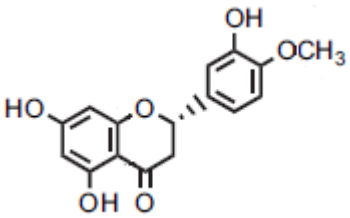
Naringin[c]



Naringenin[c]



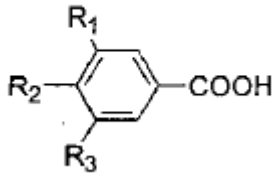
Hesperidin[c]



Hesperetin[b]

Non-flavonoids

Phenolic acids



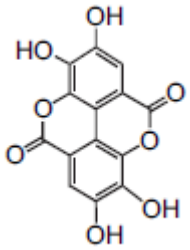
Benzoic acids

Gallic acid[d] $R_1 = R_2 = R_3 = OH$

Protocatechuic acid[d] $R_1 = H$, $R_2 = R_3 = OH$

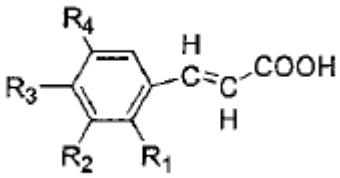
Vanillic acid[d] $R_1 = H$, $R_2 = OH$, $R_3 = OCH_3$

Syringic acid[d] $R_2 = OH$, $R_1 = R_3 = OCH_3$



Ellagic acid[b]

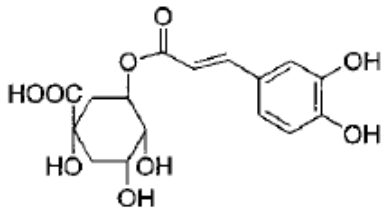
Hydrocinnamic acids



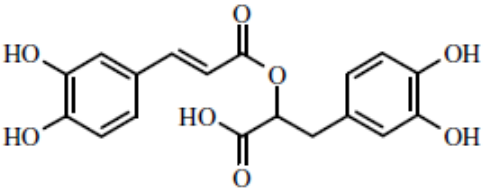
Ferulic acid[d] $R_1 = R_2 = H$, $R_3 = OH$, $R_4 = OCH_3$

p-Coumaric acid[d] $R_1 = R_2 = R_4 = H$, $R_3 = OH$

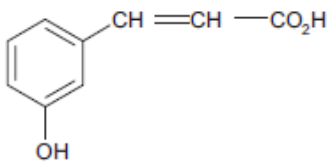
Caffeic acid[d] $R_1 = R_2 = H$, $R_3 = R_4 = OH$



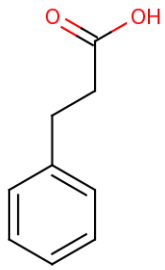
Chlorogenic acid[d]



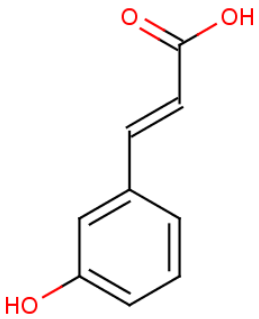
Rosmarinic acid[e]



m-coumaric acid[c]



Hydrocinnamic acid[f]



Trans-3-hydroxycinnamic acid[g]

[a] Packer et al., 1999

[b] Del Rio et al., 2010

[c] Dastmalchi et al., 2008

[d] Robards et al., 1999

[e] Toth et al., 2003

[f] Biological Magnetic Resonance Data Bank: Metabolomics a

[g] Biological Magnetic Resonance Data Bank: Metabolomics b

APPENDIX B

CALIBRATION CURVES

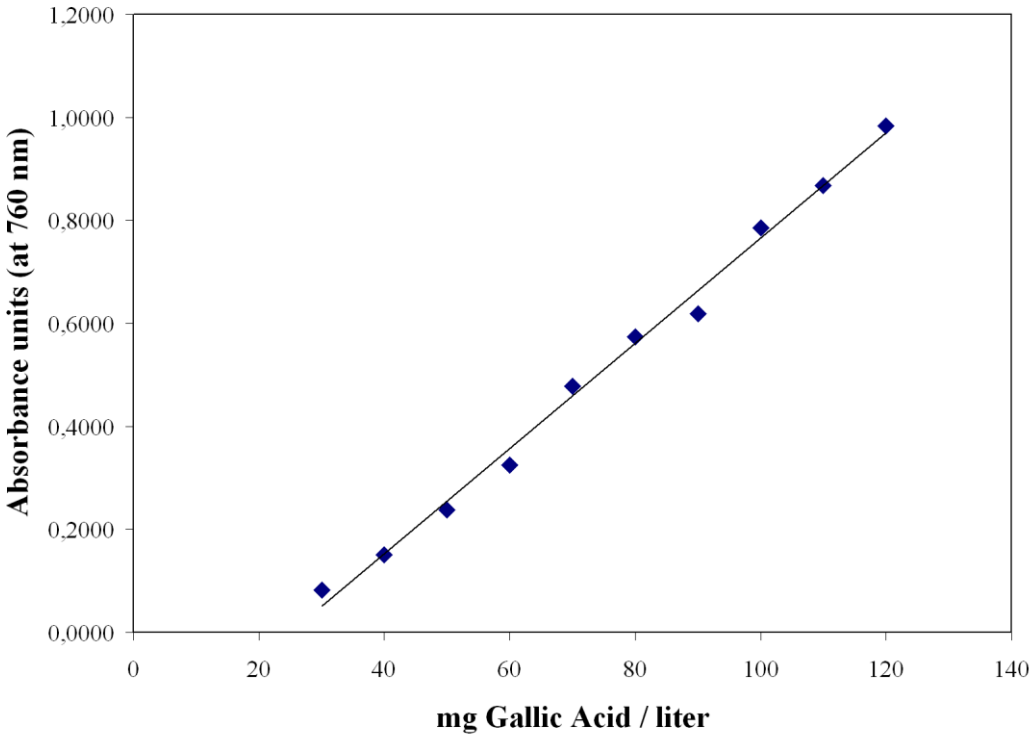


Figure B.1. Calibration curve prepared by gallic acid in water for determination of total phenolic contents

Absorbance (760 nm) = 0.0102 * (mg GA / l) – 0.2559 (B.1)

$R^2 = 0.9939$

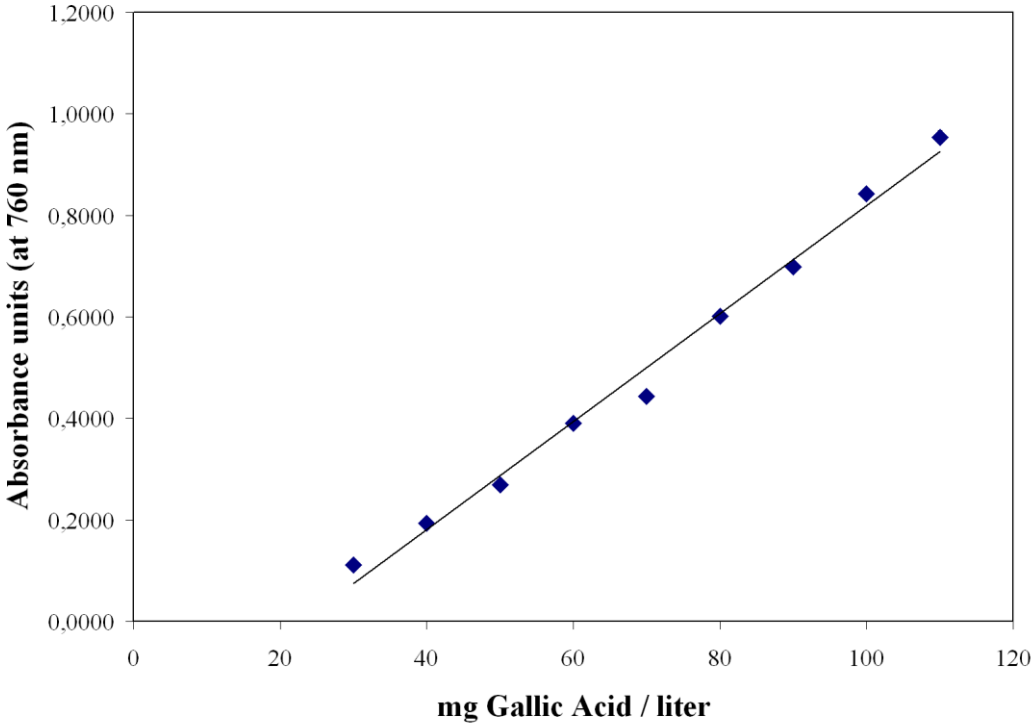


Figure B.2. Calibration curve prepared by gallic acid in ethanol for determination of total phenolic contents

Absorbance (760 nm) = 0.0106 * (mg GA / l) – 0.2449 (B.2)

$R^2 = 0.9903$

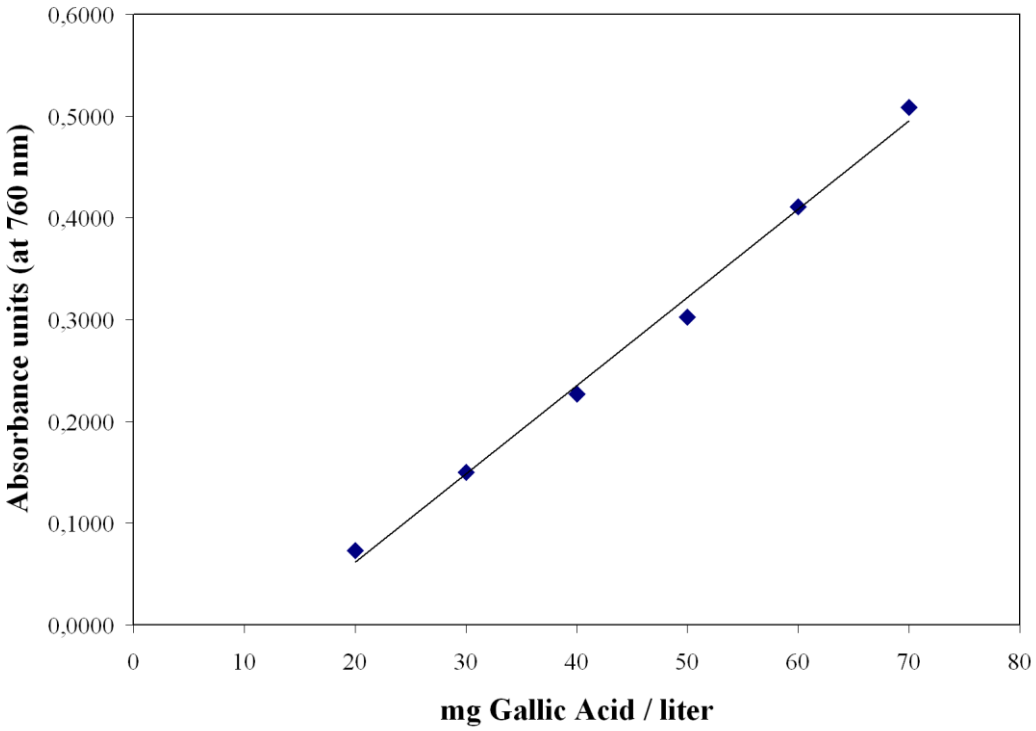


Figure B.3. Calibration curve prepared by gallic acid in ethanol:water mixture (50:50 v/v) for determination of total phenolic contents

Absorbance (760 nm) = 0.0087 * (mg GA / l) – 0.1118 (B.3)

R^2 = 0.9944

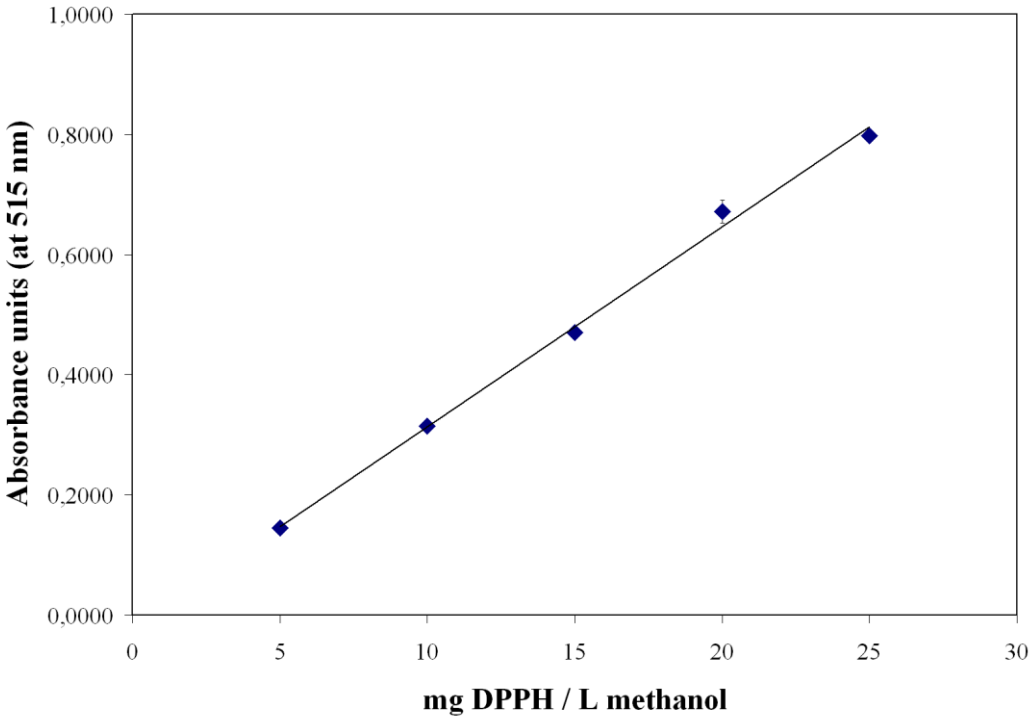


Figure B.4. Calibration curve prepared by DPPH radical in methanol for determination of antioxidant activity

Absorbance (515 nm) = 0.0332 * (mg DPPH / l) – 0.0189 (B.4)

$R^2 = 0.9965$

APPENDIX C

EXPERIMENTAL DATA

Table C.1. Experimental data of TPC for microwave extraction of nettle with water

solid to solvent ratio	time (min)	TPC	
		1st run	2nd run
1:10	5	13.8	15.0
	10	15.4	15.4
	15	17.6	16.4
	20	15.7	16.2
1:20	5	21.5	22.8
	10	22.0	24.2
	15	21.8	23.8
	20	23.5	24.8
1:30	5	23.2	26.3
	10	23.0	26.3
	15	24.7	26.0
	20	25.1	27.6

Table C.2. Experimental data of TPC for conventional extraction of nettle with water (according to time, with 1:20 solid to solvent ratio)

time	TPC	
	1st run	2nd run
15 min	11.4	12.1
20 min	19.4	18.1
30 min	21.6	21.5
1 h	22.6	21.9
4 h	20.7	21.9
6 h	20.8	20.0
8 h	18.7	20.9
10 h	19.6	18.7

Table C.3. Experimental data of TPC for conventional extraction of nettle with water (30 min)

solid to solvent ratio	TPC	
	1st run	2nd run
1:10	20.8	21.7
1:20	21.6	21.5
1:30	27.0	25.8

Table C.4. Experimental data of TPC for ultrasound extraction of nettle with water

power (%)	solid to solvent ratio	time (min)	TPC	
			1st run	2nd run
50	1:10	5	11.5	11.4
		10	11.3	12.7
		20	11.9	12.4
		30	13.0	11.8
	1:20	5	13.9	14.1
		10	16.0	16.6
		20	17.1	16.7
		30	17.5	17.3
	1:30	5	16.6	16.4
		10	19.0	18.7
		20	19.6	19.7
		30	21.3	19.9
80	1:10	5	12.6	13.2
		10	14.4	14.5
		20	17.8	16.1
		30	18.1	17.5
	1:20	5	14.2	14.1
		10	17.8	17.2
		20	18.7	19.5
		30	21.2	20.2
	1:30	5	16.0	16.9
		10	20.6	18.4
		20	21.0	21.2
		30	22.5	25.2

Table C.5. Experimental data of TPC for maceration of nettle with water at 40°C for 24 h

solid to solvent ratio	TPC	
	1st run	2nd run
1:10	18.8	16.2
1:20	19.1	19.2
1:30	26.0	25.3

Table C.6. Experimental data of TPC for microwave extraction of nettle with 1:10 solid to solvent ratio and with ethanol and ethanol-water mixture (50:50 v/v)

	TPC	
time (min)	ethanol	ethanol-water mixture (50:50 v:v)
5	0.82	8.20
10	0.86	6.73
15	1.11	6.07
20	0.91	6.47

Table C.7. Experimental data of TPC for microwave extraction of melissa with water

solid to solvent ratio	time (min)	TPC	
		1st run	2nd run
1:10	5	123.7	118.7
	10	110.3	107.3
	15	106.0	109.8
	20	116.6	109.3
1:20	5	111.9	121.6
	10	117.4	111.5
	15	134.0	126.1
	20	102.1	110.0
1:30	5	138.4	153.3
	10	145.5	145.8
	15	143.0	145.0
	20	144.3	138.7

Table C.8. Experimental data of TPC for conventional extraction of melissa (according to time, with 1:20 solid to solvent ratio)

time	TPC	
	1st run	2nd run
30 min	103.0	119.7
1 h	118.2	126.7
2 h	114.8	101.9
4 h	111.4	114.3

Table C.9. Experimental data of TPC for conventional extraction of melissa (30 min)

solid to solvent ratio	TPC	
	1st run	2nd run
1:10	87.0	88.6
20:1	103.0	119.7
1:30	122.1	116.8

Table C.10. Experimental data of TPC for ultrasound extraction of melissa

power (%)	solid to solvent ratio	time (min)	TPC	
			1st run	2nd run
50	1:10	5	86.7	79.3
		10	77.0	79.3
		20	98.1	86.8
		30	95.8	87.8
	1:20	5	76.2	72.1
		10	86.1	85.2
		20	89.2	87.0
		30	90.3	93.4
	1:30	5	85.8	78.8
		10	98.9	101.2
		20	107.3	103.7
		30	97.0	114.3
80	1:10	5	64.6	70.7
		10	78.4	76.2
		20	88.0	87.9
		30	85.1	98.5
	1:20	5	65.1	62.8
		10	77.8	88.4
		20	78.7	86.1
		30	85.6	99.8
	1:30	5	86.6	80.1
		10	88.0	95.8
		20	100.1	105.6
		30	103.2	103.6

Table C.11. Experimental data of TPC for maceration of melissa with water at 40°C for 24 h

solid to solvent ratio	TPC	
	1st run	2nd run
1:10	87.0	88.6
1:20	103.0	119.7
1:30	122.1	116.8

Table C.12. Experimental data of TPC for microwave extraction of melissa with 1:10 solid to solvent ratio and with ethanol

time (min)	TPC
5	15.4
10	13.0
15	30.1
20	27.8

APPENDIX D

HPLC CHROMATOGRAMS

D.1. Nettle extracts

1 Gallic acid
2 Caffeic acid
3 Chlorogenic acid
4 p-Coumaric acid
5 Naringenin
6 Naringin

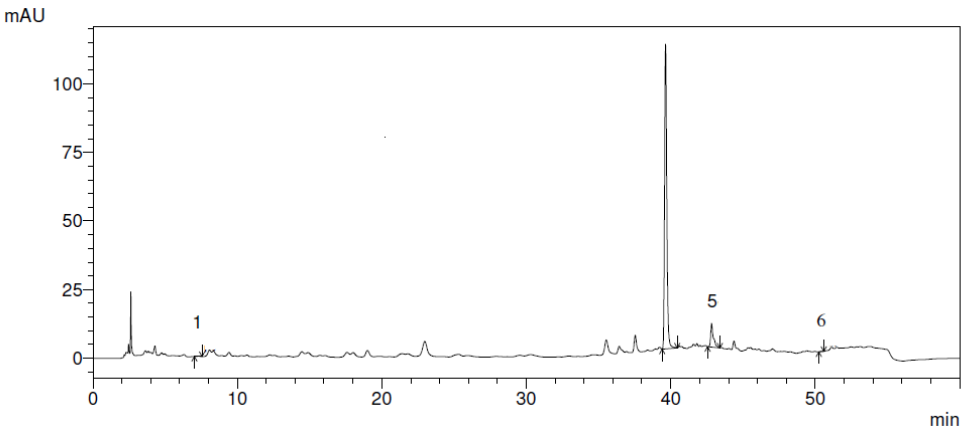


Figure D.1. HPLC chromatogram of nettle for microwave extraction at 280 nm

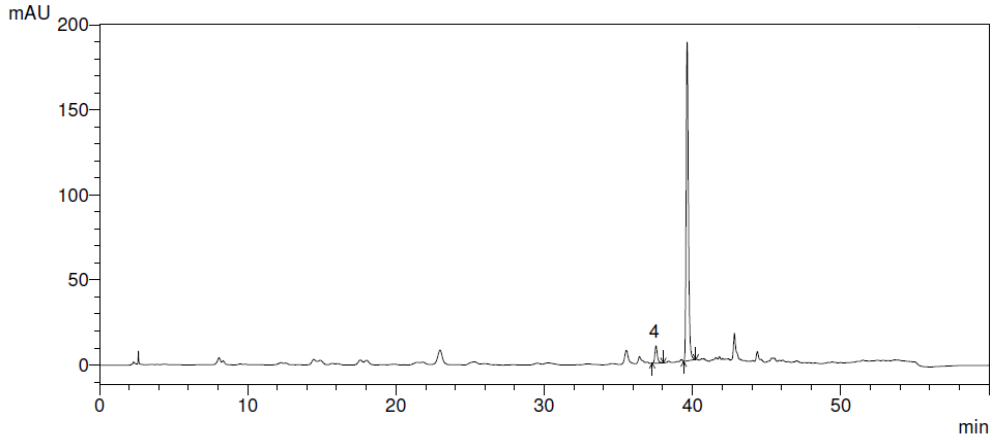


Figure D.2. HPLC chromatogram of nettle for microwave extraction at 310 nm

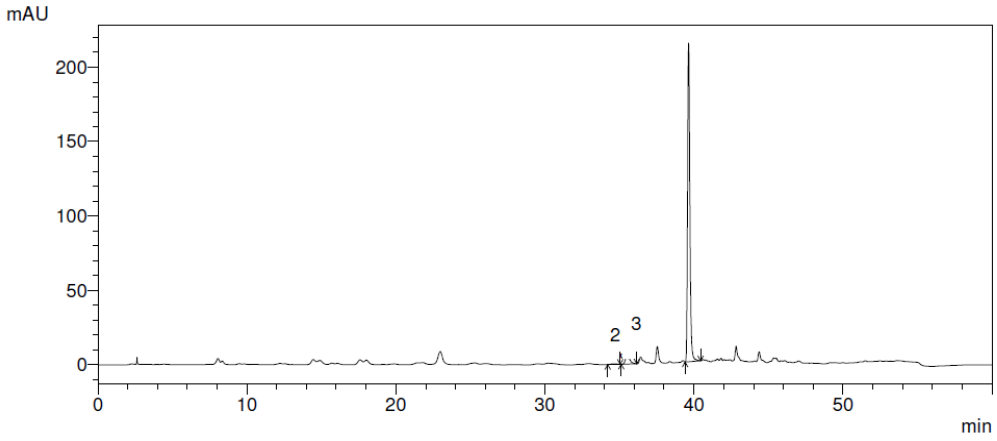


Figure D.3. HPLC chromatogram of nettle for microwave extraction at 330 nm

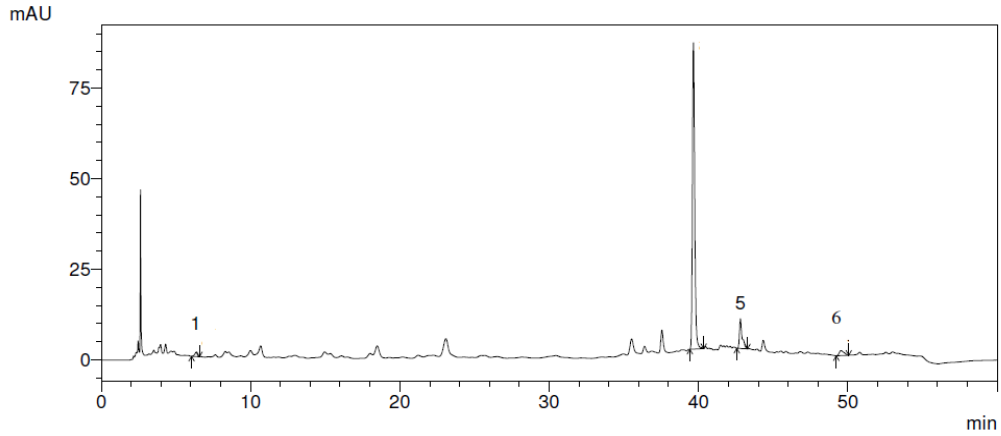


Figure D.4. HPLC chromatogram of nettle for conventional extraction at 280 nm

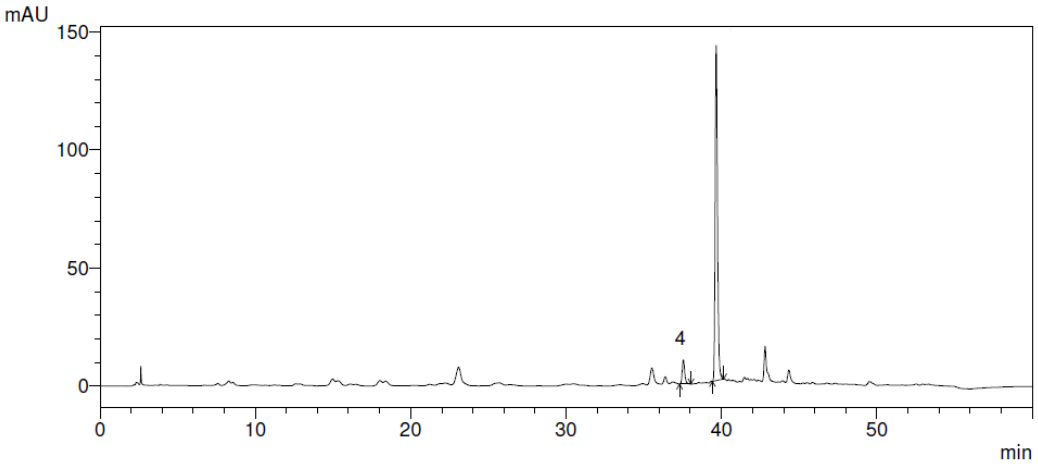


Figure D.5. HPLC chromatogram of nettle for conventional extraction at 310 nm

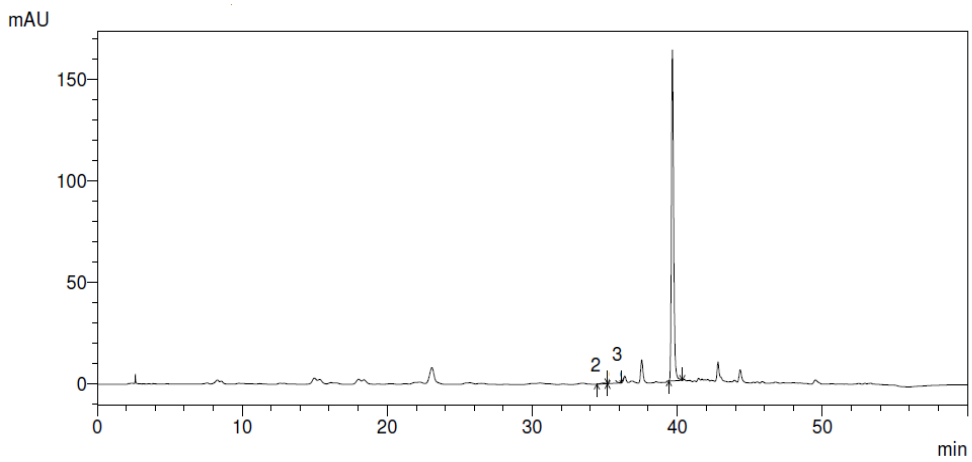


Figure D.6. HPLC chromatogram of nettle for conventional extraction at 330 nm

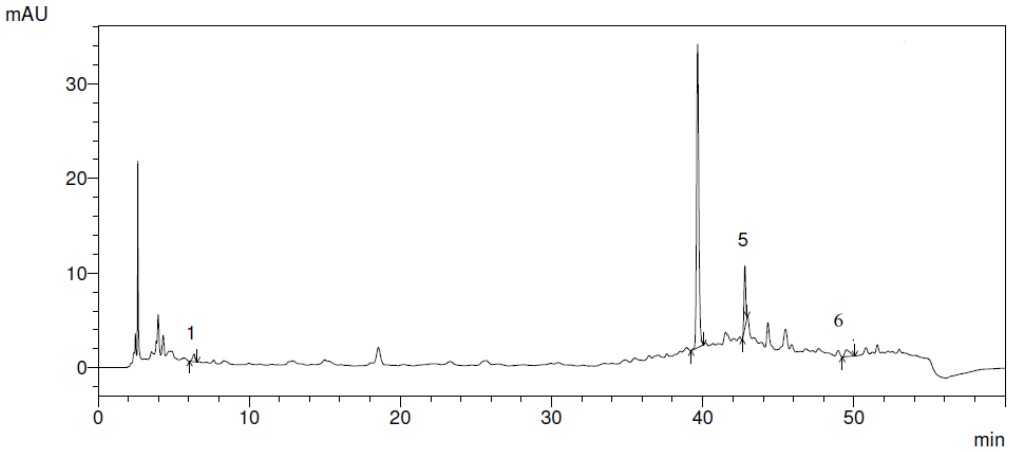


Figure D.7. HPLC chromatogram of nettle for ultrasound extraction at 280 nm

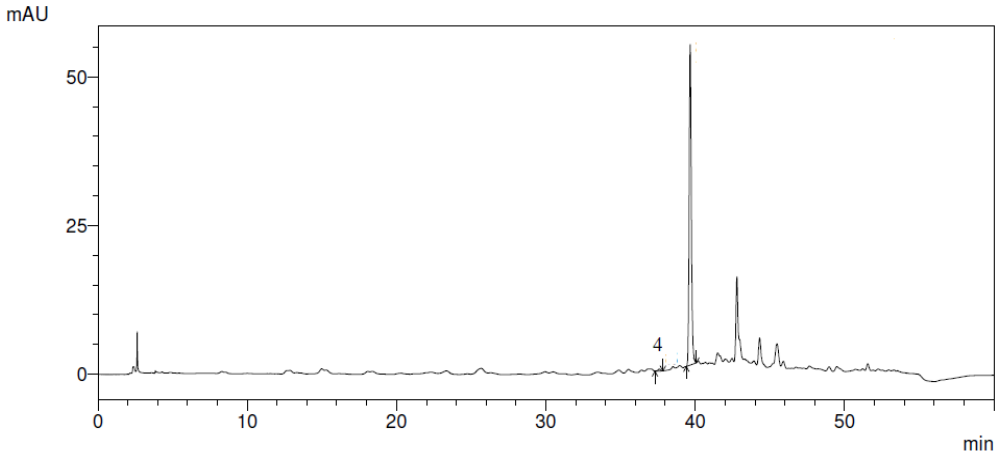


Figure D.8. HPLC chromatogram of nettle for ultrasound extraction at 310 nm

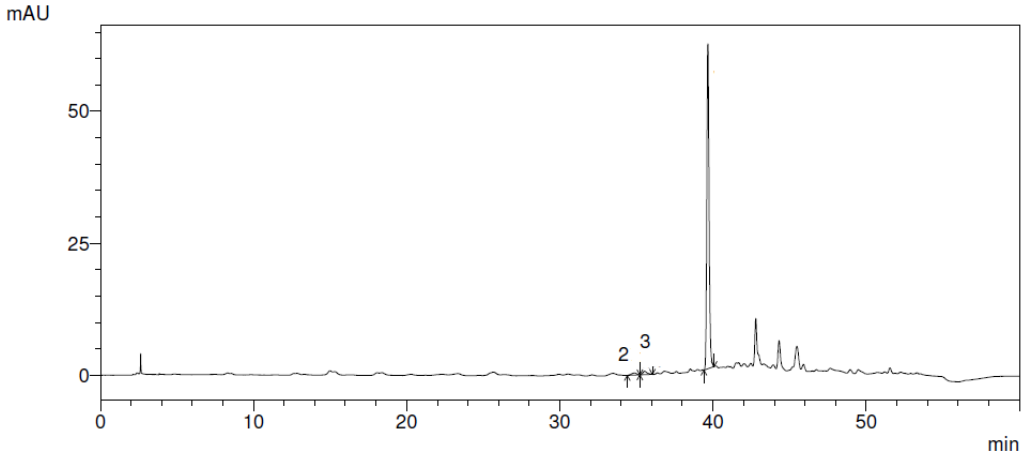


Figure D.9. HPLC chromatogram of nettle for ultrasound extraction at 330 nm

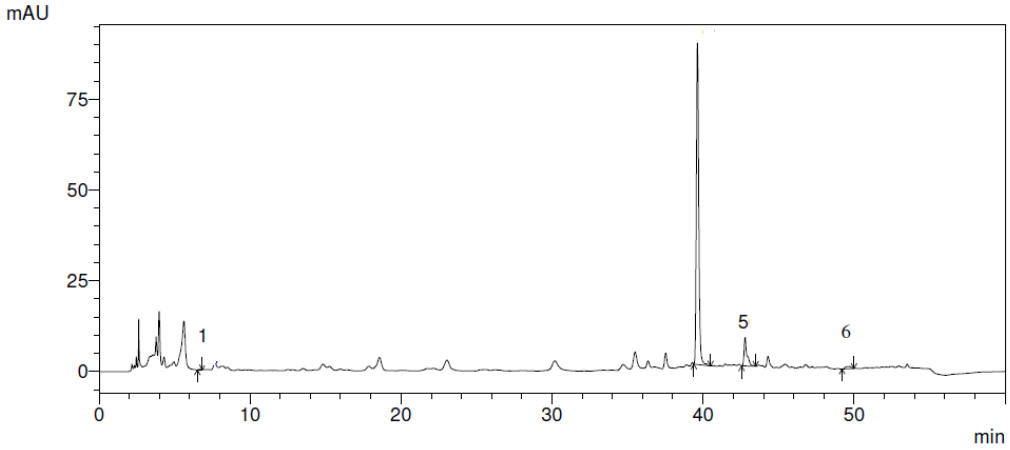


Figure D.10. HPLC chromatogram of nettle for maceration at 280 nm

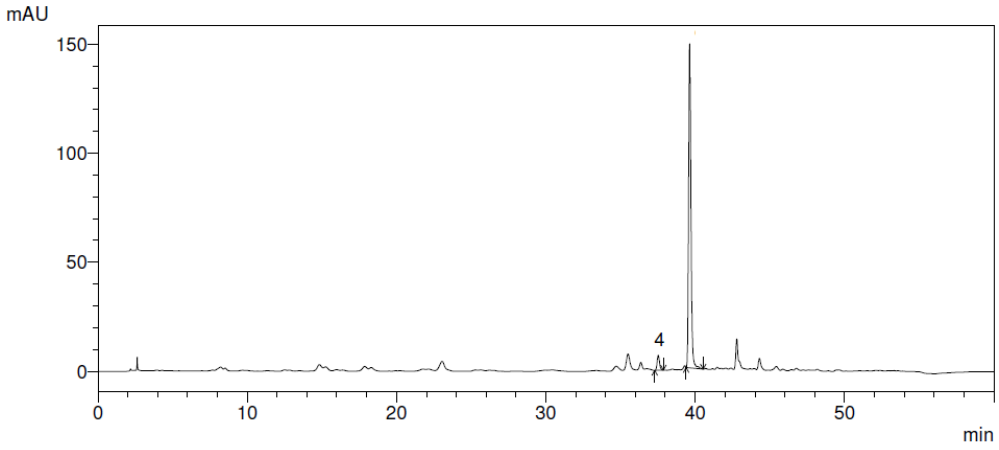


Figure D.11. HPLC chromatogram of nettle for maceration at 310 nm

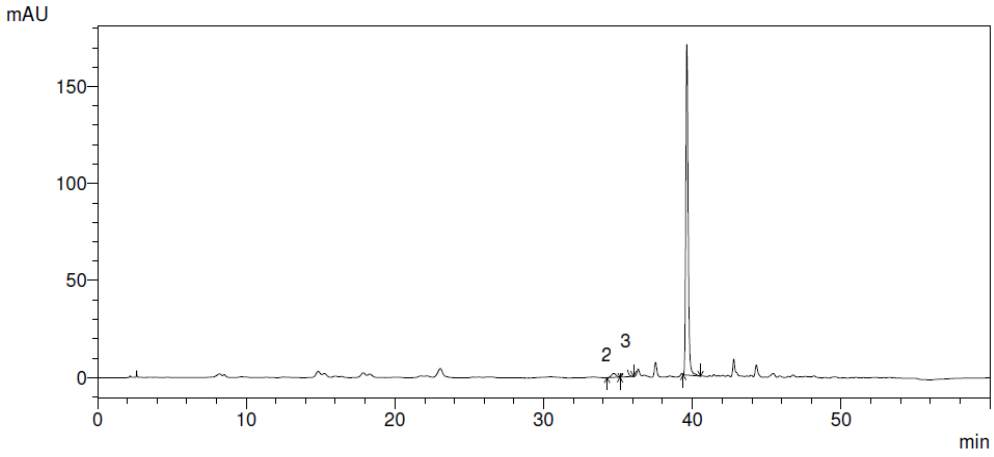


Figure D.12. HPLC chromatogram of nettle for maceration at 330 nm

D.2. Melissa extracts

1	Catechin
2	Caffeic acid
3	Vanillic acid
4	Syringic acid
5	p-Coumaric acid
6	Naringenin
7	Trans-3-hydroxycinnamic acid
8	Naringin
9	Rosmarinic acid
10	Hydrocinnamic acid
11	Hesperetin

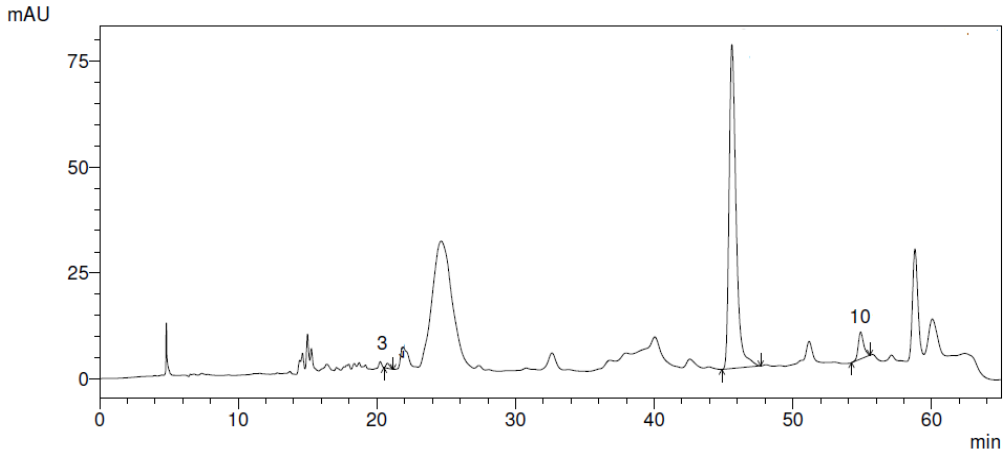


Figure D.13. HPLC chromatogram of melissa for microwave extraction at 260 nm

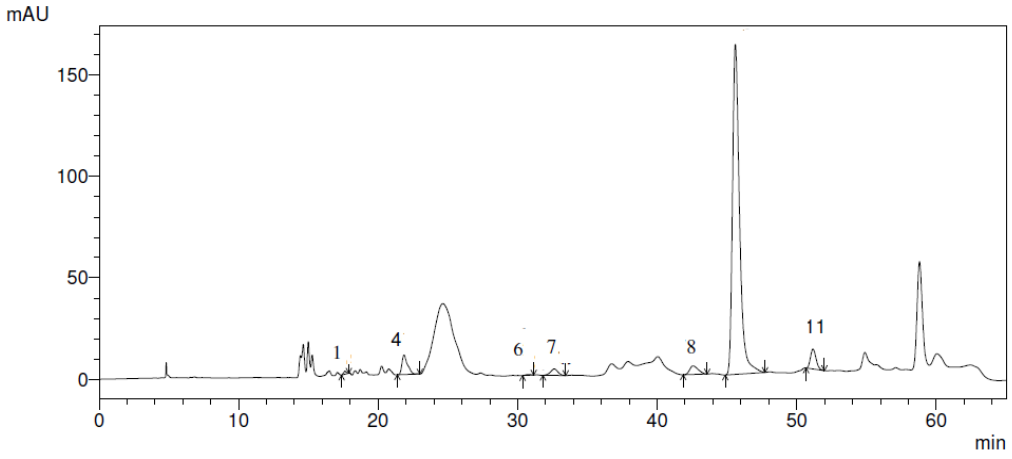


Figure D.14. HPLC chromatogram of melissa for microwave extraction at 280 nm

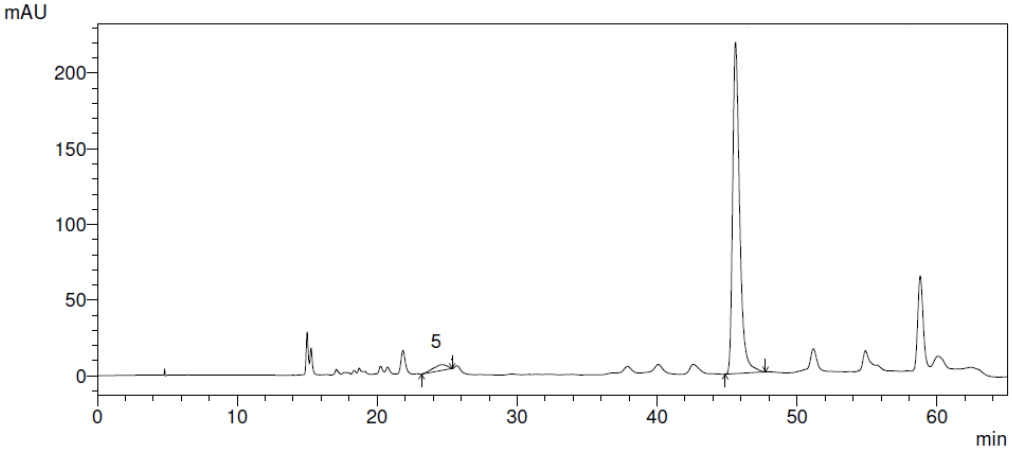


Figure D.15. HPLC chromatogram of melissa for microwave extraction at 310 nm

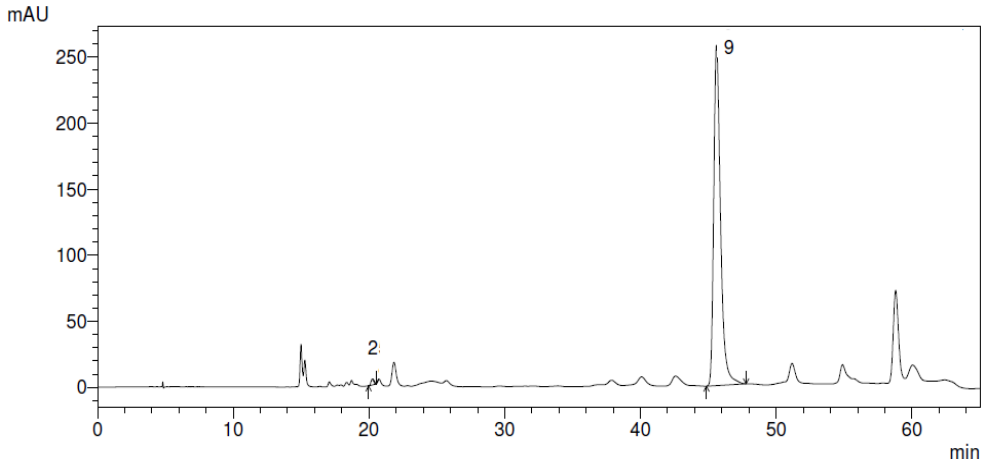


Figure D.16. HPLC chromatogram of melissa for microwave extraction at 330 nm

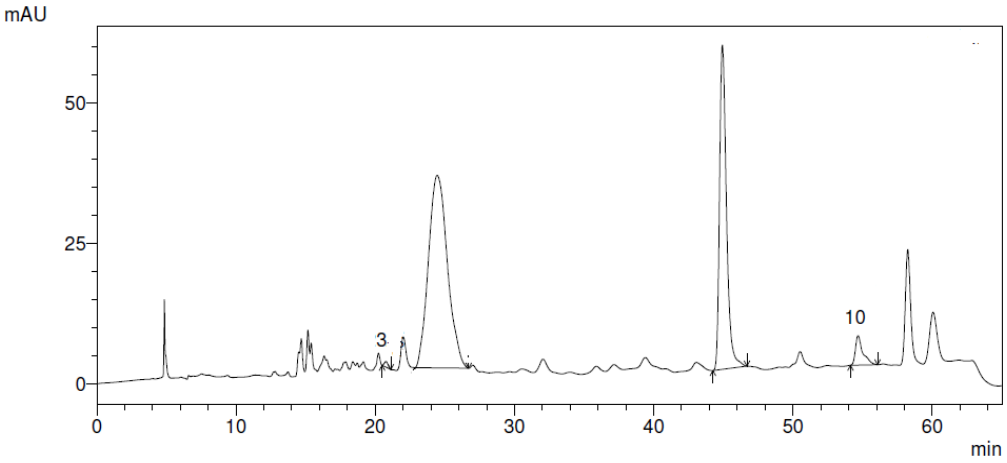


Figure D.17. HPLC chromatogram of melissa for conventional extraction at 260 nm

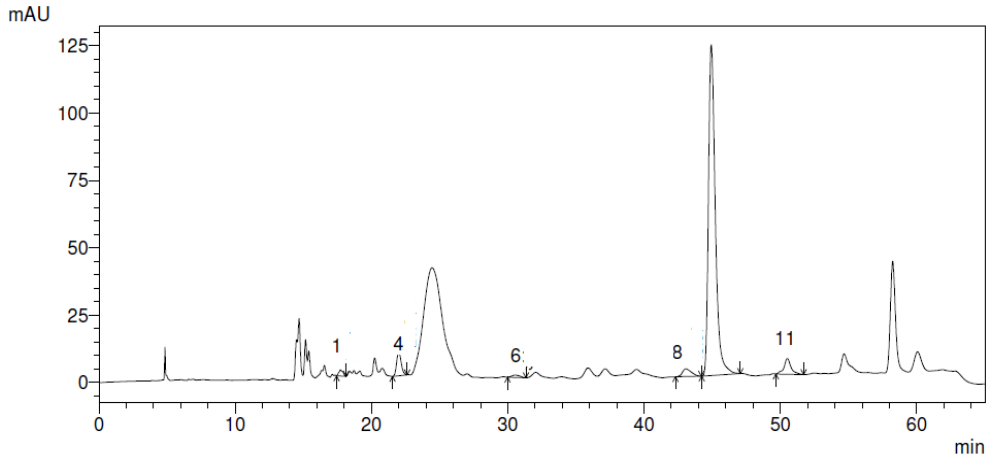


Figure D.18. HPLC chromatogram of melissa for conventional extraction at 280 nm

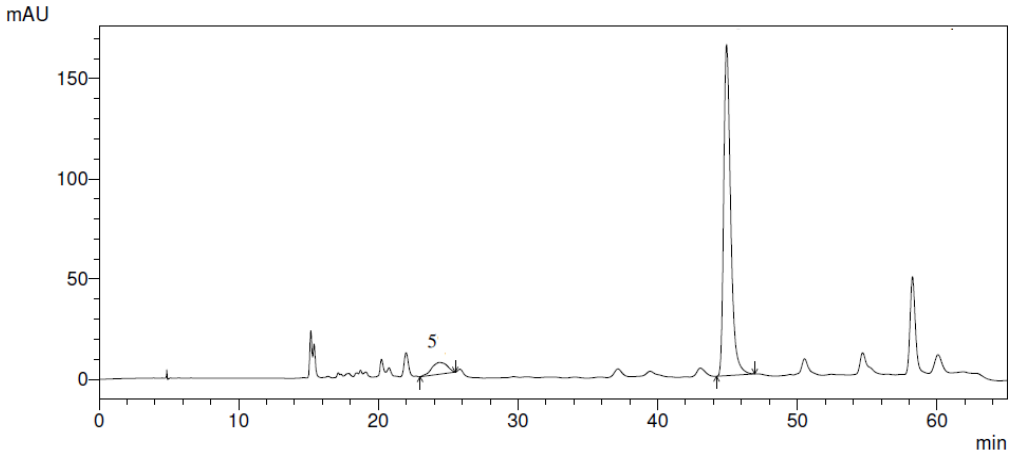


Figure D.19. HPLC chromatogram of melissa for conventional extraction at 310 nm

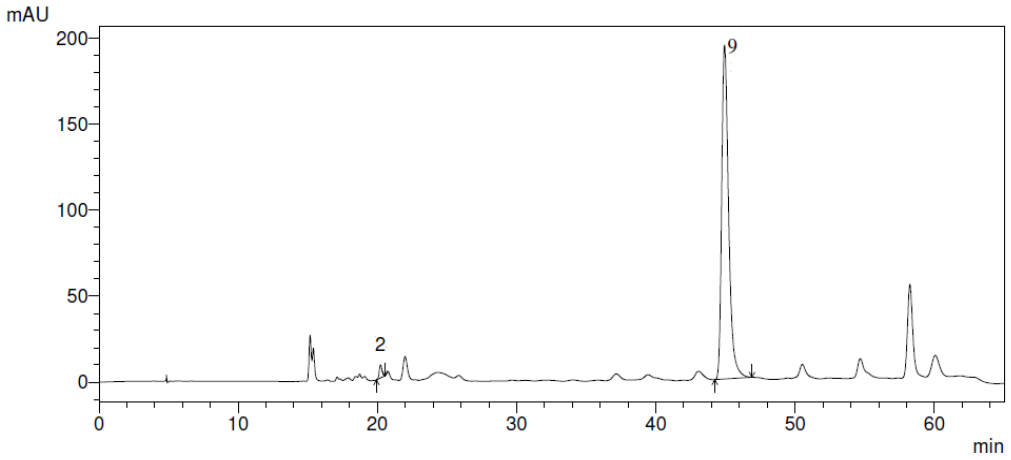


Figure D.20. HPLC chromatogram of melissa for conventional extraction at 330 nm

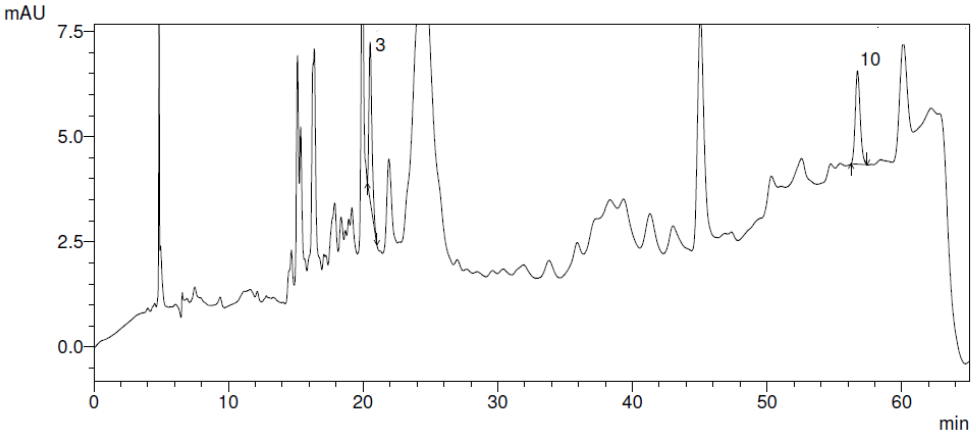


Figure D.21. HPLC chromatogram of melissa for ultrasound extraction at 260 nm

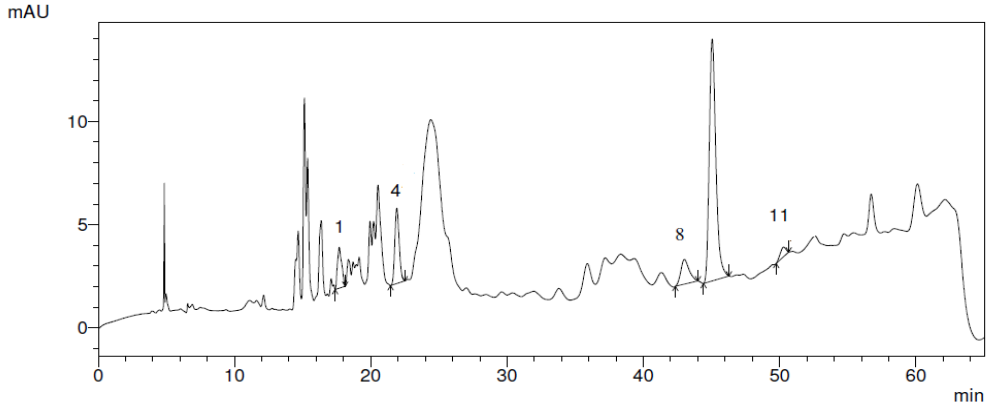


Figure D.22. HPLC chromatogram of melissa for ultrasound extraction at 280 nm

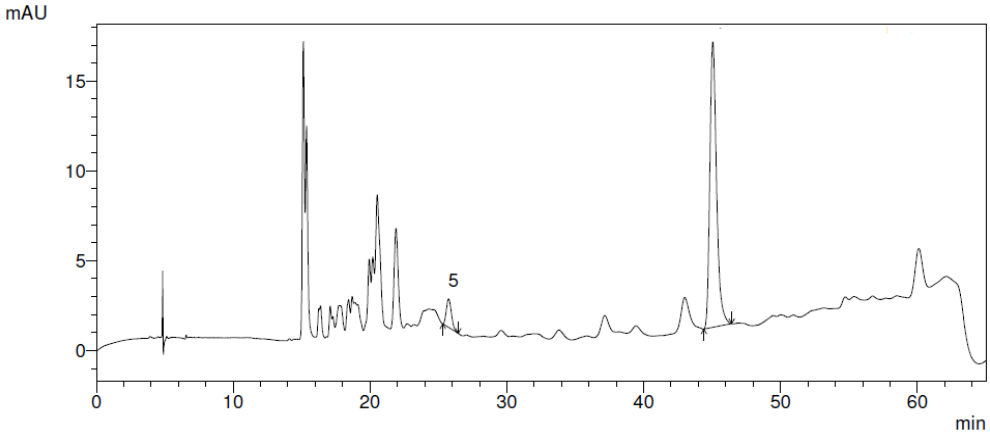


Figure D.23. HPLC chromatogram of melissa for ultrasound extraction at 310 nm

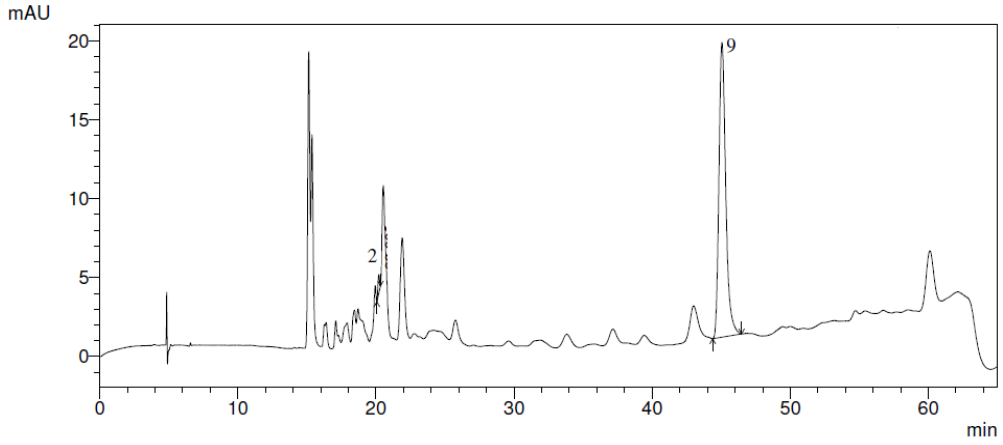


Figure D.24. HPLC chromatogram of melissa for ultrasound extraction at 330 nm

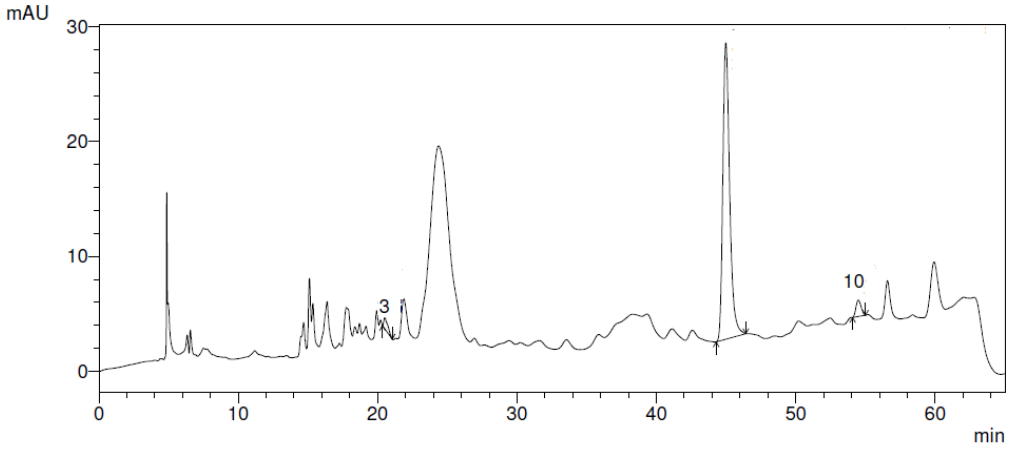


Figure D.25. HPLC chromatogram of melissa for maceration at 260 nm

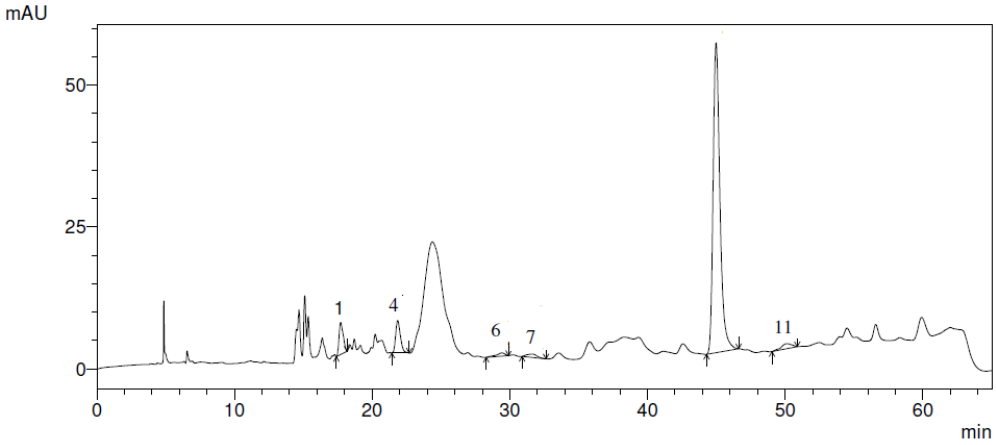


Figure D.26. HPLC chromatogram of melissa for maceration at 280 nm

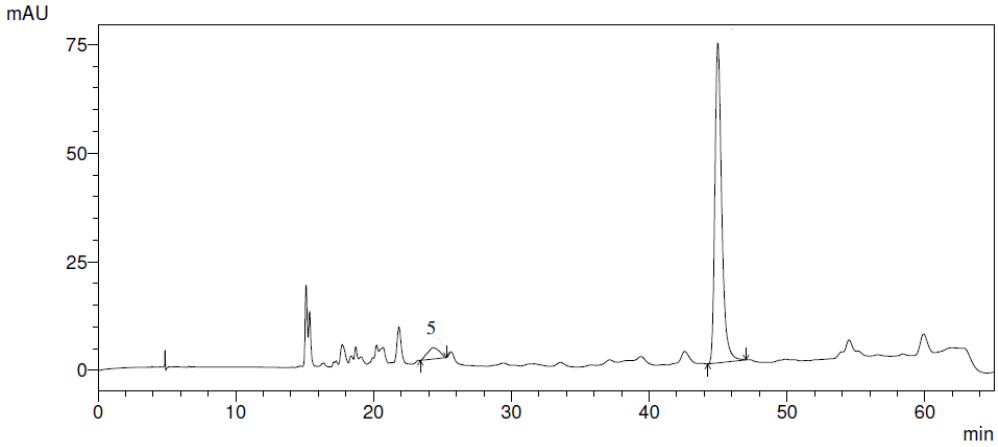


Figure D.27. HPLC chromatogram of melissa for maceration at 310 nm

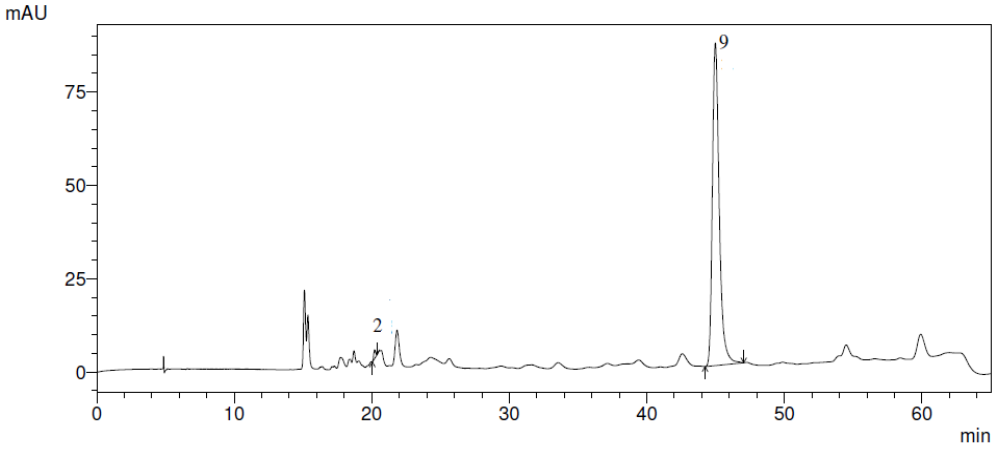


Figure D.28. HPLC chromatogram of melissa for maceration at 330 nm

APPENDIX E

STATISTICAL ANALYSIS RESULTS

Table E.1. Microwave extracts of nettle

X1 solid to solvent ratio (1, 1:10; 2, 1:20; 3, 1:30)

X2 time (1, 5 min; 2, 10 min; 3, 15 min; 4, 20 min)

The SAS System

The GLM Procedure

Class Level Information

Class	Levels	Values
X1	3	1 2 3
X2	4	1 2 3 4

Number of Observations Read	24
Number of Observations Used	24

Dependent Variable: Y

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	413.0970833	82.6194167	55.99	<.0001
Error	18	26.5591667	1.4755093		
Corrected Total	23	439.6562500			

R-Square	Coeff Var	Root MSE	Y Mean
0.939591	5.692820	1.214705	21.33750

Source	DF	Type I SS	Mean Square	F Value	Pr > F
X1	2	402.8725000	201.4362500	136.52	<.0001
X2	3	10.2245833	3.4081944	2.31	0.1108

Source	DF	Type III SS	Mean Square	F Value	Pr > F
X1	2	402.8725000	201.4362500	136.52	<.0001
X2	3	10.2245833	3.4081944	2.31	0.1108

Duncan's Multiple Range Test for Y

Alpha	0.05	
Error Degrees of Freedom	18	
Error Mean Square	1.475509	
Number of Means	2	3
Critical Range	1.276	1.339

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	X1
A	25.2750	8	3
B	23.0500	8	2
C	15.6875	8	1

Duncan's Multiple Range Test for Y

Alpha	0.05
Error Degrees of Freedom	18
Error Mean Square	1.475509

Number of Means	2	3	4
Critical Range	1.473	1.546	1.592

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	X2
A	22.1500	6	4
B A	21.7167	6	3
B A	21.0500	6	2
B	20.4333	6	1

Table E.2. Conventional extracts of nettle for determination of time

X1 time (1, 15 min; 2, 20 min; 3, 30 min; 4, 1h; 5, 4 h; 6, 6 h; 7, 8 h; 8, 10 h)

Class Level Information

Class	Levels	Values
X1	8	1 2 3 4 5 6 7 8

Number of Observations Read	16
Number of Observations Used	16

Dependent Variable: Y

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	153.0293750	21.8613393	33.60	<.0001
Error	8	5.2050000	0.6506250		
Corrected Total	15	158.2343750			

R-Square	Coeff Var	Root MSE	Y Mean
0.967106	4.164509	0.806613	19.36875

Source	DF	Type I SS	Mean Square	F Value	Pr > F
X1	7	153.0293750	21.8613393	33.60	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
X1	7	153.0293750	21.8613393	33.60	<.0001

Duncan's Multiple Range Test for Y

Alpha	0.05
Error Degrees of Freedom	8
Error Mean Square	0.650625

Number of Means	2	3	4	5	6	7	8
Critical Range	1.860	1.938	1.982	2.008	2.024	2.034	2.039

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	X1
A	22.2500	2	4
B A	21.5500	2	3
B A	21.3000	2	5
B A C	20.4000	2	6
B C	19.8000	2	7
C	19.1500	2	8
C	18.7500	2	2
D	11.7500	2	1

Table E.3. Conventional extracts of nettle for determination of solid to solvent ratio

X1 solid to solvent ratio (1, 1:10; 2, 1:20; 3, 1:30)

Class Level Information

Class	Levels	Values
X1	3	1 2 3

Number of Observations Read	6
Number of Observations Used	6

Dependent Variable: Y

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	33.42333333	16.71166667	44.37	0.0059
Error	3	1.13000000	0.37666667		
Corrected Total	5	34.55333333			

R-Square	Coeff Var	Root MSE	Y Mean
0.967297	2.660687	0.613732	23.06667

Source	DF	Type I SS	Mean Square	F Value	Pr > F
X1	2	33.42333333	16.71166667	44.37	0.0059

Source	DF	Type III SS	Mean Square	F Value	Pr > F
X1	2	33.42333333	16.71166667	44.37	0.0059

Duncan's Multiple Range Test for Y

Alpha	0.05	
Error Degrees of Freedom	3	
Error Mean Square	0.376667	
Number of Means	2	3
Critical Range	1.953	1.960

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	X1
A	26.4000	2	3
B	21.5500	2	2
B	21.2500	2	1

Table E.4. Ultrasound extracts of nettle

X1 power (1, 50%; 2, 80%)

X2 solid to solvent ratio (1, 1:10; 2, 1:20; 3, 1:30)

X3 time (1, 5 min; 2, 10 min; 3, 20 min; 4, 30 min)

Class Level Information

Class	Levels	Values
X1	2	1 2
X2	3	1 2 3
X3	4	1 2 3 4

Number of Observations Read	48
Number of Observations Used	48

Dependent Variable: Y

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	464.0329167	77.3388194	57.56	<.0001
Error	41	55.0918750	1.3437043		
Corrected Total	47	519.1247917			

R-Square	Coeff Var	Root MSE	Y Mean
0.893875	6.909321	1.159183	16.77708

Source	DF	Type I SS	Mean Square	F Value	Pr > F
X1	1	57.4218750	57.4218750	42.73	<.0001
X2	2	270.3804167	135.1902083	100.61	<.0001
X3	3	136.2306250	45.4102083	33.79	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
X1	1	57.4218750	57.4218750	42.73	<.0001
X2	2	270.3804167	135.1902083	100.61	<.0001
X3	3	136.2306250	45.4102083	33.79	<.0001

Duncan's Multiple Range Test for Y

Alpha	0.05
Error Degrees of Freedom	41
Error Mean Square	1.343704

Number of Means	2
Critical Range	.6758

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	X1
A	17.8708	24	2
B	15.6833	24	1

Duncan's Multiple Range Test for Y

Alpha	0.05
Error Degrees of Freedom	41
Error Mean Square	1.343704

Number of Means	2	3
Critical Range	.8277	.8703

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	X2
A	19.5625	16	3
B	17.0063	16	2
C	13.7625	16	1

Duncan's Multiple Range Test for Y

Alpha	0.05
Error Degrees of Freedom	41
Error Mean Square	1.343704

Number of Means	2	3	4
Critical Range	0.956	1.005	1.037

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	X3
A	18.7917	12	4
B	17.6417	12	3
C	16.4333	12	2
D	14.2417	12	1

Table E.5. Maceration extracts of nettle

X1 solid to solvent ratio (1, 1:10; 2, 1:20; 3, 1:30)

Class Level Information

Class	Levels	Values
X1	3	1 2 3

Number of Observations Read	6
Number of Observations Used	6

Dependent Variable: Y

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	74.26333333	37.13166667	30.69	0.0101
Error	3	3.63000000	1.21000000		
Corrected Total	5	77.89333333			

R-Square	Coeff Var	Root MSE	Y Mean
0.953398	5.296950	1.100000	20.76667

Source	DF	Type I SS	Mean Square	F Value	Pr > F
X1	2	74.26333333	37.13166667	30.69	0.0101

Source	DF	Type III SS	Mean Square	F Value	Pr > F
X1	2	74.26333333	37.13166667	30.69	0.0101

Duncan's Multiple Range Test for Y

Alpha	0.05	
Error Degrees of Freedom	3	
Error Mean Square	1.21	
Number of Means	2	3
Critical Range	3.501	3.512

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	X1
A	25.650	2	3
B	19.150	2	2
B	17.500	2	1

Table E.6. Comparison of optimum results of four methods for nettle extracts

X1 method (1, microwave extraction; 2, conventional extraction; 3, ultrasound extraction; 4, maceration)

Class Level Information

Class	Levels	Values
X1	4	1 2 3 4

Number of Observations Read	8
Number of Observations Used	8

Dependent Variable: Y

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	7.50375000	2.50125000	1.00	0.4807
Error	4	10.05500000	2.51375000		
Corrected Total	7	17.55875000			

R-Square	Coeff Var	Root MSE	Y Mean
0.427351	6.307234	1.585481	25.13750

Source	DF	Type I SS	Mean Square	F Value	Pr > F
X1	3	7.50375000	2.50125000	1.00	0.4807

Source	DF	Type III SS	Mean Square	F Value	Pr > F
X1	3	7.50375000	2.50125000	1.00	0.4807

Duncan's Multiple Range Test for Y

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	2.51375

Number of Means	2	3	4
Critical Range	4.402	4.498	4.521

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	X1
A	26.400	2	2
A	25.650	2	4
A	24.650	2	1
A	23.850	2	3

Table E.7. Microwave extracts of melissa

X1 solid to solvent ratio (1, 1:10; 2, 1:20; 3, 1:30)

X2 time (1, 5 min; 2, 10 min; 3, 15 min; 4, 20 min)

Class Level Information

Class	Levels	Values
X1	3	1 2 3
X2	4	1 2 3 4

Number of Observations Read	24
Number of Observations Used	24

Dependent Variable: Y

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	5144.709167	1028.941833	30.14	<.0001
Error	18	614.435833	34.135324		
Corrected Total	23	5759.145000			

R-Square	Coeff Var	Root MSE	Y Mean
0.893311	4.712679	5.842544	123.9750

Source	DF	Type I SS	Mean Square	F Value	Pr > F
X1	2	4953.157500	2476.578750	72.55	<.0001
X2	3	191.551667	63.850556	1.87	0.1708

Source	DF	Type III SS	Mean Square	F Value	Pr > F
X1	2	4953.157500	2476.578750	72.55	<.0001
X2	3	191.551667	63.850556	1.87	0.1708

Duncan's Multiple Range Test for Y

Alpha	0.05
Error Degrees of Freedom	18
Error Mean Square	34.13532

Number of Means	2	3
Critical Range	6.137	6.439

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	X2
A	144.250	8	3
B	114.963	8	2
B	112.713	8	1

Duncan's Multiple Range Test for Y

Alpha	0.05
Error Degrees of Freedom	18
Error Mean Square	34.13532

Number of Means	2	3	4
Critical Range	7.087	7.436	7.656

Means with the same letter are not significantly different.

Duncan Grouping		Mean	N	X1
A		127.933	6	1
B	A	124.833	6	3
B	A	122.967	6	2
B		120.167	6	4

Table E.8. Conventional extracts of melissa for determination of time

X1 time (1, 30 min; 2,1 h; 3, 2 h; 4, 4 h)

Class Level Information

Class	Levels	Values
X1	4	1 2 3 4

Number of Observations Read	8
Number of Observations Used	8

Dependent Variable: Y

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	222.8400000	74.2800000	1.13	0.4371
Error	4	262.9800000	65.7450000		
Corrected Total	7	485.8200000			

R-Square	Coeff Var	Root MSE	Y Mean
0.458688	7.128201	8.108329	113.7500

Source	DF	Type I SS	Mean Square	F Value	Pr > F
X1	3	222.8400000	74.2800000	1.13	0.4371

Source	DF	Type III SS	Mean Square	F Value	Pr > F
X1	3	222.8400000	74.2800000	1.13	0.4371

Duncan's Multiple Range Test for Y

Alpha	0.05		
Error Degrees of Freedom	4		
Error Mean Square	65.745		

Number of Means	2	3	4
Critical Range	22.51	23.00	23.12

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	X1
A	122.450	2	2
A	112.850	2	4
A	111.350	2	1
A	108.350	2	3

Table E.9. Conventional extracts of melissa for determination of solid to solvent ratio

X1 solid to solvent ratio (1, 1:10; 2, 1:20; 3, 1:30)

Class Level Information

Class	Levels	Values
X1	3	1 2 3

Number of Observations Read	6
Number of Observations Used	6

Dependent Variable: Y

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	1081.290000	540.645000	10.48	0.0443
Error	3	154.770000	51.590000		
Corrected Total	5	1236.060000			

R-Square	Coeff Var	Root MSE	Y Mean
0.874788	6.763294	7.182618	106.2000

Source	DF	Type I SS	Mean Square	F Value	Pr > F
X1	2	1081.290000	540.645000	10.48	0.0443

Source	DF	Type III SS	Mean Square	F Value	Pr > F
X1	2	1081.290000	540.645000	10.48	0.0443

Duncan's Multiple Range Test for Y

Alpha	0.05	
Error Degrees of Freedom	3	
Error Mean Square	51.59	
Number of Means	2	3
Critical Range	22.86	22.93

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	X1
A	119.450	2	3
A	111.350	2	2
B	87.800	2	1

Table E.10. Ultrasound extracts of melissa

X1 power (1, 50%; 2, 80%)

X2 solid to solvent ratio (1, 1:10; 2, 1:20; 3, 1:30)

X3 time (1, 5 min; 2, 10 min; 3, 20 min; 4, 30 min)

Class Level Information

Class	Levels	Values
X1	2	1 2
X2	3	1 2 3
X3	4	1 2 3 4

Number of Observations Read	48
Number of Observations Used	48

Dependent Variable: Y

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	5182.190833	863.698472	29.02	<.0001
Error	41	1220.165833	29.760142		
Corrected Total	47	6402.356667			

R-Square	Coeff Var	Root MSE	Y Mean
0.809419	6.213899	5.455286	87.79167

Source	DF	Type I SS	Mean Square	F Value	Pr > F
X1	1	210.840833	210.840833	7.08	0.0111
X2	2	1988.571667	994.285833	33.41	<.0001
X3	3	2982.778333	994.259444	33.41	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
X1	1	210.840833	210.840833	7.08	0.0111
X2	2	1988.571667	994.285833	33.41	<.0001
X3	3	2982.778333	994.259444	33.41	<.0001

Duncan's Multiple Range Test for Y

Alpha	0.05
Error Degrees of Freedom	41
Error Mean Square	29.76014

Number of Means	2
Critical Range	3.180

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	X1
A	89.888	24	1
B	85.696	24	2

Duncan's Multiple Range Test for Y

Alpha	0.05
Error Degrees of Freedom	41
Error Mean Square	29.76014

Number of Means	2	3
Critical Range	3.895	4.096

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	X2
A	96.875	16	3
B	83.763	16	1
B	82.738	16	2

Duncan's Multiple Range Test for Y

Alpha	0.05		
Error Degrees of Freedom	41		
Error Mean Square	29.76014		

Number of Means	2	3	4
Critical Range	4.498	4.729	4.881

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	X3
A	96.200	12	4
A	93.208	12	3
B	86.025	12	2
C	75.733	12	1

Table E.11. Maceration extracts of melissa

X1 solid to solvent ratio (1, 1:10; 2, 1:20; 3, 1:30)

Class Level Information

Class	Levels	Values
X1	3	1 2 3

Number of Observations Read	6
Number of Observations Used	6

Dependent Variable: Y

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	1059.343333	529.671667	33.63	0.0088
Error	3	47.250000	15.750000		
Corrected	5	1106.593333			

R-Square	Coeff Var	Root MSE	Y Mean
0.957301	4.885466	3.968627	81.23333

Source	DF	Type I SS	Mean Square	F Value	Pr > F
X1	2	1059.343333	529.671667	33.63	0.0088

Source	DF	Type III SS	Mean Square	F Value	Pr > F
X1	2	1059.343333	529.671667	33.63	0.0088

Duncan's Multiple Range Test for Y

Alpha	0.05	
Error Degrees of Freedom	3	
Error Mean Square	15.75	
Number of Means	2	3
Critical Range	12.63	12.67

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	X1
A	91.100	2	2
A	90.150	2	3
B	62.450	2	1

Table E.12. Comparison of four methods for melissa extracts

X1 method (1, microwave extraction; 2, conventional extraction; 3, ultrasound extraction; 4, maceration)

Class Level Information

Class	Levels	Values
X1	4	1 2 3 4

Number of Observations Read	8
Number of Observations Used	8

Dependent Variable: Y

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	3233.790000	1077.930000	16.27	0.0105
Error	4	264.930000	66.232500		
Corrected Total	7	3498.720000			

R-Square	Coeff Var	Root MSE	Y Mean
0.924278	7.173500	8.138335	113.4500

Source	DF	Type I SS	Mean Square	F Value	Pr > F
X1	3	3233.790000	1077.930000	16.27	0.0105

Source	DF	Type III SS	Mean Square	F Value	Pr > F
X1	3	3233.790000	1077.930000	16.27	0.0105

Duncan's Multiple Range Test for Y

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	66.2325

Number of Means	2	3	4
Critical Range	22.60	23.09	23.21

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	X1
A	145.850	2	1
B	111.350	2	2
B	105.500	2	3
B	91.100	2	4

Table E.13. Comparison of antioxidant activities of four methods for nettle extracts

X1 method (1, microwave extraction; 2, conventional extraction; 3, ultrasound extraction; 4, maceration)

Class Level Information

Class	Levels	Values
X1	4	1 2 3 4

Number of Observations Read	8
Number of Observations Used	8

Dependent Variable: Y

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	2.58083750	0.86027917	2548.98	<.0001
Error	4	0.00135000	0.00033750		
Corrected Total	7	2.58218750			

R-Square	Coeff Var	Root MSE	Y Mean
0.999477	0.475475	0.018371	3.863750

Source	DF	Type I SS	Mean Square	F Value	Pr > F
X1	3	2.58083750	0.86027917	2548.98	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
X1	3	2.58083750	0.86027917	2548.98	<.0001

Duncan's Multiple Range Test for Y

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	0.000337

Number of Means	2	3	4
Critical Range	.05101	.05212	.05239

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	X1
A	4.48000	2	4
B	4.15500	2	1
C	3.86500	2	2
D	2.95500	2	3

Table E.14. Comparison of antioxidant activities of four methods for melissa extracts

X1 method (1, microwave extraction; 2, conventional extraction; 3, ultrasound extraction; 4, maceration)

Class Level Information

Class	Levels	Values
X1	4	1 2 3 4

Number of Observations Read	8
Number of Observations Used	8

Dependent Variable: Y

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	98.47270000	32.82423333	1146.70	<.0001
Error	4	0.11450000	0.02862500		
Corrected Total	7	98.58720000			

R-Square	Coeff Var	Root MSE	Y Mean
0.998839	0.621220	0.169189	27.23500

Source	DF	Type I SS	Mean Square	F Value	Pr > F
X1	3	98.47270000	32.82423333	1146.70	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
X1	3	98.47270000	32.82423333	1146.70	<.0001

Duncan's Multiple Range Test for Y

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	0.028625

Number of Means	2	3	4
Critical Range	.4697	.4800	.4825

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	X1
A	30.6400	2	1
A	30.5800	2	2
B	25.2150	2	4
C	22.5050	2	3

APPENDIX F

GC CHROMATOGRAMS

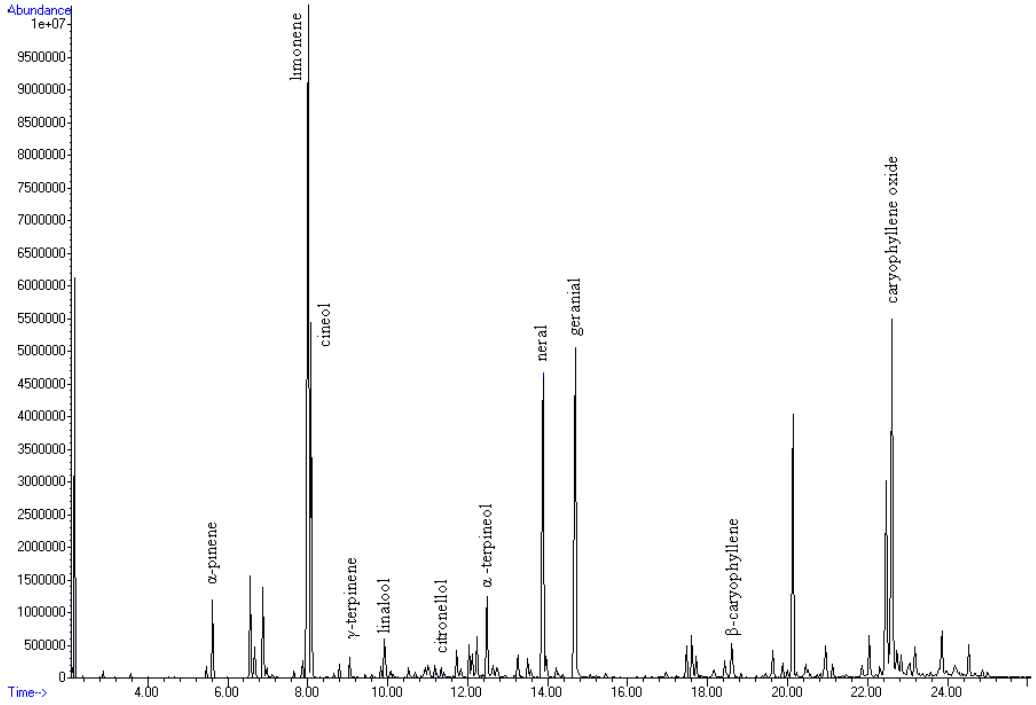


Figure F.1. GC Chromatogram of essential oil of melissa obtained by hydrodistillation

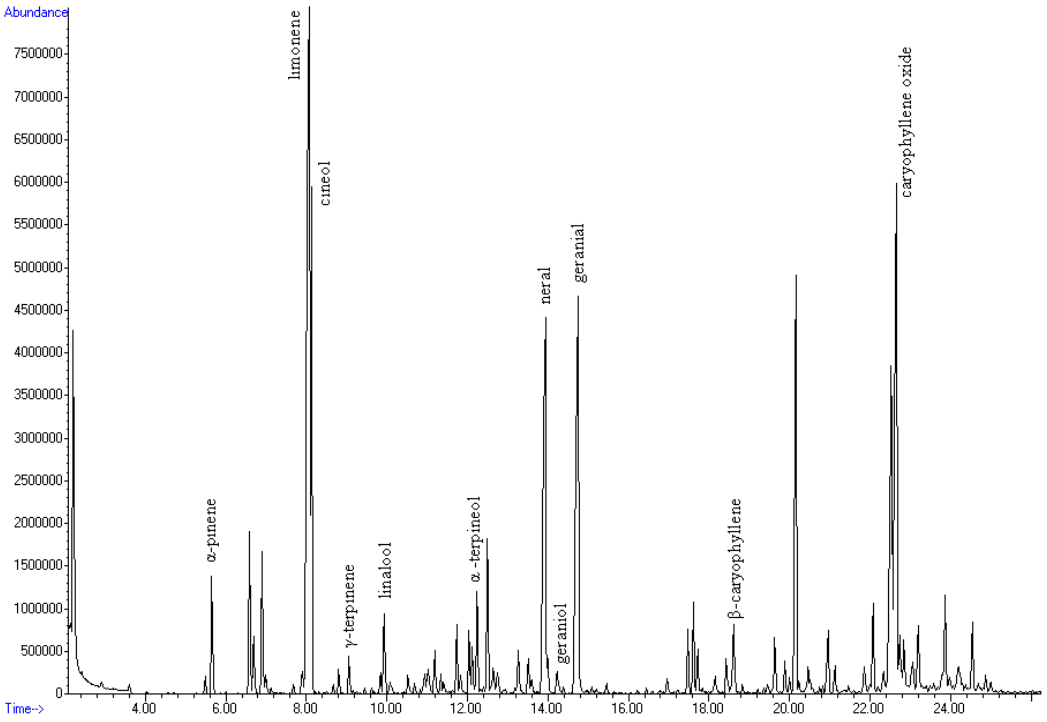


Figure F.2. GC Chromatogram of essential oil of melissa obtained by microwave extraction

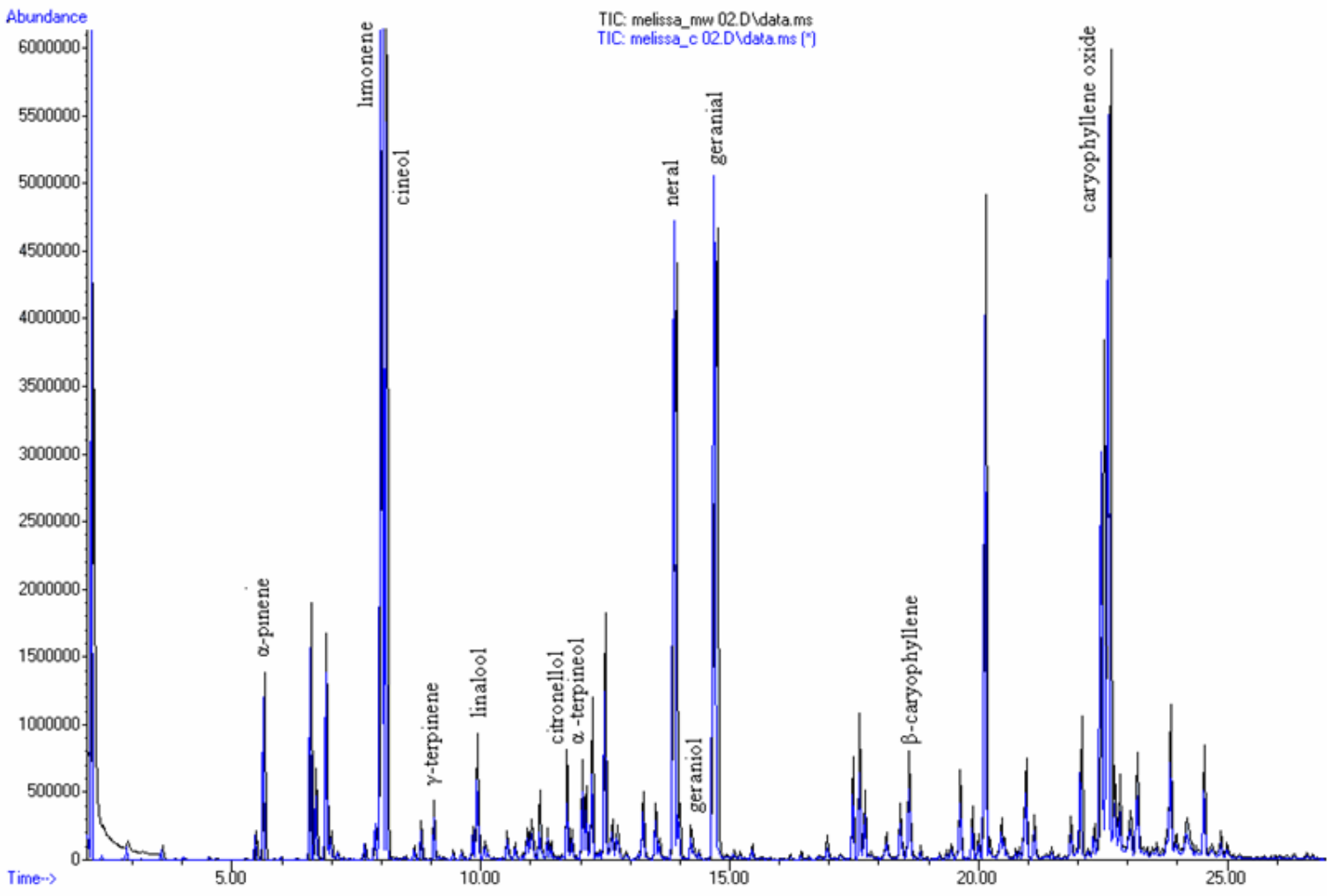


Figure F.3. Overlaying of hydrodistillation and microwave extraction GC chromatograms