DETERMINATION OF PHENOLIC COMPOUND PROFILES AND ANTIOXIDANT EFFECT OF BLACK ELDERBERRY, ECHINACEA AND ICELAND MOSS EXTRACTS ON LATE-RELEASE SOFT LOZENGE

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DETERMINATION OF PHENOLIC COMPOUND PROFILES AND ANTIOXIDANT EFFECT OF BLACK ELDERBERRY, ECHINACEA AND ICELAND MOSS EXTRACTS ON LATE-RELEASE SOFT LOZENGE

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ABSTRACT

DETERMINATION OF PHENOLIC COMPOUND PROFILES AND ANTIOXIDANT EFFECT OF BLACK ELDERBERRY, ECHINACEA AND ICELAND MOSS EXTRACTS ON LATE-RELEASE SOFT LOZENGE

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Medicinal plants contain different bioactive compounds that have immune system stimulant, anti-inflammatory, antibacterial, antiviral, antifungal, anticancer and wound healing effects. *Echinacea purpurea* L., *Sambucus nigra* L. and *Cetraria islandica* L. are known with their high antioxidant content and health promoting properties. The main objective of the study was to determine the total phenolic content, total flavonoid content and antioxidant capacity of each plant extract with water and 75 % ethanol as solvents.

The extraction efficiency was significantly higher in ethanol extracts according to total phenolic content, total flavonoid content, 1-diphenyl-2-picryl-hydrazyl (DPPH) and copper reducing antioxidant capacity (CUPRAC) analysis ($p<0.05$). Among all extracts, the highest result of radical scavenging capacity was found in ethanol extract of *Sambucus nigra* L. as 901.62±24.53 mg TEAC/ g dry extract. Total flavonoid and total phenolic contents of the same extract were determined as

840.54±13.46 mg RE/ g dry extract and 339.68±1.47 mg GAE/ g dry extract, respectively.

The selected phenolic compounds were quantified and phenolic profiles were determined by HPLC analysis for all extracts. By using the selected plant powders, the late-release soft lozenge product was formulated. Loss of bioactive compounds was examined by using spectrophotometric and chromatographic techniques. In lozenge product, the highest phenolic content was obtained with *Sambucus nigra* L. (2.33±0.11 mg GAE/ g dry extract. Results confirmed that the lozenge application caused a significant loss in antioxidant amount for all plant extracts. In order to obtain optimum antioxidant effectiveness from the lozenge products with functional properties, it is needed to provide a suitable recipe with optimized usage of plant extracts.

Keywords: Phenolic Content, Antioxidant Capacity, Medicinal Plant, Soft Lozenge

ÖZ

KARA MÜRVER, EKİNEZYA VE İZLANDA YOSUNU EKSTRAKTLARININ FENOLİK MADDE PROFİLLERİNİN BELİRLENEREK GEÇ SALINIMLI YUMUŞAK PASTİL ÜRÜNÜNDE ANTİOKSİDAN ETKİLERİNİN BELİRLENMESİ

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Tıbbi bitkiler, bağışıklık sistemi uyarıcı, antienflamatuar, antibakteriyel, antiviral, antifungal, antikanser ve yara iyileştirici etkileri olan farklı biyoaktif bileşikler içerirler. *Echinacea purpurea* L., *Sambucus nigra* L. ve *Cetraria islandica* L., yüksek antioksidan içeriği ve sağlığa katkı sağlayıcı özellikleri ile bilinir. Çalışmanın temel amacı, her bir bitki ekstraktının farklı çözücüler (%75 etanol ve su) ile toplam fenolik içeriği, toplam flavonoid içeriği ve antioksidan kapasitesini belirlemektir.

Toplam fenolik içerik, toplam flavonoid içeriği, 1-difenil-2-pikril-hidrazil (DPPH) ve bakır indirgeyici antioksidan kapasitesi (CUPRAC) analizlerine göre, etanol ekstraktlarında ekstraksiyon verimi anlamlı olarak daha yüksek bulunmuştur ($p<0.05$). Tüm ekstraktlar arasında en yüksek radikal süpürücü etki sonucu, 901.62±24.53 mg TEAC/g ekstrakt (KM) olarak *Sambucus nigra* L. etanol ekstraktı için bulunmuştur. Aynı bitkinin toplam flavonoid ve toplam fenolik içerikleri, sırasıyla 840.54±13.46 mg RE/ g extract (KM) ve 339.68 ± 1.47 mg GAE/ g ekstrakt (KM) olarak en yüksek olarak belirlenmiştir.

Çalışmada, seçilen fenolik bileşiklerin niceliği belirlenerek tüm ekstraktlar için HPLC analizi ile bitkilerin fenolik profilleri çıkarılmıştır. Seçilen bitki tozları kullanılarak geç salınımlı yumuşak pastil ürünü formüle edilmiş ve biyoaktif bileşiklerdeki olası kayıplar hem spektrofotometrik hem de kromatografik yöntemlerle incelenmiştir. Pastil ürünü uygulamalarında en yüksek fenolik içerik *Sambucus nigra* L. (2.33±0.11 mg GAE/g ekstraktı (KM) ile elde edilmiştir. Sonuçlar, tüm bitki ekstraktlarının pastil uygulamalarında antioksidan miktarında ve etkilerinde önemli kayıplara neden olduğunu göstermiştir. Fonksiyonel özelliklere sahip yumuşak pastil ürününden optimum antioksidan etkisinin elde edilebilmesi için tüm bitkilerin kullanım miktarlarının optimize edilerek uygun bir reçete geliştirilmesi gerekmektedir.

Anahtar Kelimeler: Fenolik İçerik, Antioksidan Kapasitesi, Tıbbi Bitki, Yumuşak Pastil

To my beloved family…

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LIST OF ABBREVIATIONS

ABBREVIATIONS	
GA	Gum Arabic
SN	*Sambucus nigra* L. (elderberry) dry extract powder
EP	*Echinacea purpurea* L. dry extract powder
CI	*Cetraria islandica* L. dry extract powder
SNE	Ethanol extract of *Sambucus nigra* L.
EPE	Ethanol extract of *Echinacea purpurea* L.
CIE	Ethanol extract of *Cetraria islandica* L.
SNW	Water extract of *Sambucus nigra* L.
EPW	Water extract of *Echinacea purpurea* L.
CIW	Water extract of *Cetraria islandica* L.
SNP	Water extract of *Sambucus nigra* L. pastille application
EPP	Water extract of *Echinacea purpurea* L. pastille application
CIP	Water extract of *Cetraria islandica* L. pastille application
TPC	Total Phenolic Content
TFC	Total Flavonoid Content
TAC	Total Antioxidant Capacity

DPPH	1,1-diphenyl-2-picrylhydrazyl
CUPRAC	Copper Reducing Antioxidant Capacity
ABTS salt	2,2’ azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium
HPLC	High-performance liquid chromatography
RP HPLC	Reversed Phase High-performance liquid chromatography
GAE	Gallic acid equivalent
RE	Rutin equivalent
TEAC	Trolox equivalent antioxidant capacity
DW	Dry weight
FM	Fresh matter
FW	Fresh weight
R	Correlation coefficient

CHAPTER 1

INTRODUCTION

Healthcare products are moving away from disease-focused products such as pharmaceuticals, into wellness-promoting products including herbs and nutraceuticals (Aziz, Sarmidi, Kumaresan, Taher, & Foo, 2004). Due to the insufficient nutrition, the oxygen free radical production and the antioxidant production mechanisms in human body can become unbalanced. Antioxidants reduce the oxidative damage of human body and they are used as protective agents (Dawidowicz, Wianowska, & Baraniak, 2006). Many antioxidants are effective in neutralization and adsorption of free radicals, degrading peroxides by quenching singlet and triplet oxygen (Zheng & Wang, 2001). The phenolic compounds are specified with their reducing function on these free radicals which determine their antioxidant effectiveness.

Herbs are known as dietary plants and they contain high phenolic content and antioxidant capacity. Dietary plant phenolic substances are known with their health promoting role in human body and due to their functional properties, they are gaining the interest of consumers in a growing manner (Rauha et al, 2000). Many studies proceed on characterizing compounds in extracts of herbs, which are in numerous variety. Phenolic compounds are found as a diversified collection of secondary metabolites with their known existence in most of the fruits and vegetables. The phenolic contents of the medicinal plants are specified mostly as flavonoids. The

function of flavonoids is to prevent free radicals by inhibiting enzymes which are incorporated in the synthesis of free radicals (Stanković et al., 2016).

With the aim of replacing the synthetic additives with natural components rich in polyphenolics such as phenolic acids, flavonoids, tannins, *etc*.; researchers started to investigate nature. The medicinal plants which are collected from nature, extracted and applied in supplements to promote good health by preventing diseases rather than treating diseases (Agalar, 2019).

1.1 Phenolic Compounds

Antioxidants are identified as inhibiting or delaying the oxidation of lipids or other molecules. These agents inhibit the initiation or propagation of oxidizing chain reactions. Antioxidants are natural or synthetic and the natural antioxidants are more preferable due to their medicinal properties and efficiency in food applications. Plants as natural sources are also cheaper and safer (Stanković et al., 2016). Herbs are the best known source of antioxidants, due to their wide usage in medicine and nutrition.

The most important plant phenolic compounds are polyphenols which are classified as phenolic acids, flavonoids and tannins. The polyphenols are known with their redox properties in human metabolism, such as neutralizing free radicals, quenching singlet (or triplet) oxygen and peroxide decomposition (Agalar, 2019). Singleton and Rossi (1965) revealed that, several medicinal plants have more antioxidant capacity than ordinary fruits and vegetables, due to high amount of phenolic compounds found in their structure.

Phenolic compounds are the secondary metabolites in plants and their role is to protect the plant against viruses, bacteria, insects and other harmful outsource effects such as UV light (Embuscado, 2015). Phenolic compounds are classified due to phenol ring number and the binding components (Heleno, Martins, Queiroz, & Ferreira, 2015). The denomination is made based on a basic phenolic skeleton structure and the number of regulating carbon atoms attached (Robards, Prenzler, Tucker, Swatsitang, & Glover, 1999). The compounds with two or more hydroxyl group attached to benzene rings, are called polyphenols (Vermerris & Nicholson, 2008). When carbon structure of a phenolic compound is C_6-C_1, the related class is called phenolic acids. When the aromatic compound have 15 carbons, the structure is C_6-C_3-C_6 and they are called flavonoids (Figure 1.1) (Vermerris et al., 2008).

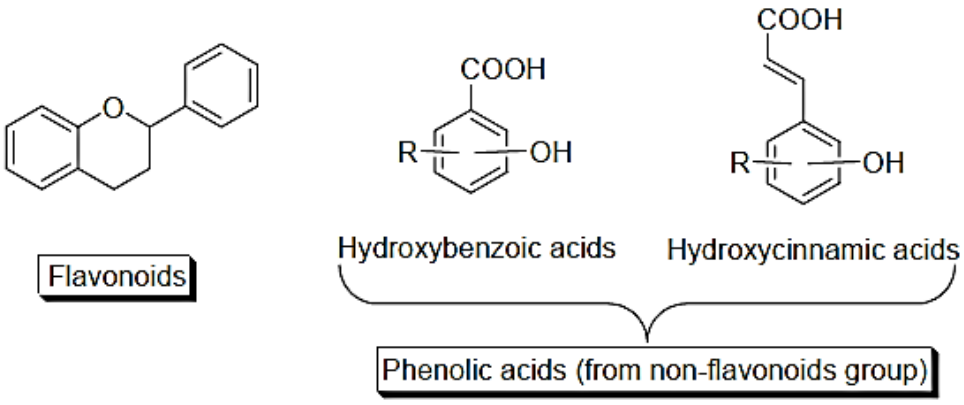

Figure 1.1 Chemical structures of flavonoids and phenolic acids (Vermerris et al., 2008).

The occurrence of the phenolic compounds in food material can be divided into three major groups. First group is made up of simple phenols and phenolic acids, second group is hydroxycinnamic acid derivatives and lastly the flavonoids (Ho, 1992). Major classes of phenolic compounds in plants can be specified as phenolic acids

(C_6-C_1), which are commonly symbolized by gallic, p-hydroxybenzoic, protocatechuic, vanillic and syringic acids (Lattanzio, 2013).

1.1.1 Phenolic acids

Phenolic acids are the subgroups of natural phenolic antioxidants (Shahidi & Ambigaipalan, 2015). They are mostly used in foods to increase the antioxidant properties. In herbal applications, their anti-inflammatory, antimicrobial, anti-allergenic and anti-viral properties are known (Veberic, Jakopic, Stampar, & Schmitzer, 2009; Tsai, Chiou, Chan, Sung, & Lin, 2012; Ercisli, Tosun, & Akbulut, 2009). The most known phenolic acid examples that are found in antioxidant and antimicrobial metabolisms are hydroxybenzoic acids and hydroxycinnamic acids (Senica, Stampar, Veberic, & Mikulic-Petkovsek, 2016).

Although phenolic acids are phenols that have one carboxylic acid in structure, when describing metabolites of plants, they belong to a specific class of organic acids. Commonly, caffeic, p-coumaric, vanillic, ferulic, and protocatechuic acids are found in all plant types. Others like gentisic and syringic acids are found in specific foods or plants (Robbins, 2003). Mainly caffeic acid derivatives are well known to reduce free radicals in DPPH (2,2-diphenyl-1-picrylhydrazyl) and AAPH (2,2'-Azobis (2-amidinopropane) dihydrochloride) radical scavenging assays (Veeren et al., 2021). In another study, the antiradical effects of most common phenolic acids were compared with DPPH and ABTS (2,2’ azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) assays and gallic acid was reported to have highest value of scavenging efficiency among caffeic, vanillic, salicylic and p-hydroxybenzoic acids (Karamac, Kosiñska, & Pegg, 2005).

1.1.2 Flavonoids

Based on the location of the aromatic ring's connection to the benzopyrano moiety, the natural products are categorized as the flavonoids (2-phenylbenzopyrans), the isoflavonoids (3-benzopyrans) and the neoflavonoids (4-benzopyrans). Flavonoids are mostly found in plants tissues, inside the cells or on the surfaces of various plant organs (Lattanzio, 2013). They are the natural group of polyphenols which can be directly related with radical scavenging capacity, contributes to enzyme inhibition during free radical production (Stanković, 2016). Their subgroups are known as flavonols, flavanols, isoflavonoids, flavonones and flavones (Senica et al., 2016). With their known lipoxygenase and cyclooxygenase enzyme-inhibiting properties, flavonoids play an important role in free radical and catalytic metal ion formation inhibition (Embuscado, 2015).

1.2 Medicinal Plants

Medicinal plants are used widely in most of the drugs and alternative products with health benefits. Their role in preventing and treating diseases is significant. People are getting more interested in medicinal plants as a result of their therapeutic efficacy and low toxicity (Li, Wong, Cheng, & Chen, 2008). They are generally adapted in most of the food supplements which are used generally as daily intake.

The antioxidant potential of the most known medicinal plants *Sambucus nigra* L., *Echinacea purpurea* L. and *Cetraria islandica* L. are known in literature with their high phenolic content. The phenolic content studies are generally related with the most distinct abilities such as; radical scavenging, chelating metal transition ions, reducing power, protection of lipids and biomolecules against oxidation, inhibition of pro-oxidant enzymes and activation of enzymatic defense system (Li et al, 2008)

1.2.1 *Sambucus nigra* L. (Elderberry)

In recent years, one of the most attractive plants due to its medicinal properties, has been *Sambucus nigra* L. (SN). It belongs to the *Caprifoliaceae* family and its common names are elder, common elder, black elder, European elder and elderberry (Agalar, 2019).

The creamy-white flowers and glossy, purplish-black berries have been used for therapeutic purposes and are traditionally consumed to prevent or diminish the effect of several diseases. For its high antioxidant and antiviral activity, SN is used as traditional medicine. There is a significant effect of elderberry in curing acute and chronic rhinosinusitis and respiratory viral infections such as cold and herpes (Mikulic-Petkovsek, Schmitzer, Slatnar, Stampar, & Veberic, 2012; Cejpek, Maloušková, Konečný, & Velíšek, 2009). According to different studies, both elderberry fruit and flower are high in polyphenols (Cejpek et al., 2009; Veberic et al., 2009).

Berry fruits are well-known for their health benefits since they contain micronutrients and phytochemicals such as macronutrients, organic acids and phenolics (Mikulic-Petkovsek et al., 2012). They are particularly rich in flavonoids, including kaempferol, astragalin, quercetin, quercetin-3-O glucoside, rutin, isoquercitrin, and hyperoside (Agalar, 2019).

SN species have a various content of the flavonols, phenolic acids, tannins and procyanidins. Similar with most of the anthocyanin rich berries, the elderberry is also vitamin rich. Agalar (2019), reported the different vitamin contents in 100 g of fresh berries; the mostly found ones are vitamin B2 (65 mg), vitamin C (18-26 mg), folic acid (17 mg), biotin (1.8 mg), β-carotene (0.36 mg) and vitamin B6 (0.25 mg). Specifically, cyanidin-3-glucoside and cyanidin-3-sambubioside are found as

anthocyanins in most of the elderberry fruits (Agalar, 2019; Senica et al., 2016). Veberic et al. (2009) were also reported the presence of cyanidin 3-sambubioside-5-glucoside, cyanidin 3,5-diglucoside and cyanidin 3-rutinoside as anthocyanins in elderberry species. The anthocyanin content in SN genotypes was previously found as 200-1000 mg cyanidin-3-glucoside equivalent (cy-3-glu) /100g in fresh matter (Ercisli et al., 2009). In the same study, the total phenolic in fresh matter was reported in the range of 371-432 mg gallic acid equivalent (GAE)/g. Due to the high amount of anthocyanin and phenolic contents in elderberry genotypes, they contribute to prevention of cardiovascular disease, cancer, inflammatory disease and diabetes (Veberic et al., 2009). SN species are also known with their high nutritional value. In a study conducted by Domínguez et al. (2020), protein and fat contents are determined as 2.97g/100g fresh weight and 0.35g/100g fresh weight, respectively.

1.2.2 *Echinacea purpurea* L.

As an herbal medicine, *Echinacea purpurea* L. (EP) is used popularly in functional foods, supplements, herbal teas and some candies due to the presence of health effective compounds in its composition, which help curing and decrease indications of common colds. The active compounds in the plant parts are identified with many methods and most known ones in these compounds are cichoric acid (di-caffeoyl tartaric acid), alkamides (alkyl amides), polysaccharides and glycoproteins (Bauer, 1999). Cichoric acid is commonly known phenolic acid in most of the EP species and it is found highly in all plant parts (Molgaard, Johnsen, Christensen, & Cornett, 2003). This phenolic acid itself is known to show antioxidant activities and play active role in suppression of HIV integrate (Lee & Scagel, 2009).

EP plant extracts are used for medicinal purposes since they reduce symptoms in acute upper respiratory infections and they are efficient in alleviating colds and sore

throats (Stanisavljević, Stojičević, Veličković, Veljković, & Lazić, 2009; Tsai et al., 2012). Due to these properties, they are used for treatment of many vital diseases such as bronchitis and pneumonitis. Immunomodulatory properties and antioxidant activities of polyphenols were reported as important biological effects As shown in Figure 1.2, the polyphenols are specified as caffeic acid derivatives in EP species, included in caftaric acid, chlorogenic acid, cynarin, echinacoside and cichoric acid, which is the main compound in EP plant root part (Oniszczuk et al., 2019).

Chemical composition of EP are represented mainly as polar compounds and nonpolar compounds. The non-polar compounds are secondary metabolites such as acetylenic and alkylamides. The phytochemical composition also includes the polysachharides and glycoproteins which are classified as constituents with high molecular weight (Bruni et al., 2018). The volatile compounds in *Echinacea* species come from the essential oil content of the plant. Different plant tissues have different amount and variety of volatile compounds. Thus, the yield of the essential oil in leaves and flowers of the plant is 0.1 - 1.8% (v/w). Whereas, the roots and stems have higher yield of essential oil (0.3 - 2.4% (v/w)) (Bruni et al., 2018).

The quantification of the chemical composition in EP species was made in another study and the cichoric acid content of flowers was found 32.39 mg/100g. The rutin (3.36 mg/100g), chlorogenic acid (1.06 mg/100g), ferulic acid (0.39 mg/100g), caffeic acid (0.03mg/100g) and gallic acid (0.01mg/100g) are the most commonly found phenolic compounds in methanol extracts of EP flowers (Erenler et al., 2015). Vitamin C (ascorbic acid) analysis in extracts of EP was resulted in 0.3g/100g dry weight, which is more than total phenolic content (Mildaziene et al., 2017).

Thygesen et al. (2019), studied on leaves, stem and root of the plant and reported to find, cichoric acid has highest free radical scavenging capacity in methanol extraction of DPPH assay. The ability of cichoric acid to reduce DPPH was

compared in different plant parts and found to be highest in extracts of leaves as 41.3 mg/g dry matter (Thygesen, Thulin, Mortensen, Skibsted, & Molgaard, 2007). In another study, it was indicated that the root parts of the plant specifically contained volatile oils and pyrrolizidine alkaloids, while the aerial parts contained alkamides and polyacetylenes (Stanisavljević et al., 2009).

Most studies for the medicinal plants focus on possible loss of the active compounds due to the thermal treatment and food supplement applications used. Indeed, thermal treatment of EP types affects the total phenolic content. Heat aided mechanically processed food supplements results apparent polyphenolic matter loss in the food types rather than primitive form of the plants. Ifie et al. (2018), investigated the effect of food processing on phenolic constituents and they reported that the phenolic acids, anthocyanins, flavonoids are degraded due to the factors such as pH and oxygen. Furthermore, thermal processing and light are the other factors that affect the retention and stability of the bioactive substances.

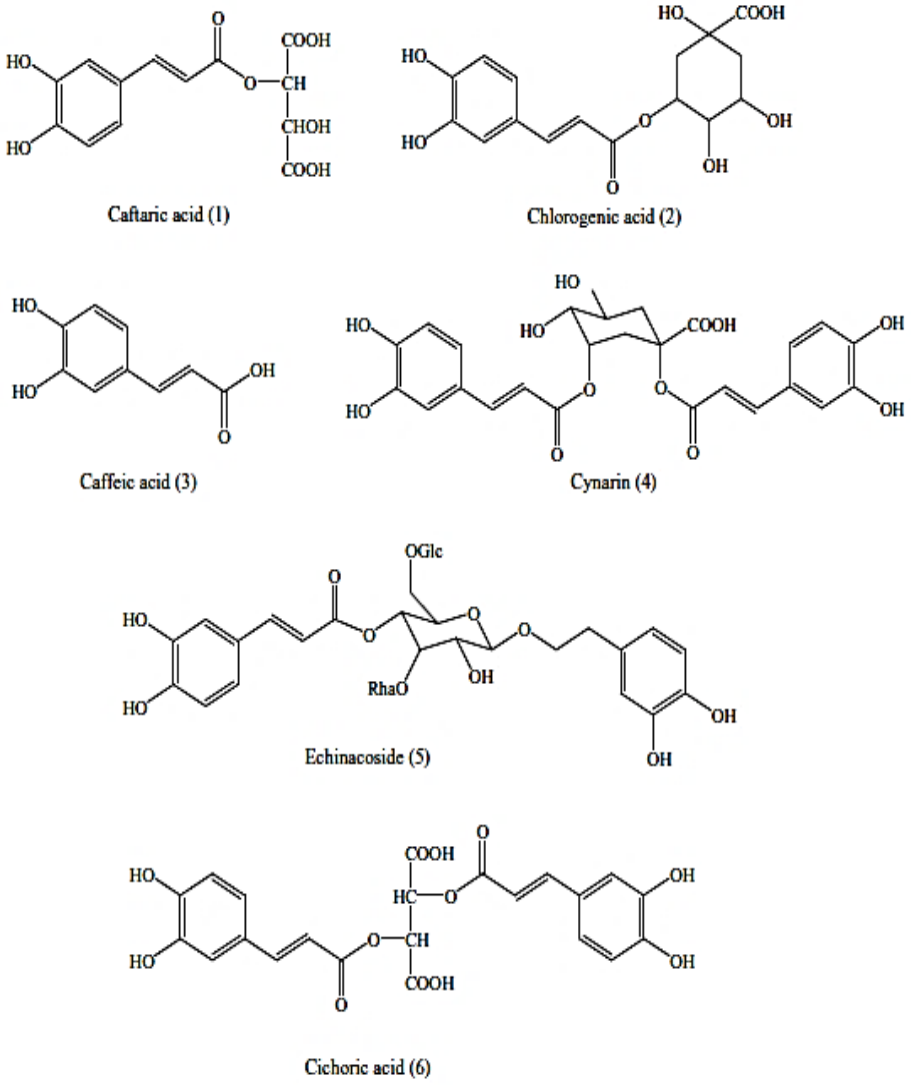

Figure 1.2 Structure of caffeic acid derivatives of *Echinacea* spp. (Oniszczuk et al., 2019)

1.2.3 *Cetraria islandica* L. (Island moss)

As a product of collective relation between algae and fungi, lichens are slow-growing composite organisms that produce lichen substances; which are mostly known as secondary metabolites (Elkhateeb & Daba, 2019). Being unique and stable, lichens are used as medicinal purposes. Some of the pharmaceutical potential of lichen types are expressed as their antimicrobial and antioxidant properties. The most significant treatment for the lichens are the chronic respiratory issues relatively bronchitis. Their metabolites are reported to show antiviral, antibiotic, antitumor properties which inhibits the enzyme activity (Elkhateeb et al., 2019; Huneck, 1999). High nutritional value permits for a healthy diet, where they contain lichesterinic acid, protolichesterinic acid, fumarprotocetraric acid. Other components in *Cetrarica islandica* spp. are arabitol, mannitol, lichenin, isolichenin and umbilicin. Vitamin content of this plant is also known to be high, including vitamin C, vitamin B1 and vitamin B12 (Grujicic et al., 2014). Lichen *Cetraria islandica* L. (CI) is also rich in minerals such as iron, copper, manganese, chromium (Cristian, Mitoi, & Brezeanu, 2013).

With their antiviral and antimicrobial activities, lichen substances are known mostly as protolichesterinic acid, pulvinic acid, physodic acid, lobaric acid, fumarprotocetraric acid and usnic acid (Kotan, Alpsoy, Anar, Aslan, & Agar, 2011). Lichens secondary metabolites are mainly depsides, depsidones, dibenzofurans, xanthones and terpene derivatives (Karagoz, Dogruoz, Zeybek, & Aslan, 2009). Due to their high reducing power and superoxide anion radical scavenging capacity, total phenol/ flavonoid content and the antioxidant capacity, lichens are good sources of natural antioxidants (Kosanić, Ranković, & Vukojević, 2010).

Iceland lichen (CI) as lichen type medicinal plant, has been used in folk medicine. With its known antioxidant capacity, superoxide anion scavenging capacity, free radical scavenging capacity and the reducing power of CI species; they are a part of pharmaceutical industry (Gulcin, Oktay, Kufrevioglu, & Aslan, 2002). Their metabolites are known to have a variety of biological actions such as antibiotic, anti-mycobacterial, antiviral, anti-inflammatory, analgesic, antipyretic, anti-proliferative and cytotoxic effects (Müller, 2001). Grujicic et al. (2014) suggest that the methanol extract of CI exhibited a relatively powerful anticancer activity.

The carbohydrate content was reported to be 82.5% of dry weight in CI lichen species. Lichenin was reported to be the highest content of carbohydrates with 50.9% on dry basis and hemicellulose (25.8%), cellulose (3.9%) and water-soluble sugars (1.9%) are other carbohydrates. The additional secondary metabolites of lichen substances were reported to be found as atranorin (1.2%), fumarprotocetraric acid (0.5 - 1.5%), gyrophoric acid (1 - 4%), salicylic acid (4 - 6%) and usnic acid (0.2 - 4%) in dry weight (Akbulut & Yıldız, 2010). When the chemical composition is investigated in detail, the most common lichen constituent of CI was found to be (+)-Protolichesterinic acid with anti-inflammatory property (Ingólfsdóttir et al., 1998).

Some of the important lichen metabolites can be specified as aliphatic acids and pulvinic acid derivatives. Also depsides and depsidones are mostly found in lichen types. Most characteristic metabolites are dibenzofuans, anthraquinones, naphthoquinonesas and epidithiopiperazinediones that are specified with their known therapeutic content (Müller, 2001).

1.3 Lozenge Formulation

Lozenges are medicated confections that are found generally in the form of soft caramel based, hard candy or compressed tablets. The soft type of lozenge is called as pastille, and the most known ingredients are gelatin, acacia, sucrose, water and also any medicine (Lieberman, Lachman, & Schwartz, 1990; Majekodunmi, 2015; Pothu & Yamsani, 2014). The pastille products are categorized under jellies and gum based confectionery products. The best known difference between the jelly type products and the hard candy products is the molding stage after cooking. Jellies and gum based confectionery products are generally rest in starch molds for 24-72 hours to obtain an ideal water content (in lozenges 0-1%; in gummies and jellies 8-22%) in the final product (Ergun et al., 2010).

The packaging of lozenges are generally in multiple packaging, where each individual unit is wrapped in polymeric moisture barrier. Since the lozenge structure is hygroscopic in nature, the packaging material should be gas tight or moisture resistant metal, glass or polyvinyl chloride. The small containers that each lozenge unit is put are sealed by aluminum foil or cellophane membrane which are specially called blisters (Majekodunmi, 2015).

The consumption of lozenges can be like keeping in mouth and sucking to be dissolved into the pharynx, or breaking down into small pieces by chewing. Lozenges dissolve slowly in the mouth or throat, making them a common delivery method for drugs which treat sore throats and cold symptoms (Umashankar, Dinesh, Rini, Lakshmi, & Damodharan, 2016). The method of consumption is based on the lozenge structure. The ingredient selection, formulation and the process parameters play an important role in final structure and texture of the product. Soft type lozenge formulations generally based on either pectin derivatives or gum based ingredients, which elongates the chewiness (Umashankar et al., 2016).

The most commonly used ingredients in the lozenge formulation are sucrose, maltose, dextrose as candy base and gum acacia, corn syrup, gelatin as binders, where all them are presented in Table 1.1.

Table 1.1 Ingredients and their examples which are used in lozenge formulations (Umashankar et al., 2016)

Ingredients	*Examples*
Candy Base 1. Sugar 2. Sugar free vehicles 3. Fillers	 Sucrose, Maltose, Lactose, Dextrose. Polyethylene Glycol (PEG) 600 and 800, Mannitol, Sorbitol. Lactose, Calcium Sulphate, Calcium Carbonate, Dicalcium Phosphate, Microcrystalline Cellulose.
Binders	Acacia, Corn Syrup, Sugar Syrup, Gelatin, Polyvinyl Pyrollidone, Tragacanth and Methylcellulose (MC).
Lubricants	Stearic Acid, Magnesium Stearate, Calcium Stearate, Polyethylene Glycol, vegetable oils and fats.
Flavoring Agents	Menthol, Eucalyptus Oil, Cherry flavor, Spearmint
Coloring Agents	Water soluble and Lakolene dyes, Food Drug and Cosmetic Colors, Orange Color paste etc.
Whipping agents	Milk protein (Casein), Egg Albumin, Gelatin, Xhantan gum, Starch, Pectin, Algin and Carrageenan.
Humectants	Glycerin, Propylene Glycol and Sorbitol.

1.3.1 Late Release Soft Lozenge

The late release soft lozenge formulation is based on a syrup containing gum arabic, maltitol and sorbitol (2:1:1 in ratio). As a binder and texture enhancer, maltodextrin and modified corn starch are included into the formulation (Table 1.2).

Table 1.2 Late release soft lozenge ingredients and their composition (%) in the formulation

Ingredients	*Composition (%)*
Gum Arabic	40
Maltitol	20
Sorbitol	20
Maltodextrin	8
Water	8
Modified corn starch	4

Gum Arabic

One of the common natural polymer which is used in food industry is gum arabic. It is mostly known in food industry with its emulsifying, stabilizing, thickening and binding functions. It is generally used as a flavor and texture enhancer in ice cream, jelly, candy, soft drink, beverage, syrup, and chewing gum industry. In pharmaceutical and herbal applications, it is used commonly in lozenge formulations (especially medicated cough drops) due to its texture enhancing and crystallization retarding properties (Patel & Goyal, 2015; Azzaoui, Hammouti, Lamhamdi, Mejdoubi, & Berrabah, 2015).

Containing at least 90% soluble dietary fiber, gum arabic is used in a variety of food and nutrient applications to include dietary fiber as well as other advantages such as moisture conservation and shelf life extension (Azzaoui et al., 2015).

Maltitol

As a non-reducing sugar and disaccharide polyol, maltitol ($C_{12}H_{24}O_{11}$; 4-O-glucopyranosyl-D-sorbitol) is an hygroscopic agent which is an alternative sweetener to sugar. It has approximately sucrose's 75-90% sweetness and related properties so it can compensate the role of sugar in the formulation (Saraiva, Carrascosa, Raheem, Ramos, & Raposo, 2020). Maltitol is less hygroscopic than sugar in its normal crystalline form due to its high crystalline purity and chemical structure.

When manufactured under defined atmospheric/climatic conditions, it is expected to increase shelf stability of products made with maltitol rather than sucrose (Saraiva et al, 2020). In gelatin or gum base lozenges, the formulation is enhanced by the addition of maltitol syrup in order to elongate the structure and keep the moisture inside to keep the texture during the shelf life. Maltitol also can be used up to 80% in hard-boiled candies, which increases clarity and makes the product more appealing (Heume & Rapaille, 1996). Since maltitol's sensorial properties are somewhat similar to those of sucrose, this disaccharide polyol is one of the most appealing replacements for conventional bulk sweeteners in non-cariogenic and/or calorie-reduced food items (Heume et al., 1996)

Sorbitol

Having the half sweetness of sucrose, sorbitol is one of the main sweetening agent with its specific flavoring characteristics and confident viscosity level in liquids. It is a six carbon sugar alcohol, where it can be found in many fruits and vegetables (Carocho, Morales, & Ferreira, 2017). Sorbitol is synthesized chemically from glucose or dextrose for the commercial formulations and its steric configuration is

similar with the glucose. In most of the structures, they are used as humectant and stabilizer (Carocho, 2017; Godswill, 2017). It can be also used in soft candy confectionery as anti-crystallizing, viscosity controlling or texture enhancer agent.
In pastille formulations, it is used as 15-20% to protect the product against loss of moisture and evolve the textural structure from harder to softer (Hashim et al., 2021).

Maltodextrin

Maltodextrins are hydrolysis products of starches with dextrose equivalent (DE) values less than 20 (Komes, Horžić, & Belščak-Cvitanović, 2015). It is used as an agent to increase the stickiness (Ergun, Lietha, & Hartel, 2010). To obtain a desirable sensory profile and a stable structure in soft lozenges, starch based agents are used in formulations. Utilizing maltodextrins with starch provide an increase in solid content of the recipe, control of sweetness and generation of a desired color.

Modified corn starch

Indigenous starches have a number of drawbacks for industrial uses, including insolubility in cold water, viscosity loss, and thickening strength after preparation (Cui, 2005). Some modifications can be made to alter the properties of starch such as solution viscosity and shelf life stability. By stabilizing the starch granules under different temperature/moisture combinations or pressure, the functionality of starch can be changed and become suitable for many food industry applications.

Due to their high water holding properties, they are added to semi solid gelly structures to enhance their structure (Burey, Bhandari, Rutgers, Halley, & Torley 2009). Modified corn starch is used in pastille formulations to provide a stable structure, as binding/thickening agent or gelling agent. For short and tender texture, it is more desirable to use modified corn starch in most of the gums, jellies and pastilles (Pothu & Yamsani, 2014).

1.4 Objectives of the Study

Since ancient times, people are searching for healthy and nutritional sources to consume to be protected from illnesses. Medicinal plants are so popular in recent years, that most of the studies investigate that traditionally known herbs, lichens and fruits due to the rich content of phenolic compounds and other antioxidants beside the nutritional value of these plants. With their known health effects, medicinal plants can be taken into body directly or with the carriers such as teas, supplements and lozenges. Compared with the direct consumption, the use of plant extracts in nutritional products decreases the unit amount of the functional compounds.

Lozenges are consumed commonly for reducing the symptoms of acute respiratory tract infections and they help to avoid new diseases like cold flu, by forming a barrier in nasal throat. Soft lozenges i.e. pastilles are known to be consumed with chewing or dissolving in a mouth. Late release soft lozenge formulations are important to increase the effective influence time of the bioactive compounds. Due to its formulation, the structure does not permit to be consumed with chewing and the release time of the bioactive compounds after dissolving are optimized to be at least in 5 min. Late dissolving not chewable structure permits the active ingredients in the medicinal plant to interact more in throat and helps to relief to symptoms of diseases.

In this study, *Sambucus nigra* L., *Echinacea purpurea* L. and *Cetraria islandica* L. plant powder extracts were investigated, by considering total phenolic content, total flavonoid content, radical scavenging activities (DPPH and ABTS methods) and qualitative and quantitative phenolic profiles.

The other objective of the study is to characterize their usage in the late release soft lozenge formulation. The results obtained from the studies were evaluated comparatively, in order to determine the possible phenolic compound losses after lozenge production. The phenolic compounds of all plants will be identified and quantified by high-performance liquid chromatography (HPLC) in ethanol and water extracts and also pastille forms of selected herbs. Based on the results obtained from the assays, the applicability of plant extracts on lozenge formulation were evaluated.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

For lozenge formulation, gum arabic (Kale Kimya, Turkey), maltitol (Sunar Mısır, Turkey), sorbitol (Sunar Mısır, Turkey), maltodextrin (Omnia Nişasta, Turkey) and modified corn starch (Cargill, Turkey) were used.

Elderberry dry extract powder (fruits of *Sambucus nigra* L.; water and ethanol extract), *Echinacea purpurea* L. dry extract powder (herbs of *Echinacea purpurea* Linn. Moench; water extract) and Iceland moss dry extract powder (whole body of lichen *Cetraria islandica* L.; water extract) were purchased from Greenutra Resource Inc. (China).

Ethanol, Folin-Ciocalteu's phenol reagent, methanol, Na_2CO_3, $NaNO_2$, $AlCl_3.6H_2O$, NaOH, $AlCl_3.6H_2O$, DPPH, ABTS, $CuCl_2$, neocuproine (Nc), ammonium acetate buffer (pH 7) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

For standard calibration curves, analytical grade gallic acid, rutin, trolox and HPLC-grade standards: cichoric acid, caftaric acid, caffeic acid, cyanidin 3-glucoside chloride, kaempferol-3-rutinoside, catechin, (-)-epicatechin were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

2.2 Methods

2.2.1 Late Release Soft Lozenge Production

Gum arabic, maltitol, sorbitol, maltodextrin and modified corn starch were mixed gently and poured into the vacuum batch cooker (Bosch, Germany). The process was applied as detailed in Figure 1.3. The cooking was held for 1 h until the temperature is 110 °C and pressure is - 0.6 bar. Brix value of the solution was measured with optical refractometer and the result was recorded as 78 °Bx (Abbe 5, Xylem Analytics, Germany) after cooking. The mix was cooled until 82 °C in cooling tray and then the dough was kneaded for 5 min until the dough temperature decreased to 55 °C. 25g dry extract (DE) powder of each plant were separately added into each 1 kg of sugar dough (0.05g dry extract powder/ 2g lozenge).

The molding was done for each plant sample in silicone and chromium shaping molds. After molding for 24 hours, the samples were removed from the molds and put on the stainless steel trays. The trays were placed inside relative humidity and temperature-controlled cabinet (SRC, Konya, Turkey) for cooling and drying. The relative humidity in cabinet was 10% RH at 15 °C. The pastille products were refrigerated in trays for 48 h. The moisture of the final product was measured according to Karl Fischer moisture analysis method (AOAC 991.02) as 2.8% (Karl Fisher, Germany). Each molded pastille product was put in blisters and kept in cool and dry place at room temperature (20 – 22 °C) until analysis.

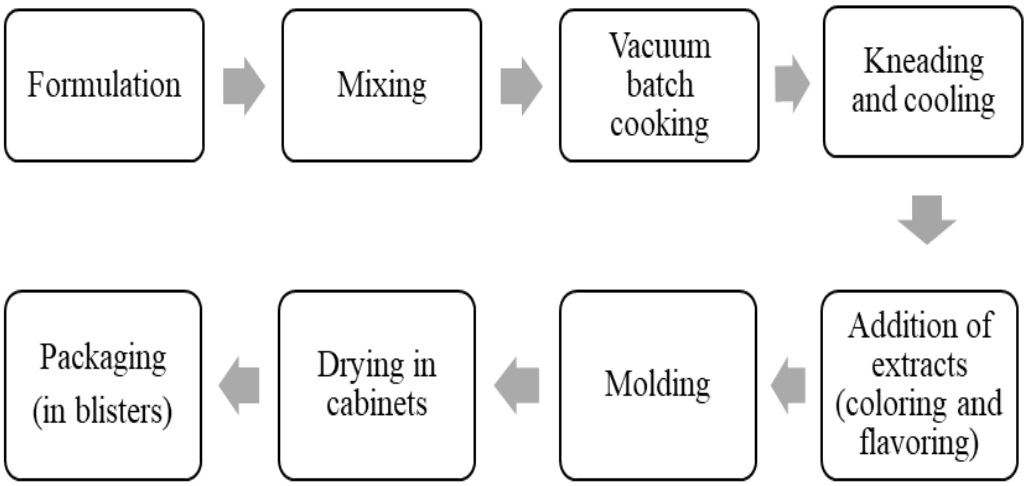

Figure 1.3 Late release soft lozenge production flow chart

2.2.2 Extraction for Analysis

For extraction of dry plant powders (SN: Elderberry dry extract powder; EP: *Echinacea purpurea* dry extract powder; CI: Iceland moss dry extract powder) and their usage in lozenges, the method of Hung & Morita (2008) was used with some modifications. Due to the known polarity and solvent efficiency on polyphenols, 75% ethanol and water were selected as solvents during extraction (Do et al., 2014; Naczk et al., 2004). Dry powder samples of SN (0.1g), EP (0.2g) and CI (0.2g) were added to 3 mL of 75% ethanol and water. The sonication was done for 15 minutes with continuous shaking in ultrasonic bath operating at 45 kHz with heating power of 600 W (USC900TH type, VWR® International Ultrasonic Cleaners, Pennsylvania, United States). The ultrasonic bath temperature was measured as 29°C after sonication. The samples were centrifuged for 10 minutes at 2500*g*. After the

upper layer was collected from the samples, the analysis was repeated to increase the extraction efficiency. Again 3 mL of 75% ethanol and 3 mL of water were added to the residue for further extraction under the same conditions. The sonication and centrifugation were repeated. The extract was collected and combined with the previous one. All extracts were hold at 4 °C until the analysis.

0.2 g lozenge sample was added to 5 ml distilled water and sonicated for 15 minutes until they dissolve. Lozenge samples were centrifuged for 10 minutes at 2500*g* and the upper layer was collected. All extracts were hold at 4 °C until the analysis.

2.2.3 Total Phenolic Content (TPC)

TPC was determined by Folin-Ciocalteu method of Singleton and Rossi (1965) with some modifications. This method is based on the reduction of Folin-Ciocalteau reagent in the presence of Na_2CO_3.

For the assay, 50 μL sample was added to 250 μL Folin-Ciocalteu reagent (0.2 N) and 3.2 mL distilled water. After 3 minutes, 1.5 mL 10% saturated Na_2CO_3 was added and the assay mixture was kept away from light and hold at ambient temperature for 2 hours. Absorbance of the samples were measured at 765 nm by using Shimadzu UV/VIS spectrophotometer (Shimadzu, UV–1700 PharmaSpec, Kyoto Japan). For the absorbance measurements, absorbance adjustment was done for the samples to obtain absorbance values lower than 1. EP and SN samples were diluted with solvent to 1/5 and 1/50, respectively. The CI samples were not diluted since the absorbance readings were lower than 1. The total phenolic content of the extracts was determined by the calibration curve obtained with 10-50 mg/ml of gallic acid in 75% ethanol, presented in Appendix A (Figure A.1). The results were expressed as mg gallic acid equivalent (GAE)/g DE.

2.2.4 Determination of Total Flavonoid Content (TFC)

TFC was determined according to the method of Dewanto et al. (2002). 0.25 mL rutin was added to 75 μL $NaNO_2$ (5%), since it is known to have higher rutin content in elderberry type red berries and medicinal plants (Grujicic et al., 2014; Milena et al, 2019; Stanković et al., 2016). After 6 min, 150 μl 10% $AlCl_3.6H_2O$ was added and then after 5 min, 0.5 ml 1 M NaOH was added. The volume was completed to 2.5 mL with distilled water. The mixture was gently mixed for 10 seconds at ambient temperature. The absorbance was measured against a blank sample with 75% ethanol at 510 nm with UV/VIS spectrophotometer. Results were expressed as mg rutin equivalent (RE)/g DE using a standard calibration curve obtained with 10-50 mg/ml rutin in ethanol. The calibration curve is in Appendix A (Figure A.2).

2.2.5 Determination of Total Antioxidant Capacity (TAC)

TAA of the samples were analyzed according to three different methods: DPPH, CUPRAC and ABTS assays. Trolox (0.01-0.10 mg /ml in 75% methanol) was used as standard for the calibration curves.

2.2.5.1 DPPH Free Radical Scavenging Capacity

DPPH radical in methanol is reduced in the presence of a hydrogen donating antioxidants. The non-radical form of DPPH-H is formed during the reaction. DPPH method was applied based on the study of Kumaran et al. (2006) and Rai et al. (2006) with some modifications. 1 mL of 0.1 mM DPPH in methanol was added to 100 mL of extract sample. Samples were stored in a place away from light for 30 min at ambient temperature. Absorbance was measured with UV/VIS Spectrophotometer at 517 nm wavelength against blank with 75% ethanol. The calibration curve is in Appendix A (Figure A.3).

2.2.5.2 Cupric Reducing Antioxidant Capacity (CUPRAC) method

This assay is based on the reduction of the cupric ion (Cu^{2+}) in copper (II) neocuproine [Cu (II)-Nc] reagent that the structure is known to be stable and both hydrophilic and lipophilic antioxidants can be detected by this rapid analysis method (Apak et al., 2004). 1 ml of 10 mM $CuCl_2.2H_2O$ in water, 1 ml neocuproine alcoholic solution (7.5 mM) and 1 ml ammonium acetate (NH_4Ac) buffer (1M, pH 7.0) solutions are mixed and added to 100 µl of extract and then 1 ml water was added. The solution was kept at ambient temperature for 30 min. Absorbance was measured with UV/VIS Spectrophotometer at 450 nm wavelength against blank with 75% ethanol. The calibration curve is in Appendix A (Figure A.4).

2.2.5.3 Total Antioxidant Capacity by ABTS method

ABTS method was carried out according to the method of Miller and Rice-Evans (1997) with some modifications. 220 mg of ABTS was dissolved in 200 mL of distilled water and 38 mg of $K_2S_2O_8$ was dissolved in 2 mL of distilled water. The solutions were mixed and stored overnight in the dark to complete radicalization. The solution was diluted with 0.05 M phosphate buffered saline (PBS) (pH 7.4) until its absorbance reaches 0.9±0.2. Then, 1 mL of ABTS solution with 0.05M PBS was added to the 100 µL of extract and the mixture was vortexed for 10 seconds at ambient temperature. Absorbance was measured with UV/VIS Spectrophotometer at 734 nm after 1 minute against 75% ethanol blank sample. The result was expressed as mg Trolox Equivalent Antioxidant Capacity (TEAC)/g DE. The calibration curve is in Appendix A (Figure A.5).

2.2.6 HPLC analysis of phenolic compounds

For the quantification of phenolic compounds, the method of Bakir et al. (2016) was applied in Waters W600 HPLC system (Milford, MA, USA) with PDA (photodiode array-Waters 996) detector and Luna - C18 (250×4.6 mm, 5 μm; Supelco Analytical, Bellefonte, PA, USA) column. Column temperature was set to 40 °C and autosampler temperature was 10 ± 5 °C. PDA detector scan interval was 200-600 nm.

The flow rate was 1 ml/min and the injection volume of 10 μL was used over a period of 50 minutes for separation. Standard calibration curves were prepared with caftaric acid, caffeic acid, chicoric acid, catechin, epicatechin, cyanidin 3-O-glucoside chloride, kaempferol 3- rutinoside. All of the standard solutions and the samples were filtered through a 0.45 μm membrane filter. 1 ml of each filtered sample was put into the vials and analyzed.

The mobile phase was distilled water with 0.1% (v/v) trifluoric acid (solvent A) and acetonitrile with 0.1% (v/v) trifluoric acid (solvent B). A linear gradient was used as in the followings:

(1) 95% solvent A and 5% solvent B (at the beginning, time = 0);
(2) 65% solvent A and 35% solvent B (at time = 45 min);
(3) 25% solvent A and 75% solvent B (at time = 47 min).

The time to return initial conditions is 54 min. Chromatograms were recorded at 280, 330, 340 and 520 nm wavelengths. Based on the retention times and UV spectra, each characteristic phenolic identification was done. The standard curves presented in Appendix C were used for quantification of phenolic compounds.

2.3 Statistical Analyses

Each analysis was performed in triplicate and the results were reported as mean value ± standard deviation. The data were analyzed by Minitab Statistical Software (18th version, Minitab Ltd., UK) by using one way analysis of variance (ANOVA) at 0.05 significance level, and Tukey's New Multiple Range Test was applied to analyze the results of the experimental data. The Range Test was applied to exact values to identify the differences between TPC, TFC and TAC which is specifically evaluated by DPPH, CUPRAC and ABTS tests ($p<0.05$). The results were evaluated statistically and the analysis tables were presented at Appendix B.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Total Phenolic Content, Total Flavonoid Content and Total Antioxidant Capacity

3.1.1 Total Phenolic Content (TPC)

The Folin–Ciocalteu reaction (reduction of the phosphotungstic-phosphomolybdic reagent) measures the reductive capacity of an antioxidant based on electron transfer and is widely used in determination of TPC of plants and their related products. Color formation by an antioxidant sample is compared to color formation of a standard compound, preferentially gallic acid and the results are reported as gallic acid equivalents. In this study, gallic acid was also used as a reference standard in TPC analysis and the calibration curve is presented in Appendix A (Figure A.1). Results for TPC analysis were expressed as mg gallic acid equivalent (GAE)/g dry weight (DW) and presented in Table 3.1.

According to Table 3.1, solvent type used in extraction was observed to affect TPC amount in samples. For EP and CI samples, the extraction with water provided more efficient extraction to obtain higher amount of phenolic compounds. In SN powder extract samples, the amount of phenolics decreased when water was used as solvent during extraction. This result refers to the fact that, extraction efficiency in red berries is higher in alcoholic extracts, since the solvents with higher water

percentages decrease anthocyanin stability (Seabra, Braga, Batista, & De Sousa, 2010).

Significantly highest TPC values were obtained in SN samples and they were followed by TPC values in EP and CI samples in decreasing order. TPC values were significantly decreased during lozenge production from plant extract powders. The decrease in TPC amount from plant extract powder to lozenge form was determined to be the highest in SN, since TPC value decreased from 178.42±11.48 to 2.33±0.11 mg GAE/g DW. This could due to high temperature and mixing during dough processing that extracts were added into the dough during lozenge production. A study reported by Senica et al. (2016) showed that the anthocyanin stability can be affected by several factors such as pH, light, oxygen etc. Moreover, heat treatment duration and temperature were found to be effective to decrease anthocyanin levels in elderberries.

In lozenges, SNP, EPP and CIP phenolic amounts were found statistically to be same ($p>0.05$). TPC value of EPE (38.48 ± 8.48^{d} mg GAE/g DW) was comparable with that of previous reports in literature. Oniszczuk et al. (2016) reported the TPC value in methanolic extract of *Echinacea purpurea* L. to be 30.54±0.95 mg GAE /g DW.

Table 3.1 Total phenolic content of samples

Samples	Total phenolic content (mg GAE/g DW)
SNE	$339.68 \pm 1.47^{Aa*}$
EPE	38.48 ± 8.48^{Ad}
CIE	2.62 ± 0.82^{Ae}
SNW	178.42 ± 11.48^{Bb}
EPW	60.77 ± 0.17^{Bc}
CIW	8.02 ± 0.12^{Be}
SNP	2.33 ± 0.11^{Ce}
EPP	1.23 ± 0.05^{Ce}
CIP	1.16 ± 0.02^{Ce}

* Data represent a mean ± SD ($n=3$); different letters within the same column shows significance difference ($p \leq 0.05$). Comparison within the same plant is expressed by uppercase letters, while comparison within all the samples is expressed by lowercase letters.

GAE: Gallic acid equivalent

SNE: Ethanol extract of *Sambucus nigra* L.

EPE: Ethanol extract of *Echinacea purpurea* L.

CIE: Ethanol extract of *Cetraria islandica* L.

SNW: Water extract of *Sambucus nigra* L.

EPW: Water extract of *Echinacea purpurea* L.

CIW: Water extract of *Cetraria islandica* L.

SNP: Water extract of *Sambucus nigra* L.; pastille application

EPP: Water extract of *Echinacea purpurea* L.; pastille application

CIP: Water extract of *Cetraria islandica* L.; pastille application

In another study, Lee et al. (2010) found the phenolic content as 444.1±4.1 mg GAE/100 g FW (fresh weight) in *E.purpurea* whole plant. When compared with these studies using the same standard (gallic acid), TPC extraction efficiency of EP samples in our study seems as higher. In another study related to the methanolic extract of the same plant, the TPC amount was determined as 60.0 ± 1.0 mg GAE/g dry extract (Mohsen & Mohammed Ali, 2012). This result is similar to TPC values of EPW samples (60.77±0.17 mg GAE/g DW) in our study. The differences in TPC results may be related with the extraction solvent type, product quality and the bioactive compound content differences in different botanical parts of the *Echinacea purpurea* plant. Thygesen at al., (2007) reported that the antioxidant properties and TPC values of *E.purpurea* herb were different in leaves, stem, rootstock, main roots and side roots.

According to the data in Table 3.1, TPC values of SNE (339.68±1.47 mg GAE/g DW) and SNW (178.42±11.48 mg GAE/g DW), were found to be meaningful when compared with the previous studies on the phenolic content in *Sambucus nigra* L. TPC of European elderberry fruits was found as 371-432 mg GAE/100g fresh matter according to the study of Ercisli et al. (2009). In a review based on the elderberry genotypes, the berries TPC values were reported in the range of 191-1790 mg GAE/100g fresh matter (Moyer, Hummer, Finn, Frei, & Wrolstad 2002). Climatic and topographical differences can also affect the berry quality and TPC values in *Sambucus nigra* L. (Atkinson & Atkinson, 2002). A similar study, in which elderberry and elderflower genotypes were compared based on their phenolic content and it was found that TPCs of elderflowers and elderberries showed a variation in the range of 15.23 - 35.57 mg GAE/g DW and 19.81 - 23.90 mg GAE/g DW, respectively (Viapiana & Wesolowski, 2017). The extract used in the study is *Sambucus nigra* berry fruit extract from China and it was previously studied that wild berries had 2 to 5 fold more total phenolics compared to cultivated elderberry plant types (Mikulic-Petkovsek et al., 2012; Veberic et al., 2015; Agalar et al., 2019).

For Island moss samples, CIE (2.62±0.82 mg GAE/g DW) and CIW (8.02±0.12 mg GAE/g DW) results were compared with different literature values. TPC value of *Cetraria islandica* L. among Bulgarian medicinal plants was found to be 40.49±0.74 μM quercetin equivalent according to the study of Ivanova et al. (2005). In another study, Ivanisova et al. (2016) investigated *C.islandica* moss and determined the total polyphenol amount as 0.37 ± 0.01 mg quercetin equivalent/g dry weight. The difference in phenolic compound's amount estimated in different studies can be related with the reference standards used in the analyses and also botanical differences in *Cetraria islandica* genotypes (Gulcin et al., 2002).

The Folin–Ciocalteu reagent reacts with any reducing reactant and measures the total reducing capacity of a sample, not only phenolic compounds. As an example, ascorbic acid was active in this reaction. Due to their reactivity toward other nonphenolic reducing compounds, this assay was suggested for measuring the antioxidant or reducing capacity of a sample rather than estimating total phenolics (Apak, Ozyurek, Guclu, & Capanoğlu, 2016; Delgado, Issaoui, & Chammem 2019; Pico, Pismag, Laudouzea, & Martinez, 2020). Polyphenolics are the most abundant antioxidants present in most medical plants and the sensitivity of ascorbic acid is very high, therefore, Folin–Ciocalteu assay gives a good approximate estimation of total phenolic content to show the effect of operational parameters in the same research study related to extraction.

3.1.2 Total Flavonoid Content (TFC)

Flavonoids are a class of polyphenols and widely found in plants. Rutin was used as a reference standard in TFC analysis. The calibration curve obtained at 510 nm, was presented in Appendix A (Figure A.2). TFC analysis were expressed as mg Rutin Equivalent (RE)/g DW and Table 3.2 shows the results.

Table 3.2 Total flavonoid content of samples

Samples	Total flavonoid content (mg RE/g DW)
SNE	$840.54 \pm 13.46^{Aa*}$
EPE	112.00 ± 4.00^{Ad}
CIE	2.80 ± 1.26^{Ae}
SNW	527.02 ± 11.26^{Bb}
EPW	329.54 ± 8.04^{Bc}
CIW	3.78 ± 0.38^{ABe}
SNP	5.24 ± 0.23^{Ce}
EPP	1.93 ± 0.27^{Ce}
CIP	1.12 ± 0.27^{Be}

* Data represent a mean ± SD (*n=3*); different letters within the same column shows significance difference ($p \leq 0.05$). Comparison within the same plant is expressed by uppercase letters, while comparison within all the samples is expressed by lowercase letters.

RE: Rutin equivalent
SNE: Ethanol extract of *Sambucus nigra* L.
EPE: Ethanol extract of *Echinacea purpurea* L.
CIE: Ethanol extract of *Cetraria islandica* L.
SNW: Water extract of *Sambucus nigra* L.
EPW: Water extract of *Echinacea purpurea* L.
CIW: Water extract of *Cetraria islandica* L.
SNP: Water extract of *Sambucus nigra* L.; pastille application
EPP: Water extract of *Echinacea purpurea* L.; pastille application
CIP: Water extract of *Cetraria islandica* L.; pastille application

Ethanol extract of SNE has higher TFC than the water extract of the same plant (SNW), but the other two plants showed the reverse results. Total flavonoid content in EP and CI water extracts showed higher results when compared to ethanol extracts. This results are in accordance with the study of Seabra et al. (2010) that

investigated the extraction efficiency by examining TPC and TFC in elderberries. They reported the highest ethanolic extraction yield in elderberry when compared with the results of extraction with water. In another study, it was concluded that the polarity of different solvents affects the phenolic and flavonoids extraction yield and the bioactive compound solubility consequently (Lim, Pang, Yusoff, Abdul Mudalip, & Gimbun, 2019).

The effect of solvent type in TPC and TFC analyses was examined on some medicinal plants, in the study proceeded by Stankovic (2011, who reported the total phenolic content in methanol and water extracts of whole herb of *Marrubium peregrinum* L. 49.27±0.815 and 46.78±0.258 mg GAE/g extract, respectively. The amount of extracted phenolics were approximately same. On the other hand, the concentrations of flavonoids in methanol and water extracts were determined as 54.77±0.8598 and 18.72±0.417 mg RE/g extract, respectively. The similar decreasing trend in flavonoid content from alcoholic extract (SNE) to water extracts (SNW) was observed in our study.

When the TFC values of samples were compared, the highest results were found in all extract forms of SN, then EP and CI. Significant decrease in TFC in pastille form was determined that the greatest loss was obtained for SN product. In lozenge samples, TFC of SNP (5.24±0.23e mg RE/g DW), EPP (1.93±0.27e mg RE/g DW) and CIP (1.12±0.27e mg RE/g DW) results were similar statistically in comparison within all the samples ($p<0.05$).

Flavonoid is one of the phenolic components, therefore phenolic content must be higher than flavonoid content. But, as observed in our study, higher flavonoid content can be determined since the assays used for the estimation of TPC and TFC are only the relative amounts equivalent of the selected standards used or limitations in the analytical methods for quantification. Also flavonoids are highly substituted they will not react with Folin reagent in the same way as simpler phenolics such as gallic acid and may give much lower equivalents of gallic acid.

3.1.3 Total Antioxidant Capacity (TAC)

All the samples were analyzed for determining total antioxidant capacity, using three known methods: ABTS, CUPRAC and DPPH assays.

3.1.3.1 Total antioxidant capacity: DPPH (2,2-diphenyl-1-picrylhydrazyl) method

Trolox was used as a reference standard in DPPH analysis. DPPH radical scavenging capacity was calculated by using the calibration curve obtained at 517 nm, presented in Appendix A (Figure A.3). The results of ethanol extracts were expressed as mg trolox equivalent antioxidant capacity TEAC/ g DW and presented in Table 3.3.

DPPH test was applied only in ethanol extracts of the samples, since the water extracts and pastille applications resulted in blurred solution when interacted with methanolic DPPH. Similar result was observed in prior studies, thus the fluctuation occured during DPPH assay measurements due to blur arising in solution (Şanlıdere Aloğlu & Öner, 2011). In another study, a similar turbidity occurred during analysis was illustrated with the difference in polarity of the solvent used (Dawidowicz, Wianowska, & Olszowy, 2012). Furthermore, Dawidowicz et al. (2012) focused on some practical problems occuring during the estimation of antioxidant capacity and found that water content of the sample decreases the reaction kinetics in DPPH assay.

Table 3.3 Total antioxidant capacity: DPPH method

Samples	Antioxidant capacity (mg TEAC/g DW)
SNE	43.48±6.85[a*]
EPE	8.89±2.12[b]
CIE	0.18±0.05[b]

* Data represent a mean ± SD (*n=3*); different letters within the same column shows significance difference ($p \leq 0.05$)
TEAC: Trolox equivalent antioxidant capacity
SNE: Ethanol (75%) extract of *Sambucus nigra* L.
EPE: Ethanol (75%) extract of *Echinacea purpurea* L.
CIE: Ethanol (75%) extract of *Cetraria islandica* L.

According to the data presented in Table 3.3, the antioxidant capacity of SNE (43.48±6.85[a] mg TEAC/g DW) was statistically different from the antioxidant capacities of EPE (8.89±2.12[b] mg TEAC/g DW) and CIE (0.18±0.05[b] mg TEAC/g DW**).** There was no significant difference between EPE and CIE capacities.

The antioxidant capacities of different lichen types were investigated in previous studies and DPPH radical scavenging capacity of *Cetraria islandica* was found to be 35% in acetone solvent and 30% in water solvent (Zambare & Christopher, 2012). The anthocyanin capacities of different mosses was also reported and the results were expressed in the range of 10±0.22 - 26±0.32 mg TEAC/g DW (Yayintas, Alpaslan, Karagul Yuceer, Yilmaz, & Sahiner, 2017). When compared with the study of Yayintas et al. (2017), the antioxidant capacity of *Cetraria islandica* L. used in our study (0.18±0.05 mg TEAC/g DW) was lower estimated.

Dawidowicz et al. (2006) studied antioxidant activity of elderberries with DPPH assay and found the percentage of inhibition (%) results between 50.25±0.80 and 67.69±1.85 in different extraction temperatures. Sidor and Gramza-Michałowska (2015) stated that, the antioxidant capacity of elder plant obtained by DPPH assay was 60.8 mg ascorbic acid equivalent (AAE)/g DW. The difference between the

results may be related with the phenolic acids used in the analyses as a reference standard. Among the data collected from the literature, the result obtained for antioxidant capacity for SNE (43.48±6.85 mg TEAC/g DW) has the lowest value. This may be related with selected berry type, geographical and altitudinal differences and also part of the plant used in the analysis (Atkinson et al., 2002; Cejpek et al., 2009).

3.1.3.2 Total antioxidant capacity: Cupric Reducing Antioxidant Capacity (CUPRAC) method

Trolox was used as a reference standard in CUPRAC analysis. Cupric reducing antioxidant capacity was calculated by using the calibration curve obtained at 450 nm, presented in Appendix A (Figure A.4). The results for the analysis were expressed as mg trolox equivalent antioxidant capacity TEAC/g DW and reported in Table 3.4.

According to the CUPRAC analysis results, the highest antioxidant capacity was found in SNE (901.62±24.53^{a} mg TEAC/g DW) and it was followed by SNW (472.08±18.10^{b} mg TEAC/g DW). The water extracts of EP and CI were found higher when compared to their ethanol extracts. Statistically similar results were observed in the study for CIE and CIW; 3.42±0.17^{e} and 16.31±0.68^{e} mg TEAC/g DW respectively.

Table 3.4 Total antioxidant capacity: CUPRAC method.

Samples	Antioxidant capacity (mg TEAC/g DW)
SNE	$901.62 \pm 24.53^{Aa*}$
EPE	91.83 ± 4.44^{Bd}
CIE	3.42 ± 0.17^{Be}
SNW	472.08 ± 18.10^{Bb}
EPW	242.01 ± 50.50^{Ac}
CIW	16.31 ± 0.68^{Ae}
SNP	4.08 ± 0.05^{Ce}
EPP	1.65 ± 0.07^{Ce}
CIP	1.16 ± 0.06^{Ce}

* Data represent a mean ± SD (*n=3*); different letters within the same column shows significance difference ($p \leq 0.05$). Comparison within the same plant is expressed by uppercase letters, while comparison within all the samples is expressed by lowercase letters.

TEAC: Trolox equivalent antioxidant capacity
SNE: Ethanol extract of *Sambucus nigra* L.
EPE: Ethanol extract of *Echinacea purpurea* L.
CIE: Ethanol extract of *Cetraria islandica* L.
SNW: Water extract of *Sambucus nigra* L.
EPW: Water extract of *Echinacea purpurea* L.
CIW: Water extract of *Cetraria islandica* L.
SNP: Water extract of *Sambucus nigra* L.; pastille application
EPP: Water extract of *Echinacea purpurea* L.; pastille application
CIP: Water extract of *Cetraria islandica* L.; pastille application

CUPRAC was used in previous studies to identify bioactive constituents in black elderberry. The antioxidant capacity of the berries used in the analyses were found to be highest in ethanol extracts, when compared to aqueous solvents (Sidor et al.,

2015; Milena et al., 2019). The extraction efficiency is in accord with the literature, thus the antioxidant capacity in SN decreased from 901.62±24.53 (SNE) to 472.08±18.10 (SNW) mg TEAC/g DW, when water solvent is used in extraction instead of ethanol. In black elderberry flowers, the radical scavenger capacity was estimated as 383.8 mg TE (trolox equivalent)/g extract for ethanol and 355.0 mg TE/g extract for water solvent extraction (Milena et al., 2019). It was concluded that 50% ethanol was a better solvent than water for *Sambucus nigra* L. To conclude, in ethanol solvent extract sample, stronger antioxidant capacity and higher content of bioactive compounds were estimated.

Gadjalova et al. reported the antioxidant capacity of *Echinacea purpurea* water extract 196.88 ± 2.88 (μM TE/g DW), which was estimated by CUPRAC assay. In another study, the water extracts of *Echinacea* flower and leaf parts were investigated with CUPRAC and the antioxidant capacity was reported 221± 1 and 283 ± 3 mg TE/g, respectively (Mohamed Sharif et al., 2021). The antioxidant capacity result obtained for EPW (242.01±50.50^{c} mg TEAC/g DW) was found valid when compared with the previous studies.

3.1.3.3 Total antioxidant capacity: ABTS method

Trolox was also used as a reference standard in ABTS analysis. Total antioxidant capacity was calculated over the calibration curve obtained at 450 nm, presented in Appendix A (Figure A.5). The results for the analysis were expressed as mg trolox equivalent antioxidant capacity TEAC/g DW and presented in Table 3.5.

Table 3.5 Total antioxidant capacity: ABTS method

Samples	Antioxidant capacity (mg TEAC/g DW)
SNE	$299.39 \pm 15.57^{Aa*}$
EPE	77.40 ± 0.62^{Ab}
CIE	14.70 ± 0.51^{Ac}
SNW	302.44 ± 5.31^{Aa}
EPW	74.72 ± 8.29^{Ab}
CIW	8.93 ± 1.18^{Bc}
SNP	0.82 ± 0.04^{Bc}
EPP	0.75 ± 0.02^{Bc}
CIP	0.72 ± 0.02^{Cc}

* Data represent a mean ± SD (*n=3*); different letters within the same column shows significance difference ($p \leq 0.05$). Comparison within the same plant is expressed by uppercase letters, while comparison within all the samples is expressed by lowercase letters.

TEAC: Trolox equivalent antioxidant capacity

SNE: Ethanol extract of *Sambucus nigra* L.

EPE: Ethanol extract of *Echinacea purpurea* L.

CIE: Ethanol extract of *Cetraria islandica* L.

SNW: Water extract of *Sambucus nigra* L.

EPW: Water extract of *Echinacea purpurea* L.

CIW: Water extract of *Cetraria islandica* L.

SNP: Water extract of *Sambucus nigra* L.; pastille application

EPP: Water extract of *Echinacea purpurea* L.; pastille application

CIP: Water extract of *Cetraria islandica* L.; pastille application

There was no significant difference in antioxidant capacities among different plant type lozenge products; SNP, EPP and CIP. In CI samples, extraction solvent type did not affect the antioxidant capacity, statistically (CIE: 14.70±0.51; CIW: 8.93±1.18 mg TEAC/g DW). Also, the loss in antioxidant capacity due to processing can be ignored since the result obtained for lozenge form of *C.islandica* (CIP: 0.72±0.02 mg TEAC/g DW) is statistically similar with the powder extracts.

According to ABTS antioxidant capacity measurement, SNW and SNE results were statistically estimated similar (SNE: 299.39±15.57; SNW: 302.44±5.31 mg TEAC/g DW). Also the antioxidant capacities of EP extracts (EPE: 77.40±0.62 and EPW: 74.72±8.29 mg TEAC/g DW) and CI extracts (CIE: 14.70±0.51 and CIW: 8.93±1.18 mg TEAC/g DW) are statistically related. The decrease in the antioxidant capacity in lozenge processing, is consistent with the ABTS assay results. Cooking process leads to a significant decrease in total antioxidant content as stated in the study of Garretson et al. (2018). The loss of antioxidants due to cooking was found between 30 - 60%. These results were parallel with the study of Xu and Chang (2008), hence they estimated the percentage loss of antioxidant amount with ABTS assay, as 33 - 50%. According to the results in the study, this loss was correlated with the boiling and steaming processes in fruits and vegetables.

3.1.4 Correlation among TPC, TFC and TAC results

The correlations among all TPC, TFC and TAC results were analyzed by Pearson's correlation analysis method and computed as Pearson's correlation coefficients (R) that were given in Table 3.6. All relations were found as statistically significant ($p<0.01$). A significant positive relation was observed between TFC and TPC (R=0.977). This result shows that phenolic groups are dominating flavonoids in all extracts. CUPRAC method shows less relation with TFC (R=0.990) than TPC (R=0.996). ABTS method shows less close relationship among other methods.

According to Table 3.7, for ethanol extracts of the plants, both DPPH and ABTS assays did not show significant correlations with TPC ($p>0.05$). However, TFC or CUPRAC can be used to predict TPC of all plant extracts due to their good correlation (1.00, $p<0.05$). The CUPRAC assay was previously found to be a reliable technique of total antioxidant capacity detection and related with total phenolic content (Apak et al., 2006). Apak et al. stated that, the cupric ion reducing antioxidant capacity of herbal plants directly refers to their polyphenol content which is identified by Folin spectrophotometric method.

According to the values indicated in Table 3.7, ABTS and DPPH antioxidant capacity methods have also a significant relation (1.00, $p<0.05$). This argument is consistent with the correlation analysis results obtained from a previous study on elderberries. The correlation coefficients (R) of total polyphenols ($R=0.98$, $p<0.001$; $R=0.98$, $p<0.001$), total anthocyanins ($R=0.93$, $p<0.001$; $R=0.90$, $p<0.001$) and total flavonols ($R=0.79$, $p<0.01$; $R=0.73$, $p<0.05$) over DPPH and ABTS assays (consequently) were evaluated in elderberries. An important relation between total phenolic content and total antioxidant capacity was found as a result of the study of Jakobek et al. (2007).

Table 3.6 Pearson's correlation analysis for TPC, TFC, CUPRAC and ABTS methods.

Methods	TPC	TFC	CUPRAC
TFC	0.977	-	-
CUPRAC	0.996	0.990	-
ABTS	0.932	0.944	0.927

The regression variance analysis is statistically significant for all values ($p<0.01$).

Table 3.7 Pearson's correlation analysis for TPC, TFC, DPPH, CUPRAC and ABTS methods for ethanol extracts of the plants (SNE, EPE, CIE).

Methods	TPC	TFC	DPPH	CUPRAC
TFC	1.000*	-	-	-
DPPH	0.996	0.998*	-	-
CUPRAC	1.000*	1.000*	0.995	-
ABTS	0.993	0.996	1.000*	0.993

*****The regression variance analysis is statistically significant for values ($p<0.05$).

3.2 Determination of Phenolic Compounds by HPLC-PDA

CI, EP and SN were analyzed with HPLC-PDA and phenolic compounds were identified. Identification was made with the retention times and areas at the maximum wavelength based on the calibration curves presented in Appendix C and the chromatograms in Appendix D.

3.2.1 *Cetraria islandica* L.

Island moss extract samples were analyzed by HPLC at the highest absorbance 280 nm and the phenolic amount was given in Table 3.8. Any phenolic type was not identified in the analyses, but the most near spectrum was defined as term of 4-hydroxy benzoic acid. Bachereau and Asta (1997), were evaluated *Cetraria islandica* (L.) Ach. with RP-HPLC and as a result of the study, 12 phenolic compounds were separated but could not be identified. In another study proceeded by Zagoskina et al. (2013), aqueous extract of *Cetraria islandica* L. was evaluated at 280 and 330 nm with densitograms and p-hydroxybenzoic acid, vanillic acid and protocatechuic acid derivatives were identified.

The chromatograms for each CI sample: plant ethanol extract, plant water extract and water extract of lozenge form were shown in Figure D.1.1, Figure D.1.2 and Figure D.1.3 consequently, in Appendix D.

CIE showed a peak in minute 3, where it can be defined as protocetraric acid according to HPLC chromatogram of the natural tallus of *Cetraria islandica* L., evaluated by Yoshimura et al. (1994). In CIW and CIP, unidentified substances were detected, thus the peaks could only be quantified as indicated in Table 3.8.

Table 3.8 *Cetraria islandica* L. phenolic amount

Sample	Phenolic amount (µg/g DW)
CIE	887
CIW	1404
CIP	16

3.2.2 *Echinacea purpurea* L.

EP samples were analyzed by HPLC at the highest absorbance, 330 nm. The identified phenolics were shown in Table 3.9, Table 3.10 and Table 3.11. Chromatograms for each extract; EPE, EPW and EPP were shown as Figure D.2.1, Figure D.2.2 and Figure D.2.3 consequently, in Appendix D.

Caftaric acid and chicoric acid were the dominant phenolics in EPE and EPW, specifically chicoric acid was only identified in EPE and EPP. Oniszczuk et al.(2016) stated that the polyphenols as most active compounds in EP and they determined cichoric, caftaric and caffeic acid by HPLC. In *Echinacea purpurea* L., chlorogenic acid, cynarin and alkamide were previously identified with two different analysis methods RP-LC PAD (reversed phase liquid chromatography photodiode array detector) and HPLC UV (high-performance liquid chromatography ultraviolet detector) (Lee & Scagel, 2009; Lema- Rumińska et al., 2019; Pellati, Benvenuti, Magro, Melegari, & Soragni, 2004).

Table 3.9 *Echinacea purpurea* L. ethanol extract phenolic compounds

Compound	Area	Retention time	Factor	Amount (μg/g DW)
Caftaric acid	180072	10.167	$1x10^7$	450
Caffeic acid	46406	13.482	$4x10^7$	30
Chicoric acid	345277	24.117	$4x10^7$	220

Table 3.10 *Echinacea purpurea* L. water extract phenolic compounds

Compound	Area	Retention time	Factor	Amount (μg/g DW)
Caftaric acid	17929	10.261	$1x10^7$	17.93
Chicoric acid	26271	24.186	$4x10^7$	6.57

Table 3.11 *Echinacea purpurea* L. lozenge form water extract phenolic compounds

Compound	Area	Retention time	Factor	Amount (μg/g DW)
Caftaric acid	1229476	10.305	$1x10^7$	3070
Caffeic acid	72487	13.623	$4x10^7$	50
Chicoric acid	2364838	24.192	$4x10^7$	1480

3.2.3 *Sambucus nigra* L.

SN samples were analyzed in the highest absorbance; 280 nm with the relative phenolic compounds shown in Table 3.12, Table 3.13 and Table 3.14. Chromatograms for each extract SNE, SNW and SNP were presented as Figure D.3.1, Figure D.3.2 and Figure D.3.3 consequently, in Appendix D.

Most dominant phenolics in elderberry were identified as catechin, epicatechin, cyanidin 3-glucoside and kaempferol 3-rutinoside. Christensen et al. (2010), analyzed the methanol extract of *Sambucus nigra* L. and found caffeic acid, quercetin-3-O-rutinoside, quercetin-3-O-glucoside, kaempferol-3-O-rutinoside, isorhamnetin-3-O-rutinoside, isorhamnetin-3-O-glucoside, quercetin and kaempferol as plant metabolites.

In another study, similar phenolic compounds were separated with HPLC DAD and ESI/MS analysis. The most common ones were quercetin 3-O-rutinoside, quercetin 3-O-glucoside, Kaempferol 3-O-rutinoside, 3,5-di-O-caffeoylquinic acid and isorhamnetin 3-O-glucoside (Barros, Dueñas, Carvalho, Ferreira, & Santos-Buelga, 2012).

Table 3.12 *Sambucus nigra* L. ethanol extract phenolic compounds

Compound	Area	Retention time	Factor	Amount (μg/g DW)
Catechin	545203	10.934	$7x10^7$	380
Epicatechin	66186	13.974	$7x10^6$	470
Epicatechin	404354	14.761	$7x10^6$	2800
Cyanidin 3-glucoside	1655540	18.974	$3x10^7$	2760
Kaempferol 3- rutinosi	27840	19.889	$9x10^6$	150

Table 3.13 *Sambucus nigra* L. water extract phenolic compounds

Compound	Area	Retention time	Factor	Amount (μg/g DW)
Catechin	32695	11.389	$7x10^7$	4
Epicatechin	13787	15.188	$7x10^6$	10
Cyanidin 3-glucoside	62455	18.414	$3x10^7$	12

Table 3.14 *Sambucus nigra* L. lozenge form water extract phenolic compounds

Compound	Area	Retention time	Factor	Amount (μg/g DW)
Catechin	93581	10.923	$7x10^7$	670
Epicatechin	17472	12.968	$7x10^6$	1250
Epicatechin	57047	14.772	$7x10^6$	4070
Cyanidin 3-glucoside	79121	19.010	$3x10^7$	1840

CHAPTER 4

CONCLUSION AND RECOMMENDATIONS

In this study, the bioactive compounds in the powder extracts of *Cetraria islandica* L., *Echinacea purpurea* L. and *Sambucus nigra* L. were investigated by spectrophotometric methods: TPC, TFC and TAC assays with two different solvent in extraction: water and 75% ethanol. All analyses were also applied to the lozenge formulations containing plant extract powders to investigate the loss of bioactive compounds in the final product. The correlation analysis was performed for comparing the efficiency and relationships of each spectrophotometric analysis method on identifying the bioactive compounds. HPLC analysis was conducted to identify and quantify the phenolic components in each sample.

The solvent efficiency of 75% ethanol extracts was found to be significantly higher than water extracts in all methods of TPC, TFC, DPPH and CUPRAC, however the solvent type used in the extraction did not demonstrate a significant change in ABTS antioxidant activity method ($p>0.05$). According to all antioxidant activity analysis methods, the lozenge processing were found to lead antioxidant loss.

All the plant powders showed a similar trend in lozenge applications, since the statistical results exhibited no significant difference ($p<0.05$) in SNP, EPP and CIP samples. Among the antioxidant activity analysis methods, the radical scavenging potentials of DPPH and ABTS were evaluated. ABTS method of antioxidant activity showed higher results than ABTS in ethanol solvent extracted samples. CUPRAC method was found to be most efficient among others, due to highest content of bioactive compounds. The correlation analysis showed that, the total phenolic and

flavonoid contents exhibited a meaningful association with CUPRAC analysis method results ($p<0.05$).

HPLC analysis used in the study helped us to isolate and identify phenolic compounds and estimate their amounts for each plant. However, it was concluded that further work is needed to be proceeded to clarify the anti-oxidative components in *Cetraria islandica* L.

The results of the study indicated that all medicinal plant samples studied were good sources of phenolics, flavonoids and showed antioxidant properties. If an effective formulation with suitable production technique is developed, their applications in confectionery is possible as functional foods.

In future studies, regarding the changes in the bioactive compounds in lozenge processing, different formulations can be studied. The present findings are the data that contribute a baseline for different studies on new lozenge formulations that deal with many diseases. All of the extracts can be applied together in a lozenge formulation in different ratios and the usage ratios can be determined with optimization studies with Response Surface Methodology (RSM). The phenolic content of different lozenge formulations with different amount of medicinal plants can be compared with spectrophotometric analysis methods and HPLC analysis method to investigate possible health promotion. For further studies, *in vitro* bioavailability of phenolic compounds in the late release soft lozenge with this invaluable medicinal plants *Cetraria islandica* L., *Echinacea purpurea* L. and *Sambucus nigra* L. can be investigated.

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APPENDICES

APPENDIX A

CALIBRATION CURVES

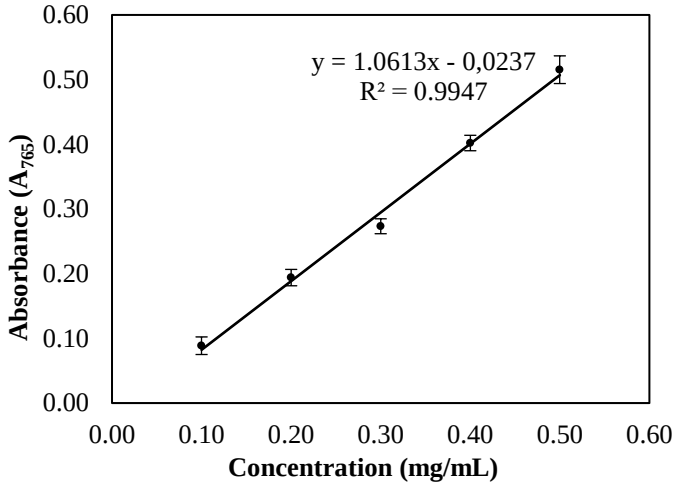

Figure A.1 Standard calibration curve of gallic acid.

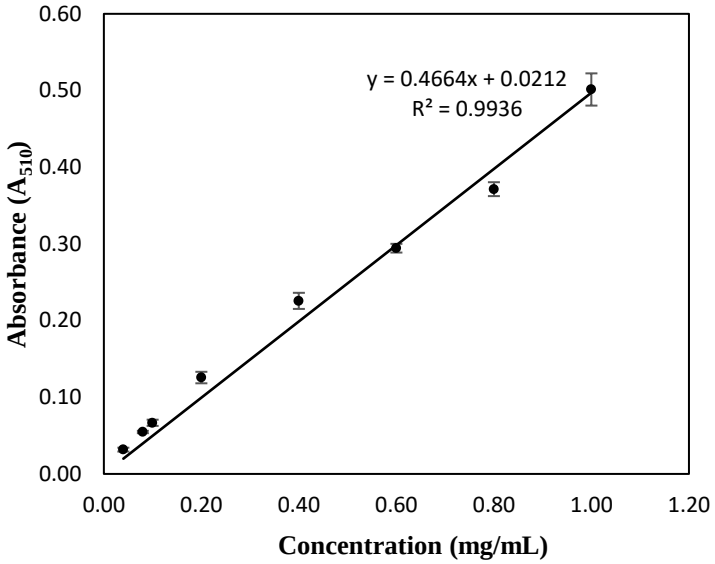

Figure A.2 Standard calibration curve of rutin.

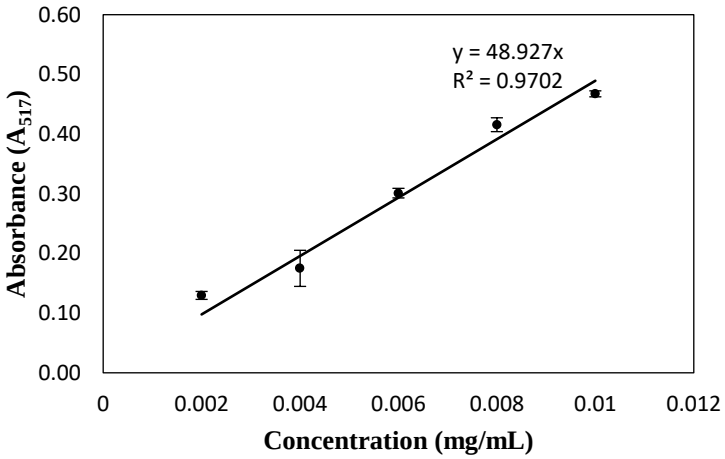

Figure A.3 Trolox standard calibration curve.

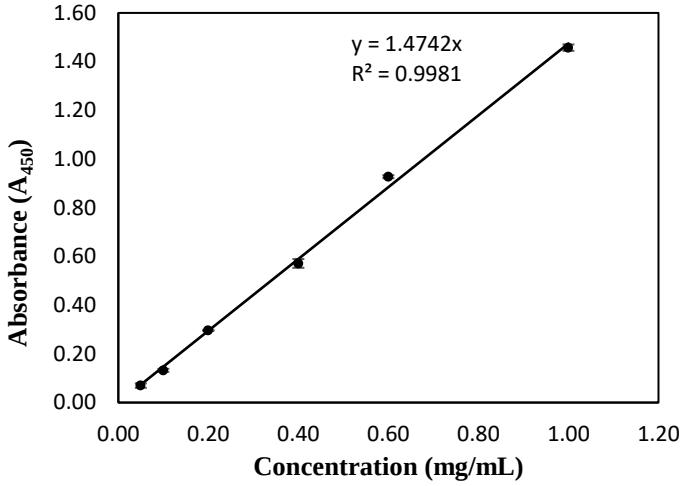

Figure A.4 Trolox standard calibration curve.

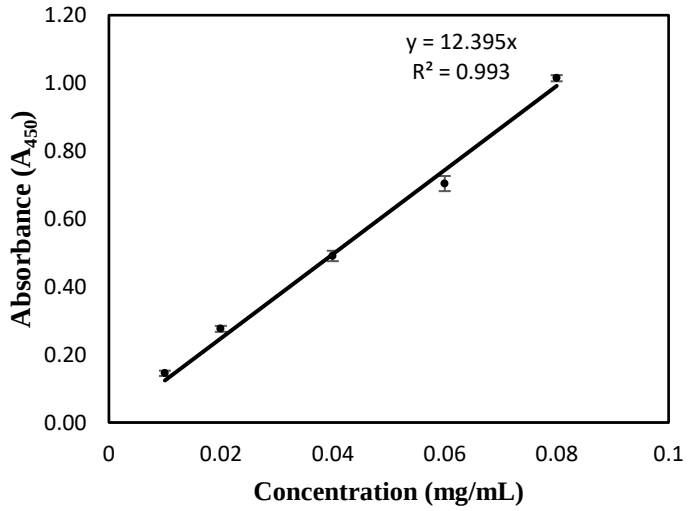

Figure A.5 Trolox standard calibration curve.

APPENDIX B

STATISTICAL ANALYSES

Table B.1.1 One way ANOVA and Tukey's comparison test for total phenolic content (TPC)

One-way ANOVA: SNE; SNW; SNP; EPE; EPW; EPP; CIE; CIW; CIP
Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	9	SNE; SNW; SNP; EPE; EPW; EPP; CIE; CIW; CIP

Analysis of Variance for TPC, using Adjusted SS for Tests

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	8	323967	40495.9	1337.88	0.000
Error	18	545	30.3		
Total	26	324512			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
5.50171	99.83%	99.76%	99.62%

Means

Factor	N	Mean	StDev	95% CI
SNE	3	339.680	1.470	(333.007; 346.353)
SNW	3	178.42	14.06	(171.75; 185.10)
SNP	3	2.3367	0.1069	(-4.3367; 9.0101)
EPE	3	38.48	8.48	(31.81; 45.15)
EPW	3	60.7700	0.1646	(54.0966; 67.4434)
EPP	3	1.2267	0.0643	(-5.4467; 7.9001)
CIE	3	2.620	0.819	(-4.053; 9.293)
CIW	3	8.0233	0.1193	(1.3499; 14.6967)
CIP	3	1.1600	0.0200	(-5.5134; 7.8334)

Pooled StDev = 5.50171

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping				
SNE	3	339.680	A				
SNW	3	178.42		B			
EPW	3	60.7700			C		
EPE	3	38.48				D	
CIW	3	8.0233					E
CIE	3	2.620					E
SNP	3	2.3367					E
EPP	3	1.2267					E
CIP	3	1.1600					E

Means that do not share a letter are significantly different.

Table B.1.2 One way ANOVA and Tukey's comparison test for total flavonoid content (TFC)

One-way ANOVA: SNE; SNW; SNP; EPE; EPW; EPP; CIE; CIW; CIP
Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	9	SNE; SNW; SNP; EPE; EPW; EPP; CIE; CIW; CIP

Analysis of Variance for TFC, using Adjusted SS for Tests

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	8	2207409	275926	5469.61	0.000
Error	18	908	50		
Total	26	2208317			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
7.10262	99.96%	99.94%	99.91%

Means

Factor	N	Mean	StDev	95% CI
SNE	3	840.54	13.46	(831.92; 849.16)
SNW	3	527.02	13.79	(518.41; 535.64)
SNP	3	5.237	0.225	(-3.379; 13.852)
EPE	3	112.00	4.00	(103.38; 120.62)
EPW	3	329.55	8.04	(320.93; 338.17)
EPP	3	1.937	0.265	(-6.679; 10.552)
CIE	3	2.800	1.260	(-5.815; 11.415)
CIW	3	3.783	0.375	(-4.832; 12.399)
CIP	3	1.120	0.270	(-7.495; 9.735)

Pooled StDev = 7.10262

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping			
SNE	3	840.54	A			
SNW	3	527.02		B		
EPW	3	329.55			C	
EPE	3	112.00				D
SNP	3	5.237					E
CIW	3	3.783					E
CIE	3	2.800					E
EPP	3	1.937					E
CIP	3	1.120					E

Means that do not share a letter are significantly different.

Table B.1.3 One way ANOVA and Tukey's comparison test for DPPH method for total antioxidant capacity (TAC)

One-way ANOVA: SNE; EPE; CIE

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	3	SNE; EPE; CIE

Analysis of Variance for DPPH Method, using Adjusted SS for Tests

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	3147.2	1573.60	91.81	0.000
Error	6	102.8	17.14		
Total	8	3250.0			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
4.13999	96.84%	95.78%	92.88%

Means

Factor	N	Mean	StDev	95% CI
SNE	3	43.48	6.85	(37.63; 49.33)
EPE	3	8.89	2.12	(3.04; 14.74)
CIE	3	0.1800	0.0500	(-5.6687; 6.0287)

Pooled StDev = 4.13999

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
SNE	3	43.48	A
EPE	3	8.89	B
CIE	3	0.1800	B

Means that do not share a letter are significantly different.

Table B.1.4 One way ANOVA and Tukey's comparison test for CUPRAC method for total antioxidant capacity (TAC)

One-way ANOVA: SNE; SNW; SNP; EPE; EPW; EPP; CIE; CIW; CIP
Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	9	SNE; SNW; SNP; EPE; EPW; EPP; CIE; CIW; CIP

Analysis of Variance for CUPRAC Method, using Adjusted SS for Tests

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	8	2306818	288352	741.26	0.000
Error	18	7002	389		
Total	26	2313820			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
19.7231	99.70%	99.56%	99.32%

Means

Factor	N	Mean	StDev	95% CI
SNE	3	901.6	24.5	(877.7; 925.5)
SNW	3	472.1	18.1	(448.2; 496.0)
SNP	3	4.0767	0.0451	(-19.8468; 28.0002)
EPE	3	91.83	4.44	(67.90; 115.75)
EPW	3	242.0	50.5	(218.1; 265.9)
EPP	3	1.6467	0.0651	(-22.2768; 25.5702)
CIE	3	3.4233	0.1650	(-20.5002; 27.3468)
CIW	3	16.307	0.685	(-7.617; 40.230)
CIP	3	1.1633	0.0551	(-22.7602; 25.0868)

Pooled StDev = 19.7231

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping				
SNE	3	901.6	A				
SNW	3	472.1		B			
EPW	3	242.0			C		
EPE	3	91.83				D	
CIW	3	16.307					E
SNP	3	4.0767					E
CIE	3	3.4233					E
EPP	3	1.6467					E
CIP	3	1.1633					E

Means that do not share a letter are significantly different.

Table B.1.5 One way ANOVA and Tukey's comparison test for ABTS method for total antioxidant capacity (TAC)

One-way ANOVA: SNE; SNW; SNP; EPE; EPW; EPP; CIE; CIW; CIP

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	9	SNE; SNW; SNP; EPE; EPW; EPP; CIE; CIW; CIP

Analysis of Variance for ABTS Method, using Adjusted SS for Tests

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	8	376190	47023.7	1239.74	0.000
Error	18	683	37.9		
Total	26	376872			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
6.15876	99.82%	99.74%	99.59%

Means

Factor	N	Mean	StDev	95% CI
SNE	3	299.39	15.57	(291.92; 306.86)
SNW	3	302.44	5.31	(294.97; 309.91)
SNP	3	0.8200	0.0400	(-6.6504; 8.2904)
EPE	3	77.397	0.625	(69.926; 84.867)
EPW	3	74.72	8.29	(67.25; 82.19)
EPP	3	0.7500	0.0200	(-6.7204; 8.2204)
CIE	3	14.703	0.505	(7.233; 22.174)
CIW	3	8.930	1.180	(1.460; 16.400)
CIP	3	0.7200	0.0200	(-6.7504; 8.1904)

Pooled StDev = 6.15876

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping		
SNW	3	302.44	A		
SNE	3	299.39	A		
EPE	3	77.397		B	
EPW	3	74.72		B	
CIE	3	14.703			C
CIW	3	8.930			C
SNP	3	0.8200			C
EPP	3	0.7500			C
CIP	3	0.7200			C

Means that do not share a letter are significantly different.

Table B.2.1 One way ANOVA and Tukey's comparison test for total phenolic content (TPC) within the same plant

One-way ANOVA: SNE; SNW; SNP
Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	3	SNE; SNW; SNP

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	170811	85405.4	1282.35	0.000
Error	6	400	66.6		
Total	8	171210			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
8.16092	99.77%	99.69%	99.47%

Means

Factor	N	Mean	StDev	95% CI
SNE	3	339.680	1.470	(328.151; 351.209)
SNW	3	178.42	14.06	(166.89; 189.95)
SNP	3	2.3367	0.1069	(-9.1925; 13.8658)

Pooled StDev = 8.16092

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
SNE	3	339.680	A
SNW	3	178.42	B
SNP	3	2.3367	C

Means that do not share a letter are significantly different.

One-way ANOVA: EPE; EPW; EPP

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	3	EPE; EPW; EPP

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	5430.1	2715.03	113.23	0.000
Error	6	143.9	23.98		
Total	8	5573.9			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
4.89668	97.42%	96.56%	94.19%

Means

Factor	N	Mean	StDev	95% CI
EPE	3	38.48	8.48	(31.56; 45.40)
EPW	3	60.7700	0.1646	(53.8523; 67.6877)
EPP	3	1.2267	0.0643	(-5.6910; 8.1443)

Pooled StDev = 4.89668

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
EPW	3	60.7700	A
EPE	3	38.48	B
EPP	3	1.2267	C

Means that do not share a letter are significantly different.

One-way ANOVA: CIE; CIW; CIP
Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	3	CIE; CIW; CIP

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	78.433	39.2165	171.84	0.000
Error	6	1.369	0.2282		
Total	8	79.802			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.477714	98.28%	97.71%	96.14%

Means

Factor	N	Mean	StDev	95% CI
CIE	3	2.620	0.819	(1.945; 3.295)
CIW	3	8.0233	0.1193	(7.3485; 8.6982)
CIP	3	1.1600	0.0200	(0.4851; 1.8349)

Pooled StDev = 0.477714

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
CIW	3	8.0233	A
CIE	3	2.620	B
CIP	3	1.1600	C

Means that do not share a letter are significantly different.

Table B.2.2 One way ANOVA and Tukey's comparison test for total flavonoid content (TFC) within the same plant

One-way ANOVA: SNE; SNW; SNP

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	3	SNE; SNW; SNP

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	1068286	534143	4313.27	0.000
Error	6	743	124		
Total	8	1069029			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
11.1282	99.93%	99.91%	99.84%

Means

Factor	N	Mean	StDev	95% CI
SNE	3	840.54	13.46	(824.82; 856.26)
SNW	3	527.02	13.79	(511.30; 542.74)
SNP	3	5.237	0.225	(-10.484; 20.958)

Pooled StDev = 11.1282

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
SNE	3	840.54	A
SNW	3	527.02	B
SNP	3	5.237	C

Means that do not share a letter are significantly different.

One-way ANOVA: EPE; EPW; EPP

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	3	EPE; EPW; EPP

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	166772	83386.2	3099.40	0.000
Error	6	161	26.9		
Total	8	166934			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
5.18690	99.90%	99.87%	99.78%

Means

Factor	N	Mean	StDev	95% CI
EPE	3	112.00	4.00	(104.67; 119.33)
EPW	3	329.55	8.04	(322.22; 336.88)
EPP	3	1.937	0.265	(-5.391; 9.264)

Pooled StDev = 5.18690

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
EPW	3	329.55	A
EPE	3	112.00	B
EPP	3	1.937	C

Means that do not share a letter are significantly different.

One-way ANOVA: CIE; CIW; CIP

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	3	CIE; CIW; CIP

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	10.883	5.4413	9.06	0.015
Error	6	3.602	0.6004		
Total	8	14.485			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.774840	75.13%	66.84%	44.04%

Means

Factor	N	Mean	StDev	95% CI
CIE	3	2.800	1.260	(1.705; 3.895)
CIW	3	3.783	0.375	(2.689; 4.878)
CIP	3	1.120	0.270	(0.025; 2.215)

Pooled StDev = 0.774840

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping	
CIW	3	3.783	A	
CIE	3	2.800	A	B
CIP	3	1.120		B

Means that do not share a letter are significantly different.

Table B.2.3 One way ANOVA and Tukey's comparison test CUPRAC method for total antioxidant capacity (TAC) within the same plant

One-way ANOVA: SNE; SNW; SNP

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	3	SNE; SNW; SNP

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	1209125	604562	1952.14	0.000
Error	6	1858	310		
Total	8	1210983			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
17.5981	99.85%	99.80%	99.65%

Means

Factor	N	Mean	StDev	95% CI
SNE	3	901.6	24.5	(876.8; 926.5)
SNW	3	472.1	18.1	(447.2; 496.9)
SNP	3	4.0767	0.0451	(-20.7846; 28.9379)

Pooled StDev = 17.5981

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
SNE	3	901.6	A
SNW	3	472.1	B
SNP	3	4.0767	C

Means that do not share a letter are significantly different.

One-way ANOVA: EPE; EPW; EPP

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	3	EPE; EPW; EPP

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	88465	44232.3	51.60	0.000
Error	6	5143	857.1		
Total	8	93607			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
29.2771	94.51%	92.67%	87.64%

Means

Factor	N	Mean	StDev	95% CI
EPE	3	91.83	4.44	(50.47; 133.19)
EPW	3	242.0	50.5	(200.7; 283.4)
EPP	3	1.6467	0.0651	(-39.7138; 43.0071)

Pooled StDev = 29.2771

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
EPW	3	242.0	A
EPE	3	91.83	B
EPP	3	1.6467	C

Means that do not share a letter are significantly different.

One-way ANOVA: CIE; CIW; CIP

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	3	CIE; CIW; CIP

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	400.408	200.204	1202.43	0.000
Error	6	0.999	0.167		
Total	8	401.407			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.408044	99.75%	99.67%	99.44%

Means

Factor	N	Mean	StDev	95% CI
CIE	3	3.4233	0.1650	(2.8469; 3.9998)
CIW	3	16.307	0.685	(15.730; 16.883)
CIP	3	1.1633	0.0551	(0.5869; 1.7398)

Pooled StDev = 0.408044

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
CIW	3	16.307	A
CIE	3	3.4233	B
CIP	3	1.1633	C

Means that do not share a letter are significantly different.

Table B.2.4 One way ANOVA and Tukey's comparison test ABTS method for total antioxidant capacity (TAC) within the same plant

One-way ANOVA: SNE; SNW; SNP
Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	3	SNE; SNW; SNP

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	180126	90063.0	998.14	0.000
Error	6	541	90.2		
Total	8	180667			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
9.49900	99.70%	99.60%	99.33%

Means

Factor	N	Mean	StDev	95% CI
SNE	3	299.39	15.57	(285.97; 312.81)
SNW	3	302.44	5.31	(289.02; 315.86)
SNP	3	0.8200	0.0400	(-12.5995; 14.2395)

Pooled StDev = 9.49900

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
SNW	3	302.44	A
SNE	3	299.39	A
SNP	3	0.8200	B

Means that do not share a letter are significantly different.

One-way ANOVA: EPE; EPW; EPP

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	3	EPE; EPW; EPP

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	11353.0	5676.48	246.69	0.000
Error	6	138.1	23.01		
Total	8	11491.0			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
4.79695	98.80%	98.40%	97.30%

Means

Factor	N	Mean	StDev	95% CI
EPE	3	77.397	0.625	(70.620; 84.173)
EPW	3	74.72	8.29	(67.94; 81.49)
EPP	3	0.7500	0.0200	(-6.0268; 7.5268)

Pooled StDev = 4.79695

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
EPE	3	77.397	A
EPW	3	74.72	A
EPP	3	0.7500	B

Means that do not share a letter are significantly different.

One-way ANOVA: CIE; CIW; CIP

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	3	CIE; CIW; CIP

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	296.269	148.135	269.69	0.000
Error	6	3.296	0.549		
Total	8	299.565			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.741133	98.90%	98.53%	97.52%

Means

Factor	N	Mean	StDev	95% CI
CIE	3	14.703	0.505	(13.656; 15.750)

CIW	3	8.930	1.180	(7.883; 9.977)
CIP	3	0.7200	0.0200	(-0.3270; 1.7670)

Pooled StDev = 0.741133

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping		
CIE	3	14.703	A		
CIW	3	8.930		B	
CIP	3	0.7200			C

Means that do not share a letter are significantly different.

APPENDIX C

HPLC CALIBRATION TABLES

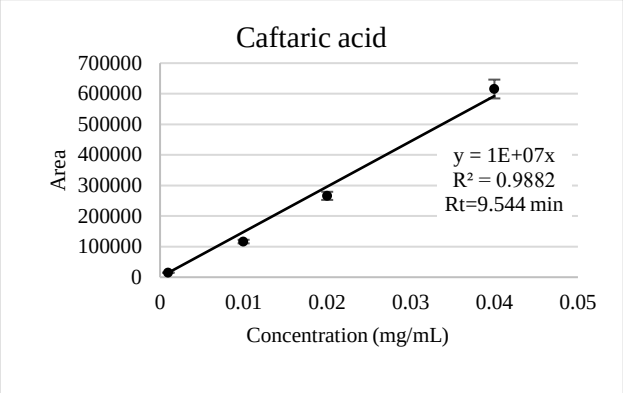

Figure C.1 Standard calibration curve of Caftaric acid.

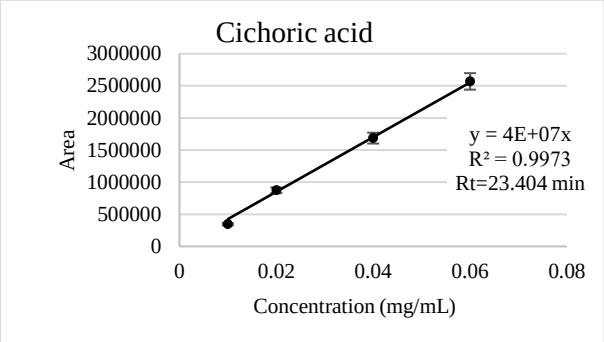

Figure C.2 Standard calibration curve of Cichoric acid.

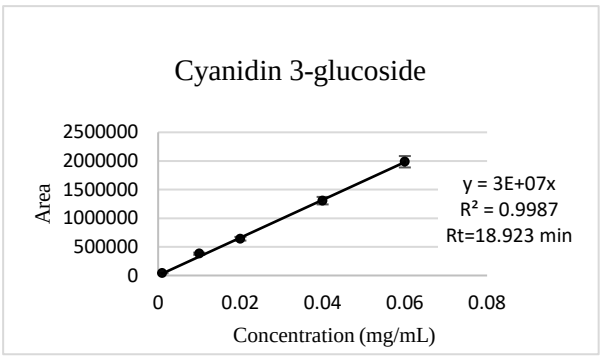

Figure C.3 Standard calibration curve of Cyanidin 3-glucoside.

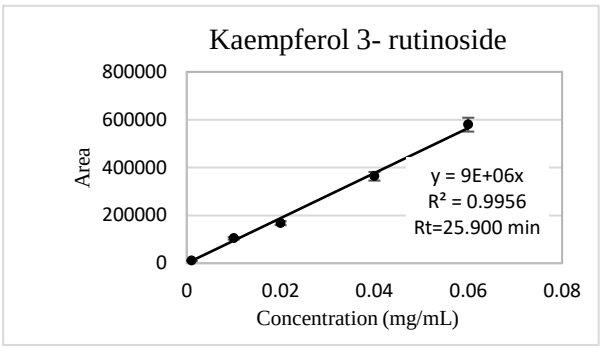

Figure C.4 Standard calibration curve of Kaempferol 3-rutinoside.

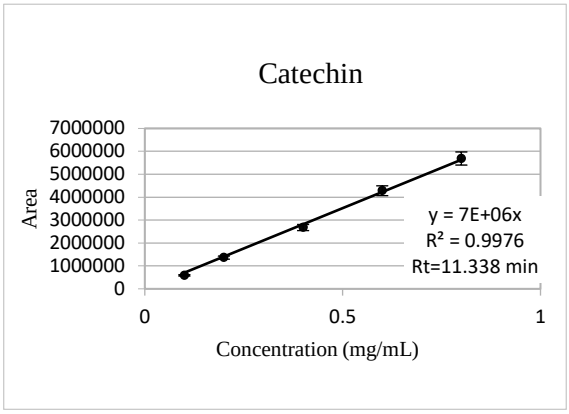

Figure C.5 Standard calibration curve of Catechin.

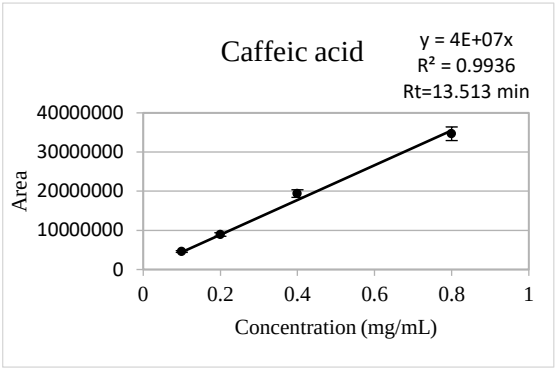

Figure C.6 Standard calibration curve of Caffeic acid.

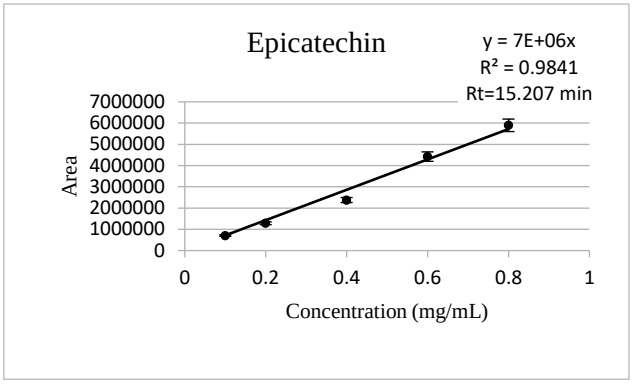

Figure C.7 Standard calibration curve of Epicatechin.

APPENDIX D

HPLC CHROMATOGRAMS

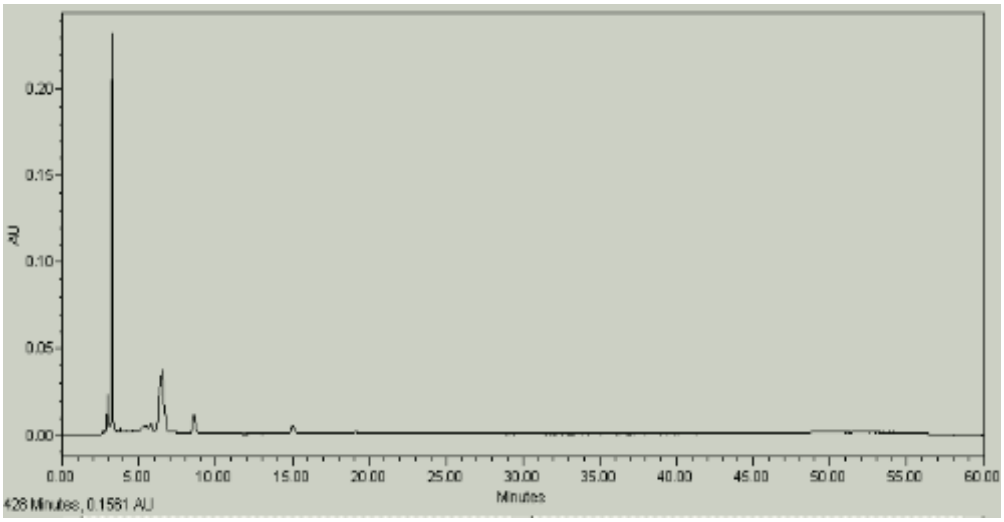

Figure D.1.1 Island moss ethanol extract (CIE) chromatogram (280 nm)

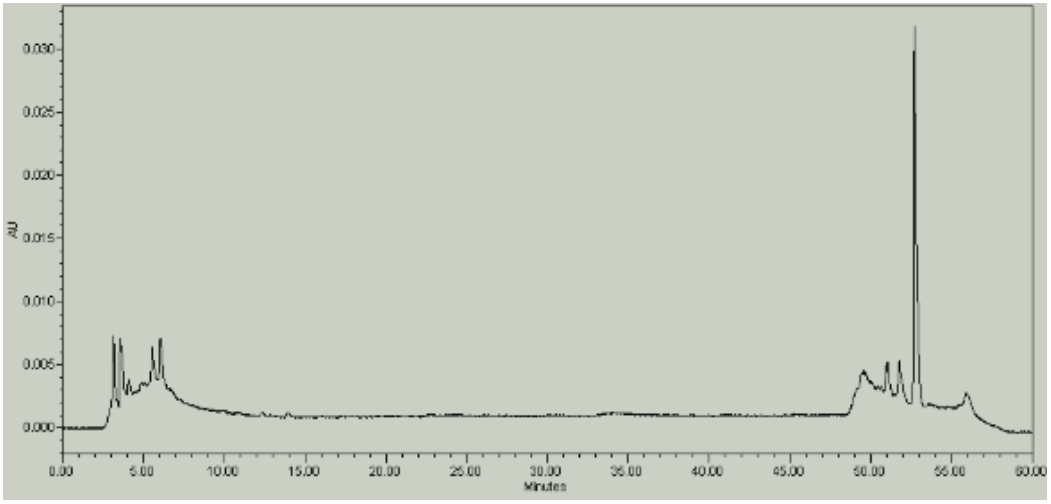

Figure D.1.2 Island moss water extract (CIW) chromatogram (280 nm)

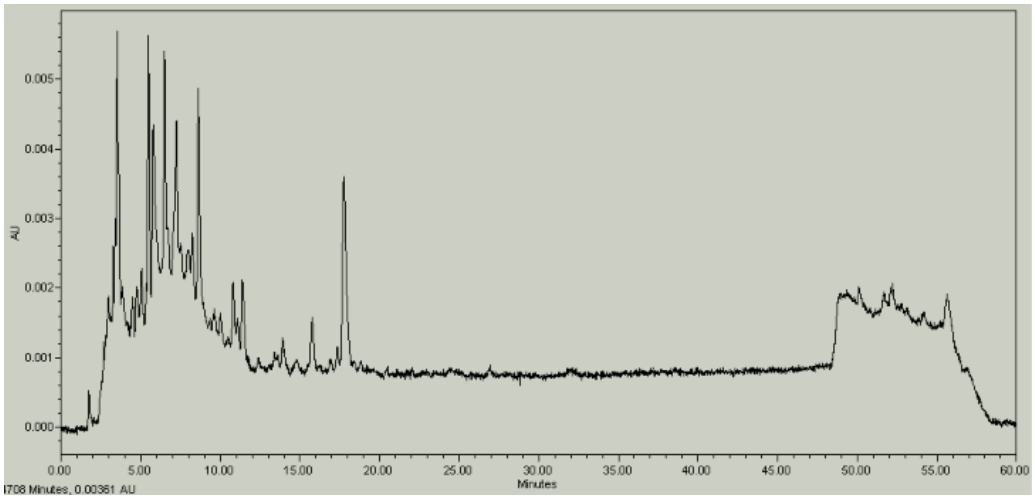

Figure D.1.3 Island moss lozenge form - water extract (CIP) chromatogram (280 nm)

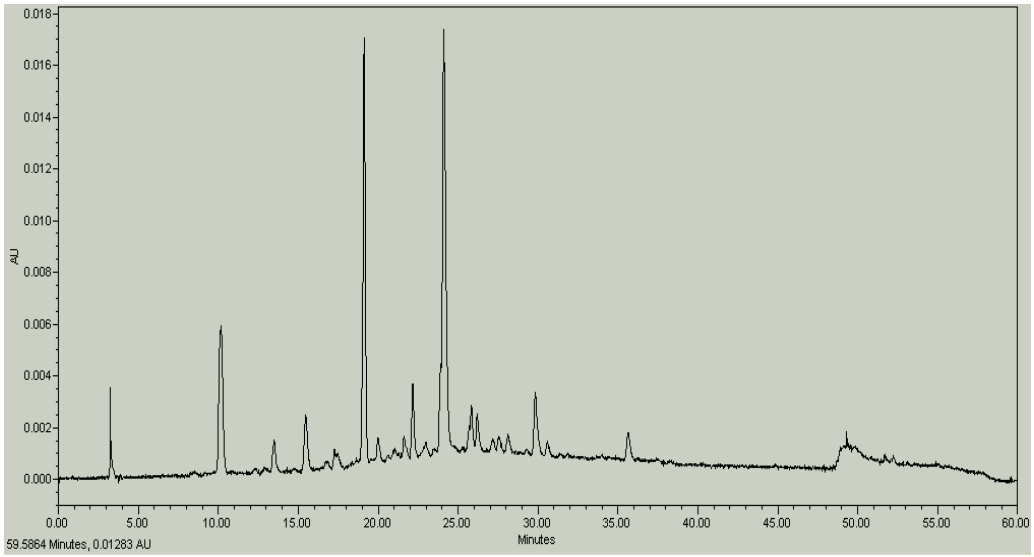

Figure D.2.1 *Echinacea purpurea* L. ethanol extract (EPE) chromatogram (330 nm)

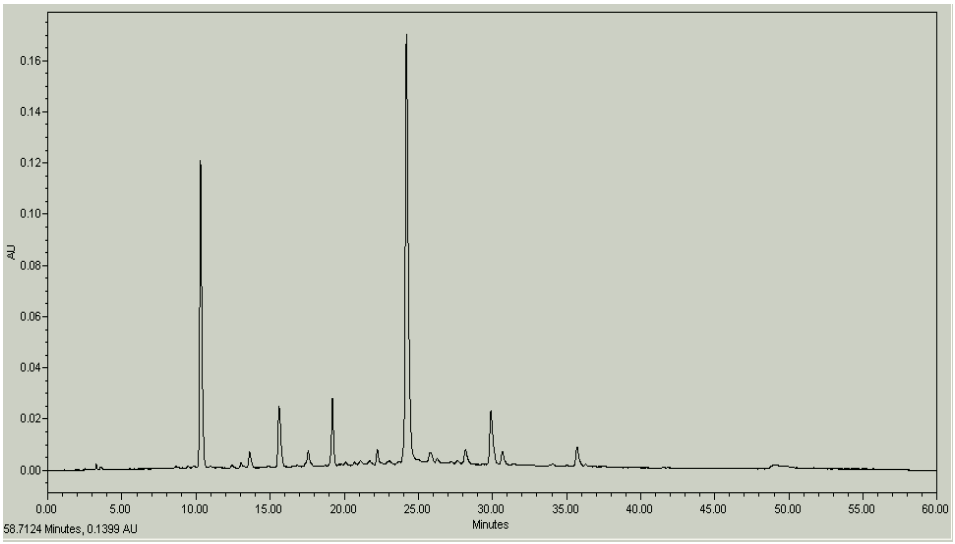

Figure D.2.2 *Echinacea purpurea* L. water extract (EPW) chromatogram (330 nm)

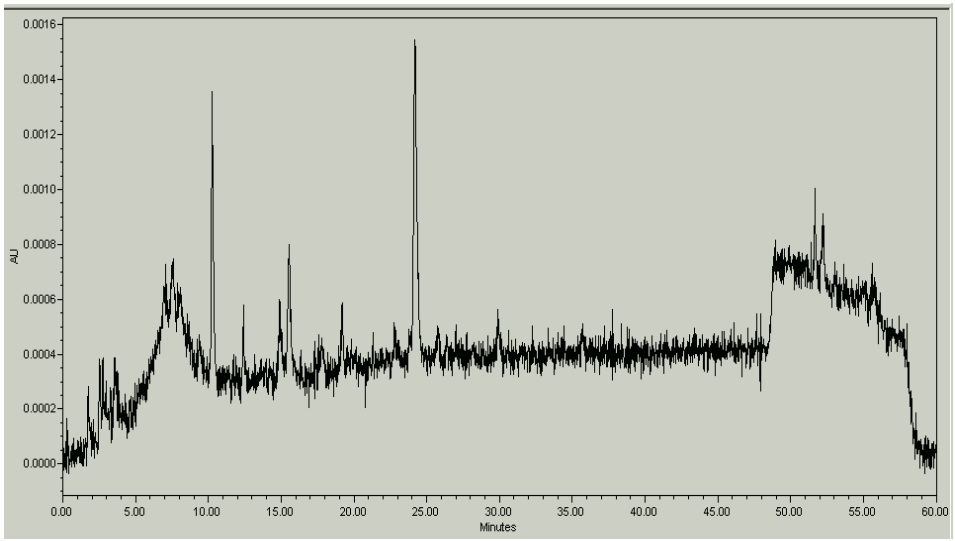

Figure D.2.3 *Echinacea purpurea* L. lozenge form - water extract (EPP) chromatogram (330 nm)

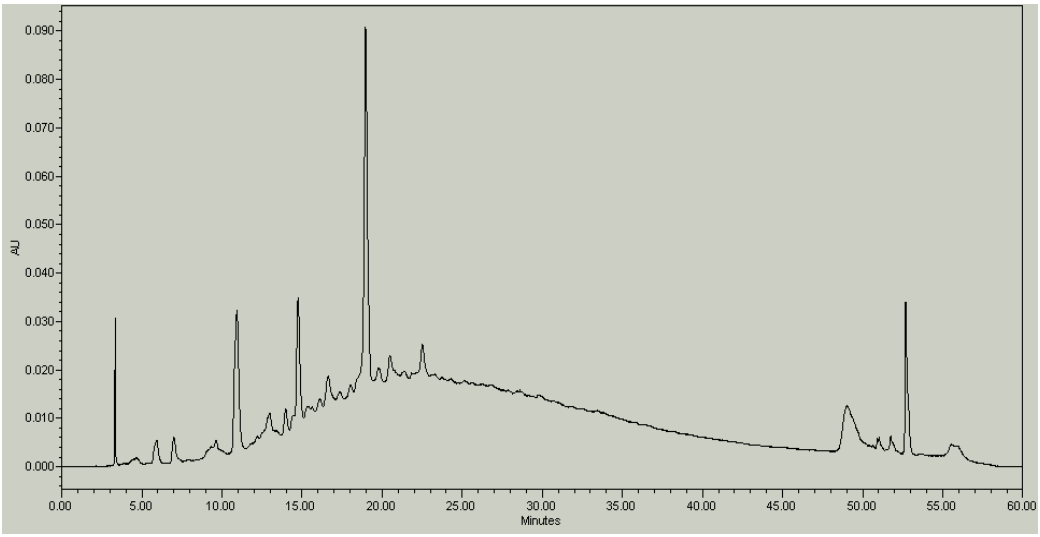

Figure D.3.1 Elderberry ethanol extract (SNE) chromatogram (280 nm)

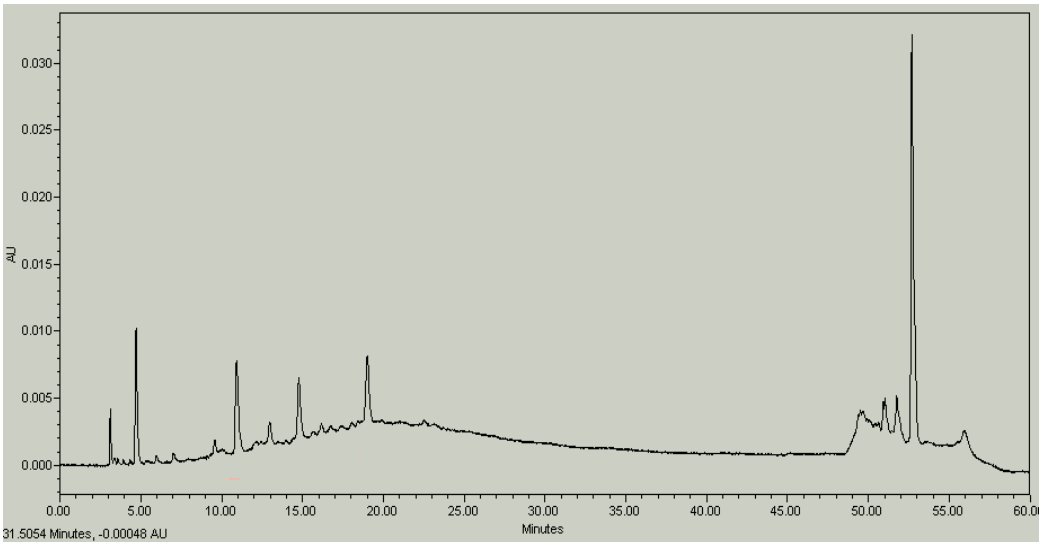

Figure D.3.2 Elderberry water extract (SNW) chromatogram (280 nm)

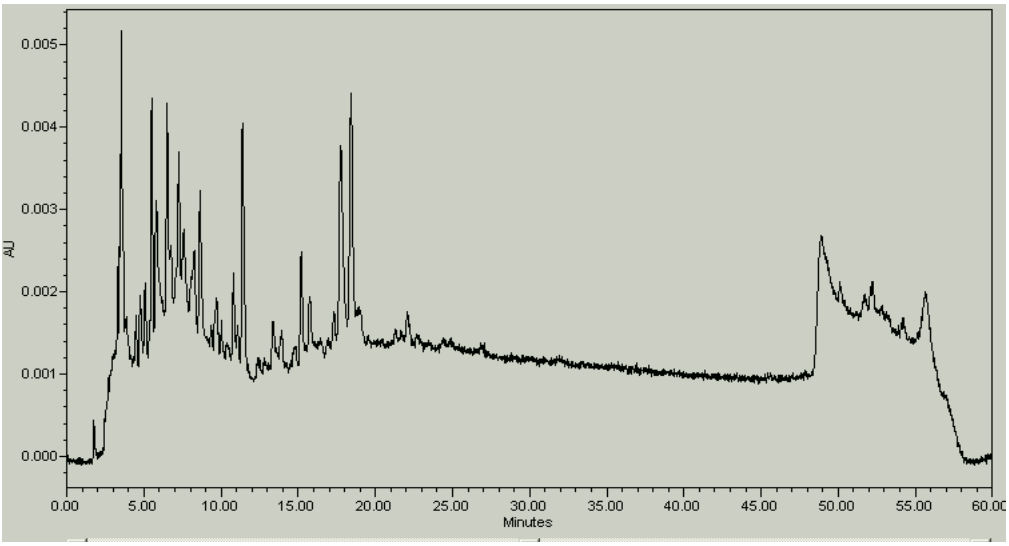

Figure D.3.3 Elderberry lozenge form - water extract (SNP) chromatogram (280 nm)