CHAPTER 5

EFFECT OF DIFFERENT SOLVENTS ON TOTAL PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY OF ELDERBERRY (*Sambucus nigra* L.) FLOWERS

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INTRODUCTION

Sambucus nigra L., which is also called elderberry and belonging to the Adoxaceae family, is a shrub-shaped (Ho et al., 2016) herb with small dark colored fruits and white flowers that matures in late summer, native to Europe, Asia and North Africa (Mandrone et al., 2014). Its creamy white flowers are used in the production of soft drinks in England, Sweden and Denmark with their distinctive scent (Ho et al., 2016). The productive parts of elderberry are the fruit and flower extract (Młynarczyk et al., 2018). Elderberry (*Sambucus nigra* L.) is a rich source of biologically active polyphenols, especially phenolic acids, flavonols and anthocyanins (Sidor and Gramza-Michałowska, 2015). These polyphenols are found in the leaves, fruits and flowers of elderberry (Veberic vd. 2009, Mandrone vd. 2014, Sidor and Gramza-Michałowska 2015). Therefore, a diet containing elderberry fruit, leaf and flower constitutes a potential protective agent against the negative effects of oxidative stress in the human body (Sidor and Gramza-Michałowska, 2015). Elderberry flowers are used in traditional medicine to treat inflammation, joint pains, skin disorders, diuretic, colds, fever and other respiratory disturbances. It has been stated in studies that elderberry flowers have pharmacological effects such as blood sugar regulator, diuretic, antibacterial, antifungal, antiviral, immunomodulation, anti-inflammatory, antioxidative (Ho et al., 2016).

Phenolic compounds are secondary metabolites in plants that can inhibit the reactive free radicals produced by lipid oxidation and chelate redox active metal ions (Rezaie et al., 2015). Phenolic compounds are

the main antioxidant components and their total contents are directly proportional to their antioxidant activity (Do et al., 2014).

Oxidation processes are very important for the living organism. The uncontrolled production of free oxygen radicals and unbalanced mechanism of antioxidant protection cause the onset of many diseases and accelerate aging (Dawidowicz et al., 2006). Antioxidants are considered protective agents that reduce oxidative damage to the human body (Dawidowicz et al., 2006; Do et al., 2014). In recent years, many plants, especially medicinal and aromatic plants, have been intensively studied due to their antioxidant activities. It is believed that the intake of food rich in natural antioxidants is associated with the prevention of degenerative diseases due to oxidative stress, especially cardiovascular diseases and cancer (Pe´rez-Jime´nez et al., 2008; Rezaie et al., 2014). Studies are being carried out on medicinal and aromatic or derived antioxidants from other plants to develop natural antioxidant formulations to replace synthetic antioxidants (Rezaie et al., 2014), that are suspected to have potentially toxic effects for food, cosmetics and other applications (Miliauskas et al., 2004).

The solubility of phenolic compounds are affected by many factors such as the type of solvent used, the degree of polymerization of phenolic compounds, interactions between phenolics and other plant components, and the structure of insoluble complexes. Therefore, there is no universal method suitable for the extraction of all plant phenolics (Rezaie et al., 2015).

There is no detailed study on the effect of different solvents on TFC and antioxidant activity of elderberry flower (*S. nigra* L.). Therefore, this study was planned to determine the effects of different extraction solvents on the total phenolic content and antioxidant activity of elderberry flower (*S. nigra* L.).

1. MATERIALS AND METHODS

1.1. Material

Elderberry (*S.nigra* L.) flowers were collected at Sivas Cumhuriyet University Campus. The collected elderberry flowers were dried in the shade. Then, after the samples were ground in a coffee grinder (SİNBO), they were passed through sieves (RETSCH) with 300 µm and 150 µm aperture sizes, and samples in this range were used in the analyses. The samples stored at + 4^{o}C before experiments.

1.2. Chemicals

The solvents used in the extraction in the study acetone, methanol and ethanol were purchased from Riedel-de Haën. Folin–Ciocalteu's reagent and sodium carbonate were purchased from Merck (Darmstadt-Germany), DPPH (2,2-diphenyl-1-icryhydrazil) from Sigma Chemical Co. (St. Louis, MO, USA).

1.3. Extraction of Samples

Water and three different concentrations (50%, 80% and 80%) of methanol, ethanol and acetone were used in the extraction of the

samples. 0.2 g sample was extracted with 10 ml of distilled water and solvents for 1 h on a horizontal shaker (250 rpm). After extraction, the samples were filtered through filter paper. Extraction was carried out in triplicate. The extracts were diluted five times with distilled water to obtain appropriate absorbance values. extracts were stored at -18 °C until analysis.

1.4. Total Phenolic Content

The total phenolic content (TPC) of the samples was determined using the ISO (14502-:2005) method. 0.5 ml of extract was mixed with 2.5 ml of Folin-Ciocalteu (10%, v/v) reagent. 0.5 mL of extract or pure water (as a blank) was mixed with 2.5 mL of Folin-Ciocalteu rea-gent (10%, v/v). 2 mL sodium carbonate solution (7.5% w/v) is added to the mixture after 5 min and shaken thoroughly. The mixture was allowed to stand for 60 min and blue color formed was measured at 765 nm against blank using a spectrophotometer (Optima SP3000). The results were calculated from the regression equation of the calibration curve [y(absorbance)=0.0116 x (gallic acid concentration), R^2 =0.999] obtained with the gallic acid working solutions prepared at different concentrations (5-50 µg/ml) and expressed as mg Gallic acid equivalent (GAE)/g dry matter (DM).

1.5. Antioxidant Activity

Antioxidant activity was determined by using DPPH method of Turkmen et al., 2009). In this method, 50 µl of extract (2 mg/ml) was mixed with 1950 µl of DPPH radical ($6x10^{-5}$ M, prepared in methanol).

In the control sample, distilled water was used instead of the extract. The mixture was vortex-mixed and let to stand at room temperature in the dark for 60 min. After absorbance at 517 nm was measured using a spectrophotometer using methanol as a blank. Antioxidant activity (inhibition %) was determined by the following equation (Yen and Duh, 1994).

$$Antioxidant\ activity\ (\text{Inhibition }\%) = \frac{Abs_{Control} - Abs_{\text{Sample}}}{Abs_{Control}} x100$$

Abs control is the absorbance of the DPPH solution without sample and *Abs sample* is the absorbance of the test sample.

1.6. Statistical Analysis

Statistical analyzes were performed with variance analysis using one-way ANOVA in the MINITAB 18 program. Results are given as the mean ± standard deviation of triplicate measurements. Means were compared by using Tukey's multiple comparison test ($p<0.05$).

2. RESULTS AND DISCUSSION

2.1. Total Phenolic Content

TPC of elderberry flowers is examined and presented in Table 1. The results indicated that TPC of *S. nigra* L. varied in the range of 3.31-59.71 mg GAE /g DM. Mikulic-Petkovsek et al., (2016) found the TPC of 40.14 mg GAE /g DM in elderberry flowers after extraction with methanol containing 3% (v/v) formic acid in an ultrasonic bath for 1

hour. They found it higher than the amount of TPC in this study (32.03 mg GAE /g DM). The TPC found by the researchers is higher than the TPC (32.03 mg GAE /g DM) obtained from 100 % methanol extraction in this study.

Table 1: Effect of different solvents on TPC (mg GAE/g DM) and antioxidant activity (inhibition %) of elderberry flowers

Solvents	TFC	Inhibition %
Water	35.54 ± 0.29	47.25 ± 0.15
Methanol		
50 %	51.89 ± 1.47[a]	74.44 ± 0.14[a]
80 %	48.43 ± 0.17[b]	73.55 ± 0.15[b]
100 %	32.03 ± 1.46[c]	35.51 ± 0.39[c]
Ethanol		
50 %	53.58 ± 0.37[a]	78.60 ± 1.04[a]
80 %	47.68 ± 0.34[b]	71.57 ± 0.48[b]
100 %	9.83 ± 0.17[c]	31.65 ± 1.27[c]
Acetone		
50 %	59.71 ± 0.39[a]	85.74 ± 0.45[a]
80 %	55.40 ± 0.35[b]	80.88 ± 0.52[b]
100 %	3.31 ± 0.47[c]	24.47 ± 0.76[c]

*: For each organic solvent, values in the same column bearing different letters are significantly different at $p<0.05$.

This difference is thought to be due to the formic acid added to the methanol solution and the extraction method. Viapiana and Wesolowski (2017) found the TPC of 15.23-35.57 mg GAE /g DM after 15 minutes extraction with boiling water in elderberry flowers from

different regions. In this study, the of TPC (35.54 mg GAE / g DM) found as a result of the extraction with water is among these values. Pavlović et al., (2013) determined the of TPC after the extraction with water at 95 ^{o}C for 15 minutes, as 42.67 mg GAE/g DM, higher than the extraction with water in this study. The reason for this difference is thought to be due to the extraction temperature and time. The temperature of the water used in the extraction is very effective in the extraction of phenolic compounds (Yang et al., 2007).

Extracting solvent significantly affected TPC of elderberry flowers extracts ($p<0.05$). All extracts prepared with 50% solvents contained highest level of TPC and followed by those with 80% and 100% solvents, respectively. The highest TPC (59.71 mg GAE /g DM) was found in 50% acetone extract. The lowest amounts of TPC were obtained with 100% acetone (3.31 mg GAE /g DM) and 100% ethanol (9.83 mg GAE /g DM) extract, respectively. The results show that with the increase in the polarity of the solvent used, more TPC is obtained. These results are in agreement with studies (Türkmen et al., 2006; Sultana et al., 2009; Bhebbe et al., 2016) showing that the aqueous forms of the solutions used in the extraction extract higher TPC.

In general, the extractability of phenolic compounds depends on the solvents used in the extraction, the solute-solvent ratio, and the polarity of the solvent (Nguyen et al., 2022). In addition, the solubility of phenolic compounds in extraction solvents is also effective on the recovery of certain phenolic compounds. Extraction efficiency also depends on the polarity of certain phenolic compounds. Not all phenolic

compounds can be extracted efficiently with organic solvents. The solubility trend is associated with the stereochemistry (the polar and the nonpolar fragment within their molecules) of phenolics and the intermolecular strength of hydrogen bonds that occur between phenolic compounds and solvents (Lopez-Perea et al., 2019). Soluble phenolic compounds are mainly distributed in cell vacuoles, while most lignin, flavonoids and insoluble phenolic compounds are found in the cell wall together with proteins and polysaccharides throughthe hydrogen bond and hydrophobic bond (Fan et al., 2015). The addition of water in solvents generates a medium polarity that facilitates the extraction (Lopez-Perea et al., 2019). Low concentration of organic solvents can reach the cells, but high concentration causes protein denaturation, preventing the dissolution of polyphenols and thus the passage of phenolic substances into the extraction solution (Chen et al. 2013).

2.2. Antioxidant Activity

DPPH is a stable organic nitrogen radical (Saha et al., 2016). DPPH react with phenols through two different mechanismis. The first is the direct separation of the H atom from the phenol, and the second is by electron transfer. Both of these pathways depends on the nature of the solvent and/or the redox potential (Saha et al., 2016; Vladimir-Knežević et al., 2011). Antioxidants that react with DPPH neutralize the free radical (Saha et al., 2016). The color of the reaction mixture changes from purple to yellow. The degree of the discoloration measures the potential of antioxidant activity (Vladimir-Knežević et al., 2011). Phenolic compounds are strong hydrogen donors to the DPPH radical

due to their ideal chemical structure (Von Gadov and Hansman, 1997; Rice-Evans and Paganga, 1997). The DPPH method is widely used to determine the antioxidant activity of plant extracts (Türkmen et al., 2006; Do et al., 2014; Saha et al., 2017; Nguyen et al., 2020). Antioxidant activity of elderberry flowers is examined and presented in Table 1. The results indicated that antioxidant activity of elderberry flowers varied in the range of 24.47%-85.74%. Extracting solvent significantly affected antioxidant activity of elderberry flowers extracts ($p<0.05$). All extracts prepared with 50% solvents contained highest level of antioxidant activity and followed by those with 80% and 100% solvents, respectively. The highest antioxidant activity (85.74%) was found in 50% acetone extract. The lowest amounts of antioxidant activity were obtained with 100% acetone (24.47%) and 100% ethanol (31.65%) extract, respectively. Antioxidant activities of samples with high TPC were also high (Table 1). It has also been shown in previous studies that there is a strong relationship between TPC and antioxidant activity (Velioglu et al. 1998; Türkmen et al., 2006; Kim and Chin 2016). A high correlation was found between TPC and antioxidant activity in elderberry flowers (Figure 1). This has proven that phenolic compounds contribute to antioxidant activity.

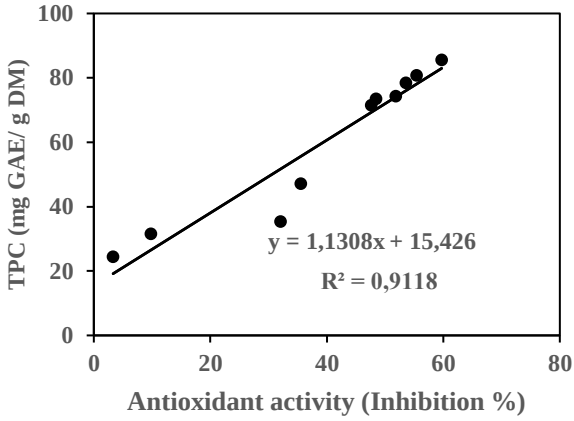

Figure 1: Correlation Between TPC Substance and Antioxidant Activity in Elderberry Flowers (*Sambucus nigra* L.)

CONCLUSIONS

In this study, the effects of different solvents on TPC and antioxidant activity of elderberry flowers (*Sambucus nigra* L.) were investigated. Extraction solvent significantly affected TPC and antioxidant activity of elderberry flowers extracts. Rankings in the TPC of extracts varied depending on the concentration of solvent. The most effective solvents for TPC extraction were 50% extracts of all solvents. These extracts also showed the highest antioxidant activity. A high correlation was obtained between the TPC of elderberry flowers extracts and their antioxidant activities. This study showed that elderberry flowers is an important source of phytochemicals with natural antioxidant properties when extracted with a suitable solvent. This study showed that elderberry flowers are an important source of phytochemicals with natural antioxidant properties when extracted with a suitable solvent.

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