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Effects of Some Extraction Parameters on Anthocyanin Content of Barberry (*Berberis vulgaris* L.) and Its Antioxidant Activity*

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Abstract: In the study, determination of the optimal conditions for anthocyanin extraction from the dried fruit of barberry (*Berberis vulgaris* L.) was aimed. For this purpose, the solvent extraction method was used. The present investigation was carried out to different extraction conditions such as different solvents (ethanol and 2% hydrochloric acid, 2% acetic acid and 2% citric acid), the concentration of ethanol (20-80%), the concentration of suitable acid (1-4%), extraction temperature (30-60 ºC), extraction time (60-240 min) and raw material and solvent ratio (1:5-1:20). The obtained extract was subjected to total phenolic content and antioxidant activity using DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. The optimum condition for anthocyanin extraction from barberry extract was as follows: using 80% ethanol and 2% citric acid (100:10) as a solvent, the extraction temperature was 30 ºC, extraction time was 120 min and the rate of fruit/solvent ratio was 1:20. In these conditions, the total anthocyanin content, the total phenolic content and antioxidant capacity were determined as 101.03±1.89 mg $100g^{-1}$ FW, 3269.05±111.11 mg gallic acid kg^{-1} and 92.41±0.25%, respectively. Antioxidant activity of barberry (*B. vulgaris* L.) extracts has been attributed to the high polyphenol content.

Keywords: Barberry, *Berberis vulgaris* L., anthocyanin, antioxidant activity

1. Introduction

Fruits are a significantly rich source of bioactive compounds. These compounds have also attracted great interest from researchers as functional food ingredients. Bioactive compounds include antioxidants such as phenolic compounds and fruit colorants (Skrovankova et al., 2015). Anthocyanin is a family of flavonoids, which are water-soluble compounds and non-toxic. Anthocyanins have a wide range of colors such as red, blue and purple in many flowers, vegetables and are used as natural food colorants due to attractive colors (Asada et al., 2015). It is known that another important feature of anthocyanins is their strong antioxidant activity (Kong et al., 2003). The principle of antioxidants is to reduce oxidative damage to the human body by scavenging free radicals due to donating an electron to the free radical (Motalleb et al., 2005). The antioxidants found in fruits have been associated with their beneficial health effects. These compounds have also been implicated in the prevention of neural and cardiovascular diseases, cancer and diabetes (Castaneda-Ovando et al., 2009). The phytochemical studies on various Berberis species confirmed the isolation of the alkaloids, tannins, phenolic compounds, sterols, and triterpenes from berberis (Mohammadzadeh et al., 2017). Barberry extract contains various flavonoids including quercetin, krysantemin, hyperosid, delphinidin-3-O-beta-D-glucoside, plargonin and petionidin-3-O-beta-D-glucoside and it also contains ascorbic acid, α-tocopherol and beta-carotene. All of these compounds are considered antioxidants (Sharifi and Poorakbar, 2015; Salehi et al., 2019). Berberine, an isoquinoline alkaloid and the main compound of *B. vulgaris*, has been used for treating diarrhea and gastrointestinal disorders for a long time. Moreover, the multiple pharmacological effects of berberine are inhibition of intestinal ion secretion and smooth muscle contraction, inhibition of ventricular tachyarrhythmias, reduction of inflammation,

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stimulation of bile secretion and bilirubin discharge (Minaiyan et al., 2011). Several studies have described that the various part of barberry has long been used in traditional medicine (Minaiyan et al., 2011; Sharifi et al., 2013; Mohammadzadeh et al., 2017). Bright red fruits of barberry are also grown as commercial plants in Iran and used as food additives in food products such as jelly desserts, milk desserts, ice cream and yogurt. Native *Berberis* species such as *B. vulgaris* L., *B. integerrima*, *B. cretica* L., and *B. crataegina* were also found in Turkey (Sonmezdag et al., 2018). Barberry fruits are rich in nutritional compounds such as carbohydrates, citric acid, minerals and vitamin C (Sharifi et al., 2013). Anthocyanin pigments are found in cells close to the surface in many fruits and vegetables (Rodriguez-Saona and Wrolstad, 2001). Acidified solvent extraction is one of the most common methods for the anthocyanin pigments extraction in fruits. Solvent extraction was generally used for the extraction of flavonoids and many compounds from plants, fruits, vegetables (Ju and Howard, 2003; Amanda et al., 2015). The most effective factors on extraction yield are the extracting solvent, liquid /solid ratio, time, and temperature of extraction (Lorenzo et al., 2018).

In this study, the determination of optimal conditions for anthocyanin extraction from dried barberry (*B. vulgaris* L.) fruits was aimed. Experiments were performed to evaluate the influence of different solvent, the concentration of solvents, temperature, time and raw material and solvent ratio on total anthocyanin extraction. Additionally, antioxidant properties such as total phenolic content and antiradical activity of the extract were determined.

2. Materials and Methods

2.1. Sample preparation

Samples of dried *B. vulgaris* L. fruit were purchased from a local market (Khoy, Iran). Barberry fruits were purged from unwanted substances then vacuum packed and stored at -20 °C until used.

2.2. Extracts preparation

The frozen fruits were homogenized with a blender. Samples were treated with ethanol at a rate of 1:10 (raw material: solvent), at 37 °C for 45 min as the first step of the experiment according to Mai and Tan (2013). Following this process, optimization of extracts was performed using different solvent [ethanol and 2% hydrochloric acid (HCL), 2% acetic acid and 2% citric acid], the concentration of ethanol (20, 40, 60 and 80%), the concentration of suitable acid (1, 2, 3 and 4%), extraction temperature (30, 40, 50 and 60 °C), extraction time (60, 120, 180 and 240 min) and raw material and solvent ratio (1:5, 1:10, 1:15 and 1:20). At the end of each extraction, the extract was subjected to filtration with Whatman paper No.1 and then the extract was centrifuged at 5000 rpm for 15 min at 4 °C to remove very small particles.

2.3. Determination of anthocyanin content

The total anthocyanins content was determined by the pH differential method Lee et al. (2005) and Damsa et al. (2014). The color changes were measured using a spectrophotometer (Aquamate 9423 AQA 2000E, Thermo Scientific, England) at 510 and 700 nm at pH 1.0 (potassium chloride buffer) and pH 4.5 (sodium acetate buffer) (Equation 1). Total monomeric anthocyanins was calculated using Equation 2. The results were expressed as mg cyanidin 3-glucoside equivalent $100g^{-1}$ fresh weight.

$$A= (A_{510nm}-A_{700nm})_{pH1.0}-(A_{510nm}-A_{700nm})_{pH4.5} \quad (1)$$

$$\%w/w= A/\varepsilon L \times MW \times DF \times V/Wt \times 100 \quad (2)$$

Where A is the absorbance of extracts; ε is the molar extinction coefficient (26900 L mol^{-1} cm^{-1}) for cyanidin-3-glucoside; L is the path length (1 cm); MW is the molecular weight (449.2 g mol^{-1}); DF is the dilution factor; V is the volume (L), and Wt is the sample weight (g).

2.4. Determination of total phenolic content

The total phenolic content analysis was performed according to the Folin-Ciocalteau method Gutfinger (1981) with very few modification. 0.2 ml of Folin-Ciocalteu was added to 0.1 ml of the extract. Then 3 ml of Na_2CO_3 (5%) were added to the above mix and incubated for one hour at 23 °C, and vortexed. The absorbance was measured using a spectrophotometer at 765 nm. A standard curve was prepared using different concentrations of gallic acid solutions to quantify the phenolic compounds (y= 0.0021x+0.03, R^2= 0.9966). The results were expressed as mg gallic acid kg^{-1} sample.

2.5. DPPH radical scavenging capacity

The radical scavenging activity was determined using the method of Brand-Williams et al. (1995) with slight modification. 200µl of samples were transferred to tubes and 3 ml of ethanol was added. Then 0.1 mM DPPH (2,2-diphenyl-1-picrylhydrazyl) solution prepared in 0.5 ml ethanol was added. The samples were stored at room temperature for 30 min in the dark, the absorbance of the solution was measured using a spectrophotometer at 517 nm. A tube containing

ethanol and DPPH solution was used as control whereas ethanol alone was used as a blank. Radical scavenging activity was calculated with the help of Equation 3.

$$\% \text{ inhibition of } DPPH^{+} = [1-(A \text{ sample}/A \text{ control})]x100 \quad (3)$$

2.6. Statistical analysis

All data were evaluated by analysis of variance (ANOVA) and differences between means were compared with Duncan's multiple range tests. Statistical analyses were performed using the SPSS version 24 statistical software (SPSS Inc., Chicago, IL, USA). All experiments were performed twice. The results are given as means±standard deviation in the figures.

3. Results and Discussion

3.1. Effect of different solvents on anthocyanin extraction

In the processing of plant extraction, ethanol, methanol, acetone and their aqueous mixtures are usually used. Acidified ethanol is an effective solvent for anthocyanin extraction from plant materials (Castaneda-Ovando et al., 2009; Devi and Joshi, 2012). The use of acid stabilizes anthocyanin in flavylium cation form which is stable in a highly acidic condition (Amanda et al., 2015). However, Thao et al. (2015) stated that organic solvent and water mixtures are more suitable for the extraction of anthocyanins. In the present study, 50% v/v ethanol and different acids (2% HCl, 2% acetic acid, and 2% citric acid) were used in the ratio of 100:10 for the anthocyanins extraction from *B. vulgaris* fruit (Figure 1). Although the highest anthocyanin content was obtained with the ethanol-HCl mixture (100 mg $100g^{-1}$), there was no significant difference between ethanol and different acid used ($p>0.05$) (Figure 1).

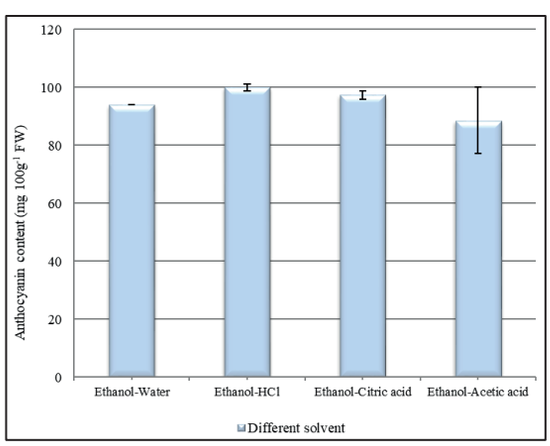

Figure 1. Effect of different solvents on the *B. vulgaris* L. anthocyanin extraction

In a study, Mai and Tan (2013) reported that in the extraction of anthocyanin from the rind of tropical fruit (*Garcinia mangostana*), the highest amount of anthocyanin (213 mg $100g^{-1}$) was obtained with a mixture of 50° ethanol and 2% hydrochloric acid. In the same study, the anthocyanin content in the extraction with water decreased considerably. In another study, anthocyanin content from barberry extracted with only citric acid was determined about 80 mg $100ml^{-1}$, whereas anthocyanin content was found about 145 mg $100ml^{-1}$ in the barberry extracted with ethanol (Sharifi and Hassani, 2012). Anthocyanin extraction is usually carried out with acidified ethanol using hydrochloric acid. However, if the obtained extract is used for food purposes, it is more appropriate to use citric acid, which is frequently used in food processing and storage (Joshi and Devi, 2014).

3.2. Effect of ethanol concentration on anthocyanin extraction

The concentration and type of solvent are the two effective factor to anthocyanin extract from a plant matrix (Devi and Joshi, 2012). The anthocyanin content with using different concentrations of ethanol (80, 60, 40 and 20%) and 2% citric acid are shown in Figure 2. Ethanol concentration had a very significant effect on anthocyanin extraction from barberry ($p<0.01$). As seen from Figure 2, ethanol showed high anthocyanin extracting power compared to water. The highest amount of anthocyanin (106.62 mg $100g^{-1}$) from barberry dried fruit was also determined in the presence of 80% ethanol and 2% citric acid.

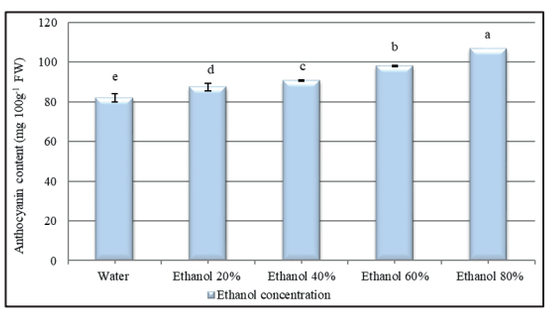

Figure 2. Effect of ethanol concentration on the *B. vulgaris* L. anthocyanin extraction

In a study on the effect of different solvents and extraction time in grape, black and red currant observed that the solubility of polar characterizes anthocyanins is the highest in methanol, slightly lower in ethanol and lowest in water. Ethanol and methanol show a weak polar character than water, and this is more effective in the degradation of

unpolar compounds present in the cells wall and seeds so that anthocyanins and polyphenols are easily separated from the cell (Lapornik et al., 2005). High ethanol concentration also causes a decrease in water-soluble components such as pectin and polysaccharide, which prevent the dissolution of anthocyanin in the solvent (Hutabarat et al., 2019). Metivier et al. (1980) reported that ethanol was more effective than water in the anthocyanin extraction from the grape pulp. In another study, Jin et al. (2019) found that the increasing ethanol volume from 0 to 40% increased anthocyanin extraction yield from lingonberry pomace.

3.3. Effect of citric acid concentration on anthocyanin extraction

In the extraction process, the membranes of the cell tissue are denatured and the solubility of the pigments is generally increased by using acidic solvents (Ju and Howard, 2003). However, the concentration of acid used is an important factor in maintaining the natural form of the pigment (Rodriguez-Saona and Wrolstad, 2001). The aim of using organic acids such as acetic acid and citric acid in the extraction of anthocyanins is to avoid damage to the anthocyanin due to hydrolysis of the glycoside bond and the acylated anthocyanins (Amanda et al., 2015). However, the excessive use of acid may destroy glycoside bonds and acyl groups. Also, the use of a large amount of acid causes the linkages with metals or co-pigments to refract during the extraction process (Castaneda-Ovando et al., 2009; Hutabarat et al., 2019). Figure 3 has shown the effect of different concentrations of citric acid on the barberry anthocyanins extraction. In this present essay, a mixture of 80% ethanol and 2% citric acid showed the highest anthocyanin content (94.52±1.17 mg $100g^{-1}$) ($p<0.05$).

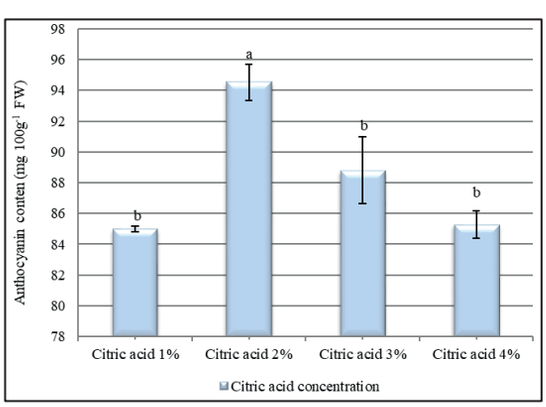

Figure 3. Effect of citric acid concentration on the *B. vulgaris* L. anthocyanin extraction

Therefore, 80% of ethanol and 2% citric acid were selected for further investigation. Mai and Tan (2013) reported that the mixture of 40° ethanol and 1.5% HCl in a tropical fruit peel showed the highest anthocyanin content and the amount of anthocyanin decreased as the HCl content increased.

3.4. Effect of temperature on anthocyanin extraction

The temperature is an important factor in the disturbance of anthocyanin during processing and storage (Sharifi and Hassani, 2012). Increased extraction temperature, hydrolysis of the 3-glycoside and pyrylium ring of anthocyanin lead to the rapid decomposition of anthocyanins (Laleh et al., 2006). A high temperature improves the solubility of the solute and the diffusion coefficient. However, phenolic compounds can be degraded at the high temperature (Spigno and De Faveri, 2007). To determine the effect of this factor, barbery extract was carried out at four different temperatures (30, 40, 50 and 60 °C). As shown in Figure 4, the difference between the means was not significant ($p>0.05$). However, anthocyanin content was found to be higher at 30 °C than at other temperatures.

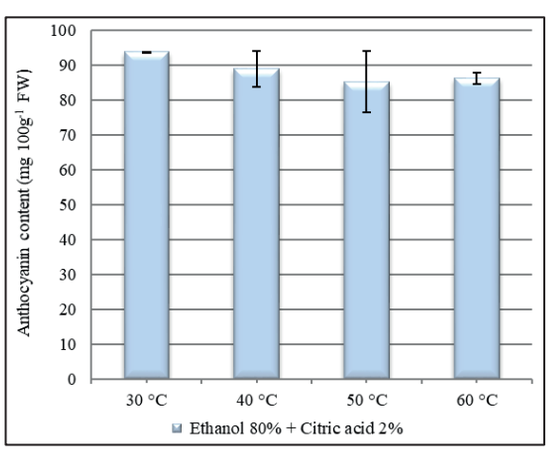

Figure 4. Effect of extraction temperature on the *B. vulgaris* L. anthocyanin extraction

As in the present study, Laleh et al. (2006) reported that *B. vulgaris* anthocyanins extracted at 5 °C had less degradation in the anthocyanin extraction from different species of Berberis using ethanol and 1% hydrochloric acid and different temperatures (5, 15, 25 and 35 °C). In contrast, Sharifi and Hassani (2012) in a study on the stability of barberry pigments was reported that the combination of ethanol-methanol solvent and 50 °C have shown the highest anthocyanin content (119.311 mg $100ml^{-1}$). Mai and Tan (2013) also reported that in the anthocyanin extraction of mangosteen performed at 40, 50, 60 and 70 °C, the highest concentration of anthocyanin was shown at the temperature of 60 °C. Some anthocyanins are

more stable than others depending on their molecular structure. The intensity and stability of the anthocyanin pigments are dependent on various factors including extracting solvent, liquid/solid ratio, time, and temperature (Laleh et al., 2006; Lorenzo et al., 2018). Considering to extraction yield of anthocyanin in this study 30 °C was selected for tasting in the later stage.

3.5. Effect of extraction time on anthocyanin extraction

The results of the extraction at different times (60, 120, 180, and 240 min), 80% of ethanol and 2% citric acid at 30 °C are shown in Figure 5. As seen from the results, the duration of extraction process is also an important factor among extraction. High temperatures and long time cause degradation of bioactive compounds in the extracts and results in low extraction yield of stable anthocyanins (Wang et al., 2016). In our study, the extraction yield of barberry increased when the time was extended from 60 min to 120 min. Accordingly, the highest amount of anthocyanin was determined 96.40±1.10 mg $100g^{-1}$ in the samples which were kept for 120 min. A decrease in anthocyanin content after 120 min was observed due to the anthocyanin decomposition. However, this decrease was not significant at 180 min ($p>0.05$). In a short time of extraction, a sufficient amount of anthocyanin was not extracted totally however anthocyanin may be oxidized in the long extraction periods (Mai and Tan, 2013). In a study, Le et al. (2019) found that the best anthocyanin content from *Carissa carandas* L. was determined at 45 min between different extraction durations (15, 30, 45, 60 and 90 min).

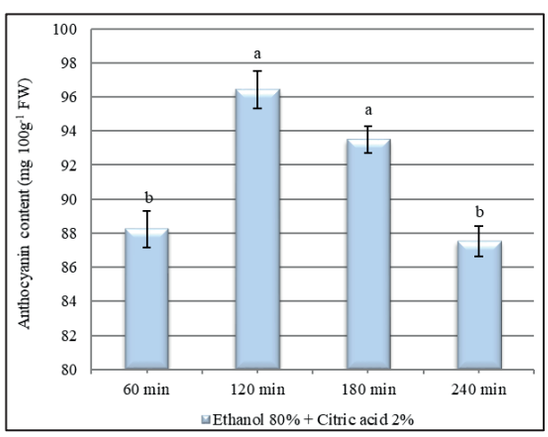

Figure 5. Effect of extraction time on the *B. vulgaris* L. anthocyanin extraction

3.6. Effect of using different rate of solvent on anthocyanin extraction

In addition to the above-mentioned conditions, raw material and solvent ratio was taken as a factor and the results of the experiments are shown in Figure 6. Accordingly, the lowest total anthocyanin content was determined in 1:5 solvent ratio (74.83±4.94 mg $100g^{-1}$) (Figure 6).

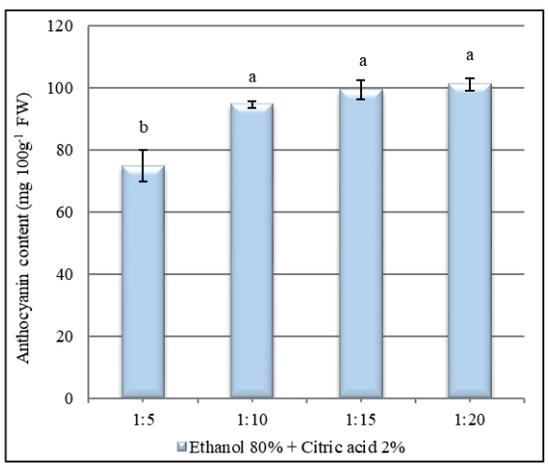

Figure 6. Effect of solvent rate on the *B. vulgaris* L. anthocyanin extraction

Ardestani et al. (2013) reported that the total anthocyanin content of *B. vulgaris* in ethanolic extract and aqueous extract was 69.006±1.65 and 62.103±1.205 mg $100g^{-1}$, respectively. A high solvent rate increased the extraction yields due to an increase in the bulk transfer rate (Hutabarat et al., 2019). In addition, Shi et al. (2005) reported that the solvent to solid ratio should be kept quite high since the concentration of polyphenols is diluted. Hutabarat et al. (2019) reported that the highest anthocyanin extraction from rabbiteye blueberry *(Vaccinium ashei)* at different liquid-to-solid ratios was obtained in the highest amount of solvent ratio 20:1. In another study, Jin et al. (2019) reported that anthocyanin extraction yield from lingonberry (*V. vitis-idaea* L.) pomace increased with increasing liquid-to-solid ratios from 5 to 25 ml g^{-1}. Thao et al. (2015) found that the best anthocyanin extraction from purple rice was obtained 116.7 mg $100g^{-1}$ in the optimal conditions such as 2:8 ethanol to water ratio, 1:10 purple rice to water ratio, 90 min extraction time, pH 2.2, and 60 °C. In another study, Devi and Joshi (2012) reported that the maximum anthocyanin content from plum was found at 50% ethanol and 0.2% citric acid with a 10:1 concentration of extracts. Mai and Tan (2013) reported that the optimal condition of anthocyanin extraction from Mangosteen was ethanol 40° as a solvent with HCl 1.5%, 1:10 the rate of rind to solvent, 60 °C extraction temperature and 40 min the extraction time.

3.7. Antioxidant activities of barberry extracts

Polyphenols, flavonoids and phenolic compounds play very important roles in the human

body due to their antioxidant properties (Pantelidis et al., 2007; Oboh and Ademosun, 2012). Studies reported that there is a positive relationship between total phenolic content and antioxidant activity in plants and herbs (Pantelidis et al., 2007; Özyazıcı et al., 2018; Rahimi et al., 2020). In the present study, under these optimum conditions (the concentration of ethanol 80% and 2% citric acid, the temperature at 30 ºC, extraction time in 120 min and the raw material/solvent ratio at 1:20), the phenolic content and antiradical activity were determined 3269.05±111.11 mg gallic acid kg^{-1} and 92.41±0.25%, respectively. Sharifi and Poorakbar (2015) reported that the maximum total phenol content of dried hybrid barberry fruits was 59.57±0.83 (mg of gallic acid $10g^{-1}$ barberry) in aqueous extract and DPPH antiradical activity was 44.62±0.99 in methanolic extract of fresh barberry. On the other hand, Ardestani et al. (2013) reported that total phenolic content was 3450 mg $100g^{-1}$ fresh fruit for *B. vulgaris.*

4. Conclusions

According to the results, the concentration of 80% ethanol and 2% citric acid, the temperature of 30 °C, extraction time of 120 min and the rate of raw material/solvent ratio at 1:20 were optimal conditions for anthocyanin extraction from barberry fruits. Barberry is a rich source of antioxidants due to its high phenolic content and radical scavenging activity. Therefore, the use of an appropriate amount of this natural antioxidant could have a positive impact on the quality and stability of foods as well as the health benefits of consumers.

Declaration of Author Contributions

Conceptualization, Methodology, Investigation, Formal Analysis, Writing-Original Draft Preparation, *R. JABERI*; Formal Analysis, Writing-Review & Editing, *G. KABAN*; Supervision, Writing-Review & Editing, *M. KAYA*. All authors declare that they have seen/read and approved the final version of the article ready for publication.

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Declaration of Conflicts of Interest

All authors declare that there is no conflict of interest related to this article.

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