

Full Length Research Paper

Investigating the antioxidant properties and rutin content of Sea buckthorn (*Hippophae rhamnoides* L.) leaves and branches

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The present study focused on the antioxidant properties and rutin content of leaves and branches of *Hippophae rhamnoides* L. (Sea buckthorn) in Turkey. Dried leaves (leaf tea), processed (PB) and unprocessed branches (UB) of Sea buckthorn (SBT) were extracted with ethanol and prepared in forms of aqueous extract (AE). All samples were analyzed for their contents of rutin, total phenolics (TPC), total flavonoids (TFC) and total antioxidant capacity by using DPPH and CUPRAC methods. TPC of leaves from ethanolic extracts (EE) were significantly higher than UB and PB extracts. The DPPH scavenging activity of extracts ranged from 41.93 ± 3.57 and 132.43 ± 6.57 mg trolox equivalent (TE)/g, and the antioxidant capacity measured with CUPRAC method were in between 129.4 ± 18.1 and 538.5 ± 34.8 mg TE/g. Both EE and AE of leaf samples had significantly higher rutin content compared to the UB and PB samples. Taking the high antioxidant and rutin content of leaves into account and with respect to their positive health effects, consumption of SBT as a herbal tea should be investigated.

Key words: Sea buckthorn, *Hippophae rhamnoides*, leaves, antioxidant, phenolic profile, rutin content.

INTRODUCTION

Sea buckthorn (SBT) (*Hippophaë rhamnoides* L.) is a species of flowering plant in the family Elaeagnaceae, deciduous shrub with good adaptability to various climate conditions and extensive genetic variability with numerous greenish-yellow flowers and bright orange, globular, ellipsoid fruit. It is native to Europe and Asia and has been domesticated in several countries (India, China, Nepal, Pakistan, Myanmar, Russia, Britain, Germany, Finland, Romania, France, etc.) (Khan et al., 2010; Yogendra Kumar et al., 2011; Pop et al., 2013). In

Turkey, SBT is widely distributed throughout North and East Anatolia and known locally as 'Yalancı igde, Cıdırgan, Dijirgan, Cıcılık' or 'Sincan Çalısı' (Aras-Tayhan, 1997). All parts of the plant are considered to be good source of large number of bioactive substances like vitamin, carotenoids, and flavonoids, organic acids, sterols and some essential amino acids. For instance, berries and seeds of SBT are well known for their antioxidative properties, attributed to hydrophilic and lipophilic compounds including ascorbic acid, flavonoids,

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Table 1. Some characteristics of the sampling sites.

Sampling site	Latitude/altitude longitude	Annual precipitation (Mm)	Annual temperature (°C)	Climate type	Sampling dates
Sivas-Sincan stream	39°54'N- 2000 m 37°59'E	794 -	2.8 -	Humid -	June 2013 August 2014

proanthocyanidins and carotenoids. The leaves of SBT are also considered for their antioxidant potential correlated to flavonoids and phenolic acids derivatives, and they have been used in some countries to make extracts, tea, animal feed, pharmaceuticals and cosmetics (Michel et al., 2012; Wani et al., 2013).

Flavonoids are the widest group of secondary metabolites involved in many biological functions in plants. They are classified into flavonols, flavones, flavanones, catechins, anthocyanidins and chalcones (Gupta et al., 2011). Rutin is one of the bioactive flavonoid compound, which is present in substantial amounts in various plants (Attanassova and Bagdassarian, 2009). Grapes and buckwheat are the most important rutin containing foods between fruits, vegetables and grain crops (Kreft et al., 2006). Rutin, a naturally occurring flavonol consisting of aglycone quercetin and a rutoside moiety in position 3 of the C ring, is found in many food substances. It has been reported to exhibit beneficial effects against several types of liver diseases (Pan et al., 2014). Within the group of flavonoids, many studies have been conducted on rutin, since this flavonoid is of great therapeutic importance. Rutin causes an increase in pancreatic lipase with consequent reduction in triacylglyceride levels in rats. It has also been described in events associated with the immune system, as seen with other flavonoids. Rutin has been shown to inhibit neoplasia induced by immunosuppression with azoxymethanol and to reduce oxidative stress in leukocytes in rheumatoid arthritis (Marcarini et al., 2011). *H. rhamnoides* is generally reported to be rich in a wide range of biologically active substances. Due to the antioxidant properties of sea buckthorn leaves the number of studies investigating their potential utilization has grown in recent years, but there are limited number of studies on SBT branches in the literature and therefore, this study was focused mainly on leaves and branches of SBT. The aim of the present study is to investigate the antioxidant properties and rutin content of sea buckthorn leaves, processed branches and unprocessed branches.

MATERIALS AND METHODS

Plant material

Wild Sea buckthorn (*H. rhamnoides* L.) were harvested from

Central Anatolia, particularly Sivas region (Table 1). The leaves and branches were separated from each other and ventilated. In order to inhibit the activation of oxidation enzyme, leaves and branches were subjected to shocking procedure. For this purpose plant materials were shocked with 90 to 100°C vapor in rotary evaporator. Output materials of the shocking unit were cooled with dry air. Cooled materials were subjected to folding procedure for 25 to 30 min. After folding unit, leaves and branches were transferred into the drying chamber and dried at 110°C. At the end of this treatment dried leaf tea and processed branch were obtained. These materials were cut into small pieces and stored in the dark at room temperature before use (up to 1 week).

Dried leaves, processed (PB) and unprocessed branches (UB) of *H. rhamnoides* were extracted with ethanol as described below, also prepared in forms of infusion of leaf tea and decoction technique was used for PB and UB. All samples were analyzed for their contents of total phenolics and total flavonoids as well as their total antioxidant capacity by using two different methods including 1,1-diphenyl-2-picrylhydrazyl (DPPH) and Copper Reducing Antioxidant Capacity (CUPRAC). The samples were also analyzed by using HPLC to identify their phenolic profiles.

Extraction procedure

Ethanolic extraction

Ethanolic extractions were carried out for dried leaves, PB and UB samples as described previously by Maheshwari et al. (2011) with slight modifications. 2.0 ± 0.01 g of each sample was extracted with 15 ml of 70% aqueous-ethanol in a cooled ultrasonic bath (Azakli, Turkey) for 15 min. After 24 h incubation, the treated samples were centrifuged (Hettich Zentrifugen Universal 32R, UK) for 10 min at 4000 rpm at 4°C and the supernatant were collected. Then 15 ml of 70% aqueous-ethanol was added to the pellet and this extraction procedure was repeated two more times. All four supernatants were combined and adjusted to a final volume of 50 ml. Prepared extracts were stored at -20°C until analysis.

Aqueous extraction

To improve the efficiency of extractions, different methods were used for aqueous extraction of leaves, UB and PB. Dried leaf tea samples were prepared in forms of infusions. For the preparation of infusions, 2 ± 0.01 g of dried leaves were weighed and dissolved in 200 ml of pure water at 90°C. After waiting for 30 min, the leaves were removed and the infusion was filtered, and further analyzed. Because of the woody structure of UB and PB, similar technique to the preparation of wood for microscopic examination was applied. 2 ± 0.01 g of UB and PB were weighed and 75 ml of water added. It was heated until boiling. At the boiling point 25 ml of pure water was added and was reboiled. This procedure repeated four more times until final volume of 200 ml. Branches were removed and extract was filtered and further analyzed (Aras-Tayhan, 1997).

Determination of total phenolic content (TP)

The TP of extracts was determined using Folin-Ciocalteu reagent according to the method modified from Velioglu et al. (2006) using gallic acid as a standard. A mixture of 100 μ l of the extract, 900 μ l of distilled water and 1.5 ml of 0.2 N Folin-Ciocalteu reagents were prepared and allowed to react for 5 min. Then 1.2 ml of 7.5% Na_2CO_3 solutions added into the reaction mixture. After incubation for 90 min at room temperature, the absorbance was measured at 765 nm using Optima SP-3000 nano spectrophotometer. The TP of extracts was expressed as mg of gallic acid equivalent (GAE) per g sample.

Determination of total flavonoid content (TF)

The TF was measured colorimetrically as described by Kim et al. (2003). Mixture of 0.25 ml of each extract, 1.25 ml of distilled water and 75 μ l of 5% NaNO_2 were prepared and allowed to react for 6 min. Then 150 μ l $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ was added and mixed. After 5 min 0.5 ml of 1 M NaOH was added. The total volume was adjusted to 2.5 ml with distilled water. Absorbance of the mixture was measured at 510 nm versus prepared blank. The TF of extracts was determined by a rutin standard curve and expressed as milligram of rutin equivalent (RE) per gram sample.

Determination of total antioxidant capacity (TAC)

Antioxidant capacity of plant extract cannot be evaluated by only a single method due to the complex nature of phytochemicals. Therefore, in this study the TAC was estimated by two different assays. The DPPH and CUPRAC assays were performed according to Kumaran and Karunakaran (2006) and Apak et al. (2005) respectively. Trolox was used as a standard and results were expressed in terms of milligram of Trolox equivalent (TE) per gram sample.

DPPH-radical scavenging activity assay

The free radical scavenging activity of sea buckthorn leaves, UB and PB aqueous and ethanolic extracts on DPPH radical were determined according to the method introduced by Kumaran and Karunakaran (2006) and Rai et al. (2006). 0.1 mM of DPPH was prepared by dissolving 3.943 mg DPPH with 100 ml ethanol. 100 μ l of each extract was mixed with 2 ml ethanolic solution of DPPH (0.1 mM). Extracts were substituted by methanol and distilled water blanks. Decolourisation of purple free radical DPPH solution was measured at 517 nm after 30 min incubation in the dark and at room temperature. A trolox calibration curve was done between 0.01 and 0.2 mg/ml. Results were expressed in mg of trolox equivalents/g of sample (mg TE/g).

Copper reducing antioxidant capacity (CUPRAC) assay

CUPRAC assay was carried out according to Apak et al. (2005) which is based on the absorbance measurement of Cu(I)-neocuproine (Nc) chelate formed as a result of the redox reaction of chain-breaking antioxidants with the CUPRAC reagent, Cu(II)-Nc, where absorbance is recorded at the maximal light-absorption wavelength of 450 nm. 10^{-2} mM of CuCl_2 solution was prepared by dissolving 0.4262 g $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ in distilled water, and diluted to 250 mL. Ammonium acetate buffer at pH =7.0, 1.0 M, was prepared by dissolving 19.27 g NH_4Ac in distilled water and diluted to 250 mL. Neocuproine (Nc) solution was prepared daily by dissolving 0.078 g Nc in 96% ethanol, and diluted to 50 mL with ethanol. 100 μ l of

extract was mixed with 1 ml of CuCl_2 solution, 1 ml of Nc solution, 1 ml of ammonium acetate buffer and 1 ml of distilled water. After 30 min absorbances were measured at 450 nm against reagent blank. Results were expressed in milligram of TE per gram of sample.

HPLC analysis of rutin and other flavonols

Rutin and major flavonols were determined following the method of Capanoglu et al. (2008). Extracts were filtered through a 0.45 μ m membrane filter and analyzed using a Waters 2695 HPLC system with a PDA (Waters 2996) detector. A Supelcosil LC-18 (25 cm \times 4.60 mm, 5 μ m column Sigma-Aldrich, Steinheim, Germany) was used. The mobile phase consisted of solvent A, Milli-Q water with 0.1% (v/v) Trifluoroacetic acid (TFA) and solvent B, acetonitrile with 0.1% (v/v) TFA. A linear gradient was used as follows: At 0 min, 95% solvent A and 5% solvent B; at 45 min, 65% solvent A and 35% solvent B; at 47 min, 25% solvent A and 75% solvent B; and at 54 min returning to initial conditions. The flow rate was 1 ml/min. Detection was done at 360 nm. Identification was based on the retention times and characteristic UV spectra. Quantification was done using external standards (rutin, quercetin-3-O-galactoside, kaempferol) as well as taking the information from the literature into account.

Statistical analysis

All the experiments were replicated three times and the data were represented as mean \pm SD. For multiple comparisons, data were subjected to statistical analysis using SPSS software (version 16.0 for Windows, SPSS Inc.) for the analysis of variance (ANOVA). Duncan's new multiple range test was used to analyze differences between treatments ($p < 0.05$).

RESULTS

Total phenolic content (TPC) and total flavonoid content (TFC)

The TPC of SBT leaves, unprocessed branches (UB) and processed branches (PB) were reported in the range of 25.8 \pm 2.9 to 75.9 \pm 6.5 mg GAE/g (Table 2). The TPC of leaves from ethanolic extracts (EE) were significantly higher than UB and PB extracts ($p < 0.05$). It was observed that TPC of PB extracts were higher than UB extracts. When considering PB and UB extracts with respect to the differences between aqueous and ethanolic extraction methods, the TPC of aqueous extracts (AE) were substantially higher than ethanolic extracts (Figure 1). This circumstance was interpreted, as decoction was a more efficient extraction technique vis-à-vis ethanolic extraction.

The TFC of SBT leaves, UB and PB were indicated in the range of 21.2 \pm 3.8 to 74.0 \pm 3.0 mg RE/g. In contrast with TPC results, TFC of all EE was higher than the AE content. The highest level of flavonoids was found in EE of leaves (74.0 \pm 3.0 mg/g) followed by UB (64.6 \pm 4.5 mg/g) and PB (59.4 \pm 6.8 mg /g). But there is no significant difference between EE of UB and PB statistically ($p > 0.05$).

Table 2. Total phenolic content (TPC), total flavonoid content (TFC), Free radical scavenging activity (DPPH) and Copper reducing antioxidant capacity (CUPRAC) of SBT ethanolic and aqueous extraction.

Variables	Sample	Total phenolics (mg GAE/g)	Total flavonoids (mg RE/g)	DPPH (mg TE/g)	CUPRAC (mg TE/g)
Leaf	Ethanolic extraction	75.9± 6.5 ^a	74.0 ± 3.0 ^a	89.6 ± 2.0 ^c	538.5 ± 34.8 ^a
	Aqueous extraction	66.9± 5.9 ^a	21.2 ± 3.8 ^e	132.4± 6.6 ^a	293.5 ± 26.7 ^b
Processed Branch	Ethanolic extraction	43.1± 5.7 ^c	59.4 ± 6.8 ^b	83.3 ± 9.8 ^c	291.3 ± 17.2 ^b
	Aqueous extraction	53.8± 8.9 ^b	45.2 ± 0.6 ^c	113.1± 6.1 ^b	235.5 ± 34.1 ^c
Unprocessed Branch	Ethanolic extraction	25.8± 2.9 ^d	64.6 ± 4.5 ^b	41.9 ± 3.6 ^e	129.4 ± 18.1 ^e
	Aqueous extraction	43.2± 3.6 ^c	30.5 ± 3.4 ^d	71.1 ± 7.9 ^d	181.7 ± 27.6 ^d

*Data represent average values ± standard deviation of three independent samples. Different letters in the columns represent statistically significant differences ($p < 0.05$).

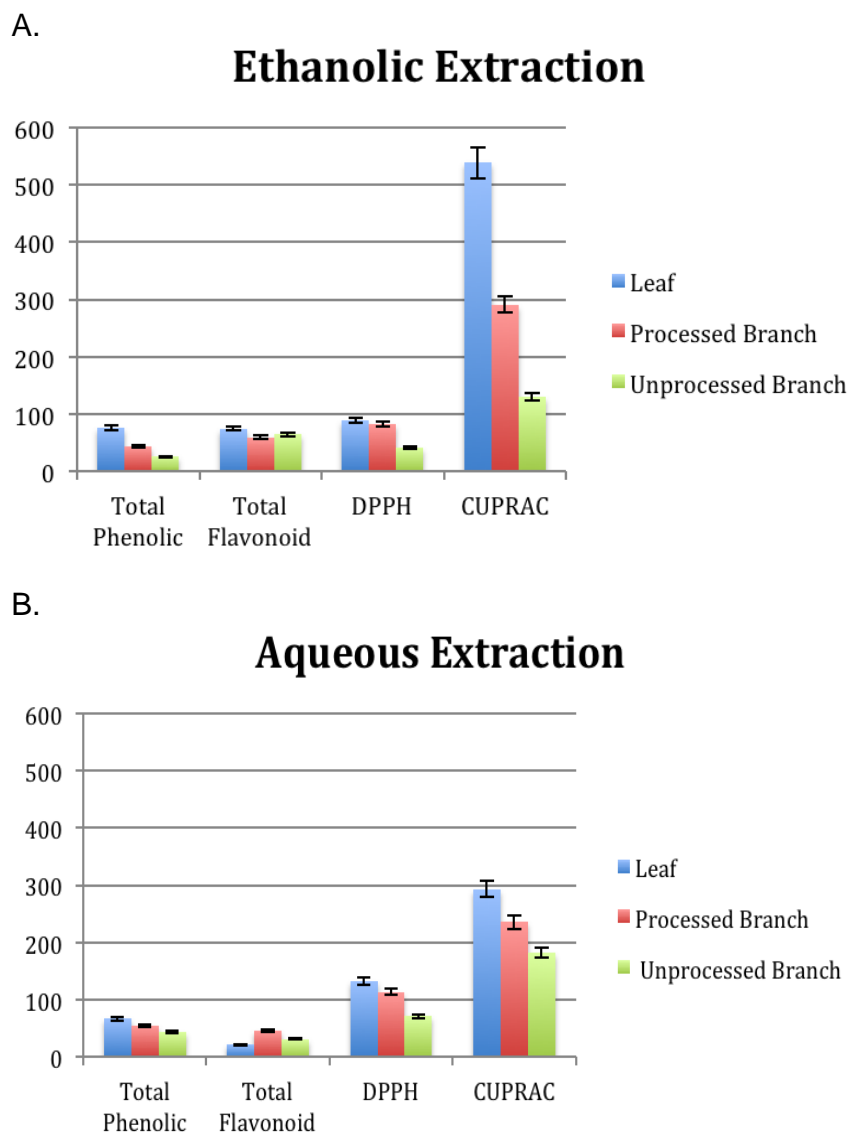
**Figure 1.** Total phenolic content (TPC), total flavonoid content (TFC), free radical scavenging activity (DPPH) and copper reducing antioxidant capacity (CUPRAC) of both ethanolic (A) and aqueous (B) extracts.

Table 3. Flavonol profile of ethanolic and aqueous extracts of SBT.

Compound ($\mu\text{g/g}$)	Leaf		Processed branch		Unprocessed branch	
	Ethanolic	Aqueous extraction	Ethanolic	Aqueous extraction	Ethanolic	Aqueous extraction
Rutin	8377 \pm 96 ^a	6939 \pm 46 ^b	1229 \pm 98 ^d	1433 \pm 47 ^c	757 \pm 13 ^e	418 \pm 108 ^f
Quercetin-3-O-galactoside	703 \pm 13 ^a	586 \pm 37 ^b	164 \pm 23 ^d	231 \pm 30 ^c	152 \pm 7 ^d	85 \pm 31 ^e
Kaempferol	129 \pm 2 ^a	65 \pm 1 ^b	Nd*	Nd	Nd	Nd

*Data represent average quantities \pm standard deviation (determined by HPLC) of three independent samples. Different letters in the rows represent statistically significant differences ($p < 0.05$). **Nd: not detected.

Antioxidant activity

To investigate the antioxidant activities of SBT leaves UB and PB two different *in vitro* assays including 1,1-diphenyl-2-picrylhydrazyl (DPPH) and Copper Reducing Antioxidant Capacity (CUPRAC) were used. The DPPH scavenging activity of extracts ranged from 41.9 ± 3.6 and 132.4 ± 6.6 mg TE/g. EE of leaf (89.6 ± 2.1 mg TE/g) had significantly higher antioxidant properties than PB (83.3 ± 9.8 mg TE/g) and UB (41.9 ± 3.6 mg TE/g). But statistically there is no significant difference between EE of leaves and PB ($p > 0.05$). This correlation was also valid for AE. When compared to extraction methods, higher scavenging activity was observed in AE of leaves, UB and PB (Figure 1). The CUPRAC values of extracts ranged from 129.4 ± 18.1 to 538.5 ± 34.8 mg TE/g. Higher antioxidant capacity was observed in EE of leaf, PB and AE of UB.

HPLC analysis

The results as shown in Table 3 indicated that the extracts are rich in rutin, Quercetin-3-O-galactoside and kaempferol.

DISCUSSION

In the present study, relatively higher phenolic compounds were observed from EE in comparison to AE of SBT leaves (Figure 1). These results were in accordance with previous studies (Upadhyay et al., 2010; Yogendra Kumar et al., 2013). Leaves were found to contain maximum TPC followed by processed branches and unprocessed branches.

In a study performed by Michel et al. (2012), different SBT extracts were evaluated for their antioxidant activity. The DPPH scavenging activity of extracts ranged from 174.8 and 528.6 mg TE/g.

In another study, Yogendra Kumar et al. (2011), evaluated antioxidant activity of the leaf extracts of SBT using DPPH and FRAP assays. The trolox equivalent

antioxidant capacity (TEAC) values reported for the extracts by using FRAP assay were in the range of 2.03 to 182.13 mg/g, while the values for the DPPH assay were 6.97 to 282.75 mg/g. In addition, according to the researchers the higher antioxidant activity exhibited by the sub critical water extracts (SWE) over the other Soxhlet and decoction extraction methods clearly demonstrated the relative advantage of SWE for obtaining formulations with high antioxidant compounds.

The profile of lipophilic antioxidants (LA) was also studied in SBT leaves, harvested in summer (June) and autumn (October) from plants of both sexes (female and male) by Górnas et al. (2014). According to this study, results indicated a greater significance of plant sex rather than harvest time; however, autumn samples of both plant sexes had a slightly higher antioxidant activity than summer samples. But an opposite phenomenon was observed in the case of isolated LA fraction from SBT leaves extracts. The higher antioxidant activity of the isolated LA fraction from 80% ethanol extracts of SBT leaves harvested in autumn, compared with the summer samples, were explained by an increase in the content of lipophilic compounds during leaf development in plants of both sexes.

According to the results, there are differences between DPPH and CUPRAC assays; higher values were obtained by the CUPRAC method. This might be related with the diversity of the reaction conditions including the required reaction time, and the wavelength at which the measurements are performed (Antolovich et al., 2002; Capanoglu et al., 2010). It is known from the literature that the chromogens of CUPRAC method have a good solubility in solvent systems (both aqueous and organic) (Apak et al., 2007), which can be the reason of high TE values. The differences are basically as a result of the non-standardized assay techniques with different radicals that is generated, time of reaction or mechanism. Especially, with regard to the antioxidant capacity tests, it will be beneficial to apply different test procedures for a full evaluation of antioxidant activity (Antolovich et al., 2002; Capanoglu et al., 2010). Moreover, in order to investigate these metabolites in detail it is recommended to use more comprehensive methods such as LC-MS.

Several authors have determined the profile of major phenolic compounds of SBT (Zu et al., 2006; Arimboor et al., 2008; Upadhyay et al., 2010; Arimboor and Arumughan, 2012; Pop et al., 2013, etc). In these studies, compounds belonging to groups of phenolic acids, flavonols, and flavones were identified. In the group of phenolic acids, gallic acid (GA) was the dominant phenolic acid for SBT leaves. Bittova et al. (2014) monitored the HPLC profiles of polyphenolic compounds in different SBT plant parts during annual growth cycle and estimation of their antioxidant potential. Their results showed that catechin, epicatechin and gallic acid were the most abundant analytes in annual green shoots and leaves, and their content varied significantly during the studied period. Besides, according to the result of Pop et al. (2013) leaves had higher levels of flavonol glycosides than berries, at average 1118 mg/100 g dry weight. On the other hand, isorhamnetin were the predominant flavonoid for berries, but rutin, quercetin-3-glucoside and kaempferol were found to be predominant in leaves.

This study also focused on the analysis of flavonols, particularly rutin, from sea buckthorn leaves and branches. In the literature, there is limited information on the content of rutin in SBT. According to our results, rutin content changed between 418 ± 108 and 8377 ± 96 $\mu\text{g/g}$. Rutin content is critical since it was reported to have positive health effects against several types of liver diseases and it has great therapeutic importance. It also causes an increase in pancreatic lipase with consequent reduction in triacylglyceride levels in rats. It has also been described in events associated with the immune system, as seen with other flavonoids (Marcarini et al., 2011). The highest rutin amounts were determined in EE of leaf (8377 ± 96 $\mu\text{g/g}$). Both EE and AE of leaf had significantly higher rutin content compared to processed branches and branches. On the other hand rutin content of all samples were significantly different ($p < 0.005$).

The quercetin-3-O-galactoside amounts of SBT leaves, processed branches and branches were reported between the range of 85 ± 31 and 703 ± 13 $\mu\text{g/g}$. The highest quercetin-3-O-galactoside amounts were determined in EE of leaf too (703 ± 13 $\mu\text{g/g}$). But there is no significant difference between EE of UB and PB ($p > 0.005$). But kaempferol was only observed in EE (129 ± 2 $\mu\text{g/g}$) and AE (65 ± 1 $\mu\text{g/g}$) leaf extracts.

HPLC results indicated that parallel to the spectrophotometric analysis results, infusion resulted with lower flavonol content. Ethanolic extraction presented the highest concentration of flavonoids except processed branches. Martins et al. (2014), evaluated and compared the antioxidant and antibacterial activities, and phenolic compounds of the infusion, decoction and hydroalcoholic extract of oregano. According to their results, both preparations, mostly decoction, gave higher antioxidant activity than the hydroalcoholic extract. The antioxidant properties seem to be related to phenolic compounds,

mainly flavonoids, since decoction presented the highest concentration of flavonoids and total phenolic compounds, followed by infusion and hydroalcoholic extract, respectively.

Conclusion

According to the results of the current study, leaves, UB and PB of SBT acquire high amounts of phenolics, flavonoids and also present some degree of antioxidant activities. Taking the high antioxidant and rutin content of leaves into account and with respect to their positive health effects, consumption of SBT in forms of an herbal tea should be investigated. Wide variations in the flavonoid profile and antioxidant activity of samples were observed which is probably related with the effect of type and variety of leaves and branches and other related factors as well as processing conditions including temperature, relative humidity, etc. Further studies should be performed in order to establish bioactive properties *in vivo* and *in vitro*.

Conflict of Interests

The authors have not declared any conflict of interests.

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