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Determination of Antioxidant Activity of Different Extracts From Bark of *Pinus* spp. grown in Giresun (Turkey) Province – Phenolic analysis by RP-HPLC-DAD

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ABSTRACT

This study aimed to investigate the phenolic compounds, total phenolic content, and antioxidant activities of *Pinus sylvestris* L. var *hamata* Steven, *Pinus pinaster* Aiton subsp. *pinaster*, and *Pinus pinea* L. bark extracts prepared with hot water. The phenolic composition and total phenolic content (TPC) of extracts were determined by Reversed Phase-High Performance Liquid Chromatography-Diode Array Detector (RP-HPLC-DAD) and Folin–Ciocâlteu method, respectively. The antioxidant activity was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH•) radical scavenging and ferric ion (III) reducing / antioxidant power (FRAP) assays. Besides, the highest total phenolic content was detected in *P. pinea* bark extract [984.46±4.08 µg mL^{-1} gallic acid equivalent (GAE) and 1163.33±4.04 µg mL^{-1} catechin equivalent (CE)] and the lowest result was detected in *P. sylvestris* bark extract (361.53±3.52 µg mL^{-1} GAE and 427.26±4.17 µg mL^{-1} CE). Among the tested materials, the most abundant phenolic compounds in *P. pinea* bark extract were catechin (3.586±0.114 mg g^{-1}) and taxifolin (1.866±0.096 mg g^{-1}). According to the antioxidant results, *P. pinea* bark extract exhibited remarkable antioxidant activity than standard BHT and Trolox [SC_{50}: 1.64310±0.00003 µg mL^{-1} for DPPH and 1428.75±5.62 µM Trolox Equivalent Antioxidant Capacity (TEAC) for FRAP]. The obtained results indicated that pine bark extracts can be used as an easily obtainable natural source of antioxidants for the food and pharmaceutical industry.

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Giresun (Türkiye) İlinde Yetiştirilen *Pinus* spp.'nin Kabuğundan Elde Edilen Farklı Ekstraktların Antioksidan Aktivitesinin Belirlenmesi – RP-HPLC-DAD ile Fenolik Analizi

ÖZET

Bu çalışmada, sıcak su ile hazırlanan *Pinus sylvestris* L. var *hamata* Steven, *Pinus pinaster* Aiton subsp. *pinaster* ve *Pinus pinea* L. kabuk ekstraktlarının fenolik bileşikleri, toplam fenolik madde içeriği ve antioksidan aktivitelerinin incelenmesi amaçlanmıştır. Ekstraktların fenolik bileşimi ve toplam fenolik içeriği sırasıyla Ters Faz-Yüksek Performanslı Sıvı Kromatografi-Diyot Array Dedektörü (RP-HPLC-DAD) ve Folin–Ciocâlteu yöntemi ile belirlendi. Antioksidan aktivite, 2,2-difenil-1-pikrilhidrazil (DPPH•) radikal temizleme ve demir (III) indirgeme / antioksidan kuvvet (FRAP) metotları ile belirlendi. Numuneler arasında en yüksek toplam fenolik içerik *P. pinea* kabuk ekstraktında [984.46±4.08 µg mL^{-1} gallik asit eşdeğeri (GAE) ve 1163.33±4.04 µg mL^{-1} kateşin eşdeğeri (CE)] ve en düşük sonuç *P. sylvestris* kabuk ekstraktında tespit edilmiştir (361.53±3.52 µg mL^{-1} GAE ve 427.26±4.17 µg mL^{-1} CE). *P. pinea* kabuk ekstraktlarında en bol bulunan fenolik bileşikler kateşin (3.586±0.114 mg g^{-1}) ve taksifolindir (1.866±0.096 mg g^{-1}). Antioksidan sonuçlarına göre, *P. pinea* kabuk ekstraktı standart BHT ve Trolox'a göre kayda değer antioksidan aktivite gösterdi [SC_{50}: DPPH için 1.64310±0.00003 µg mL^{-1} ve FRAP için

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Anahtar Kelimeler
Çam kabuğu
Antioksidan aktivite
Fenolik bileşikler
RP-HPLC-DAD
Toplam fenolik içerik

1428.75±5.62 µM Trolox Eşdeğeri Antioksidan Kapasite (TEAC)]. Elde edilen sonuçlar, çam kabuğu ekstraktlarının gıda ve ilaç endüstrisi için kolayca elde edilebilen doğal bir antioksidan kaynağı olarak kullanılabileceğini gösterdi.

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INTRODUCTION

Secondary metabolites with important functions for plants consist of many important biochemicals such as phenolic compounds, flavonoids, terpenes, and carotenoids (Gülçin, 2012; Tohma et al., 2017; Gülçin, 2020). In recent years, many studies have been conducted to obtain extracts rich in secondary metabolites and to discover their natural antioxidant properties. These products, which show different bioactivity due to their different chemical structures, have increased the interest in different industries such as medicine, cosmetics, dyes, textiles, food, and pharmaceuticals. The vast majority of plants, which are considered natural resources, are distributed in forest areas. Turkey is one of the world's most important and richest centers of plant sources, due to the presence at the junction of three different flora in terms of plant geography, geographic location, topography, water sources, the microclimate of diversity, geological structure (Avcı, 2005). This potential has enabled the local people to benefit from herbal resources in various ways from past to present. Turkey's forest area is in a rising trend from the past to the present. According to the latest data (Terzioğlu et al., 2012), Turkey has 22.6 million hectares of forest areas, and this area covers 28.6% of its land. Turkey forests, spatially 48% of pure coniferous, 33% of pure broadleaf, while the remaining portion is mixed coniferous-broadleaf forest structure. In a pine tree, while the amount of average bark ranges from 8-14% (Harkin and Rowe, 1971; Öktem, 1976), the average bark ratio of the pine species in Turkey is defined as 12.5% (Kurt and Mengeloğlu, 2006). Thus, the average is about 1,250,000 m^3 of bark left in the forest as waste material each year in Turkey. However, many studies have reported that forestry residues, especially the bark, are a rich source of secondary metabolites containing biologically active compounds (Kızılarslan and Sevgi, 2013; Çakır, 2017). In addition, pine bark extracts contain a large number of phenolic compounds such as catechins, epicatechins, taxifolin, and phenolic acids (Dróżdż and Pyrzynska, 2019; Hamad et al., 2019). The effect of pine bark extract comes from these components (D'Andrea, 2010). A group of active substances found in French maritime bark (*Pinus pinaster* Aiton subsp. *pinaster*) is called Pycnogenol and is also a trademark. It is stated that pine bark extract is good for many diseases such as cough, pertussis (Güzel et al., 2015), asthma (Özüdoğru et al., 2011), tuberculosis (Sezik et al., 1997), milk enhancer, tuberculosis, urinary tract diseases, hemorrhoids, memory weakness, wound healing, strengthening, painkiller (Everest and Öztürk, 2005) and used in treatments (Maimoona et al., 2011; Taner et al., 2014). *Pinus*, a member of Pinaceae, has three pine species grown naturally (*Pinus sylvestris* L. var. *hamata* Steven) and artificially grown (*Pinus pinea* L. and *Pinus pinaster* Aiton subsp. *pinaster*) in Giresun province in Turkey. Phenolic content and antioxidant activity of various extracts of the genus of *Pinus* members have been previously reported by a number of researchers (Yeşil-Çeliktaş et al., 2009a; Hamad et al., 2019; Skrypnik et al., 2019). However, there are very few studies in the literature about the *Pinus* species grown in northern Turkey. As is known, chemical compositions and antioxidant activities depend on many factors such as geographical region, climatic, experimental conditions, and diversity. In this respect, the aim of the study is to identify new natural antioxidant extracts or compounds. In line with this goal, phenolic composition analysis barks of three pine species (*Pinus sylvestris* L. var. *hamata* Steven*, Pinus pinaster* Aiton subsp. *pinaster,* and *Pinus pinea* L.) were quantified by Reversed Phase-High Performance Liquid Chromatography-Diode Array Detector (RP-HPLC-DAD). In order to investigate *in vitro* antioxidant activities of these samples, ferric ion (III) reducing / antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH•) tests were applied. Also, total phenolic content was determined by the Folin–Ciocâlteu method.

MATERIALS and METHOD

Reagents and chemicals

Catechin, epicatechin, taxifolin, gallic acid, vanillin, vanillic acid, ferulic acid, caffeic acid, protocatechuic acid, protocatechuic aldehyde, and ellagic acid were purchased from Sigma-Aldrich (Steinheim, Germany). Solvents in analytical purity of methanol, acetonitrile, ethyl acetate, chloroform, and acetic acid were purchased from Merck. Sodium sulfate and sodium chloride were obtained from Merck (Darmstadt, Germany). DPPH, 2,4,6-Tripyridyl-s-triazine (TPTZ), Folin–Ciocalteu reagent phenol, and Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma Aldrich (Munich, Germany). HPLC syringe filters (polyvinylidene difluoride, 0.45 µm) were purchased from ISOLAB

(Germany). The ultrapure deionized water used in the experiments was obtained from the Sartorius ultrapure water purification system.

Analysis of phenolic compounds by Reversed Phase-High Performance Liquid Chromatography-Diode Array Detector (RP-HPLC-DAD).

Stock solutions of the phenolic standards (1000 ppm) were prepared in 40% methanol-ultra pure water or %100 methanol, depending on their solubility. Analysis of phenolic compounds was performed by Thermo Scientific Dionex Ultimate™ 3000 system (Thermo Scientific, Bremen, Germany) using a reversed-phase C_{18} column (150 mm x4.6 mm, 5µ; Fortis). The mobile phase consists of A: 2% acetic acid-ultrapure water, C: 50-50% acetonitrile-ultrapure water solution in 0.5% acetic acid, and D: acetonitrile. For the separation of phenolic compounds, gradient elution with a flow rate of 0.7 mL/min was applied as followed: 0-8 min (7% C), 8-18 min (12% C), 18-23 min (23% C), 23-25 min (40% before returning to the initial conditions C), 25-35 min (45% C), 35-40 min (55% C), 40-43 min (92% C), 43-46 min (25% C). The column temperature was set to 25 ℃ and the injection volume was adjusted to 20 µL. Standards of eleven phenolic compounds (catechin, epicatechin, taxifolin, gallic acid, vanillin, vanillic acid, ferulic acid, caffeic acid, protocatechuic acid, protocatechuic aldehyde, and ellagic acid) were detected at four different wavelengths (260, 280, 308, 324 nm) comparatively. The wavelengths determined in the DAD were selected based on the wavelength of maximum absorption of phenolic standards in the literature. The Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated for the phenolic compounds in the extracts (Table 1).

Table 1. Reversed Phase-High Performance Liquid Chromatography-Diode Array Detector validation parameters
Çizelge 1. Ters Faz-Yüksek Performanslı Sıvı Kromatografisi-Diyot Array Dedektörü doğrulama parametreleri

No	Compound	RT (min.)	m[a]	R^2	LOD[b]	LOQ[b]
1	Gallic acid	7.15	1.0597	0.9973	0.0417	0.1004
2	Protocatechuic acid	13.04	2.0259	0.9965	0.0105	0.0327
3	Protocatechuic aldehyde	19.20	0.6452	0.9961	0.1140	0.3031
4	Catechin	22.17	4.6834	0.9974	0.0032	0.0089
5	Vanillic acid	24.87	1.5219	0.9964	0.0137	0.0487
6	Caffeic acid	25.58	1.0534	0.9993	0.0542	0.1312
7	Epicatechin	27.82	4.2632	0.9971	0.0030	0.0091
8	Vanillin	30.03	0.8083	0.9964	0.0762	0.2257
9	Ellagic acid	31.75	0.3390	0.9968	0.1142	0.3445
10	Taxifolin	32.94	0.8500	0.9999	0.2035	0.6334
11	Ferulic acid	33.51	1.1052	0.9947	0.0297	0.0917

[a, b]: values are given in mg L^{-1}.

Sample preparation and extraction

Analyzed pine barks (*P. pinea*, *P. sylvestris*, and *P. pinaster*) were collected from harvesting areas in the Giresun province in Turkey. The identification of the gathered plant samples was done by Dr. Mustafa KARAKÖSE. Information on *Pinus* species is given in Table 2. Each pine bark was ground separately and pulverized. The powdered sample 10 g was extracted in 150 mL of boiling water for 15 minutes. It was then left to cool to room temperature. The extract was centrifuged, and the liquid fraction was separated. Solid sodium chloride was added to the red-brown solution until saturation and high molecular weight molecules were allowed to precipitate (Masquelier, 1987). The solution was filtered to remove the precipitate. The remaining solution was extracted 3 times with 25 mL of ethyl acetate. The water and ethyl acetate phases were separated, and residual water was removed by the addition of sodium sulfate. Unlike Masquelier's method, the ethyl acetate phase was completely evaporated at 40 °C using a rotary evaporator. The resulting beige-colored solid was dissolved in a 25% (v/v) methanol/water mixture and injected into the RP-HPLC-DAD system.

Table 2. Locations, common names and collector numbers of the plant specimens
Çizelge 2. Bitki örneklerinin toplanma alanları, yaygın isimleri ve toplayıcı numaraları

Taxon name	Common name	Location	Collector no
Pinus pinaster Aiton subsp. *pinaster*	Maritime pine	4 m, Espiye district, dune afforestation area, 23.VI.2015.	M. Karaköse 1471
Pinus pinea L.	Stone pine	5 m, Espiye district, dune afforestation area, 23.VI.2015.	M. Karaköse 1472
Pinus sylvestris L. var. *hamata* Steven	Scots pine	1754 m, Alucra district, *Pinus sylvestris* forest, 23.VI.2015.	M. Karaköse 1470

Determination of total phenolic content by Folin–Ciocâlteu method

The pine bark extracts were evaluated in terms of total phenolic compounds by using the Slinkard and Singleton method with some modifications (Slinkard and Singleton, 1977). All samples were prepared by

diluting (1:4) the stock solution. A sample solution of 50 µL was diluted with 2500 µL of distilled water. The sample was mixed with 250 µL of 0.2 normal Folin–Ciocâlteu reagent by vortexing for three minutes. Then 750 µL sodium carbonate (7.5%) was added into the solution and vortexed again. After incubation for 2 h at room temperature, the absorbance of samples was measured at 765 nm. Each sample and standard concentration were studied in three parallel runs. In addition, sample and reagent blank were studied for each sample and each concentration of the standard. Total phenolic contents were expressed as µg gallic acid or catechin equivalent per mL sample using calibration graph plotted separately in the concentration range of 15.6 to 1000 µg mL^{-1} of gallic acid and catechin standards.

Ferric reducing antioxidant power assay

The FRAP method is based on the measurement of the absorbance given by the TPTZ-Fe (II) complex developed later (Benzie and Strain, 1996; Karaçelik et al., 2015). The activities of all samples were defined as micromolar Trolox Equivalent Antioxidant Capacity (TEAC). The calibration graph was constructed by using Trolox at a concentration range of 62.5-1000 µM. All samples were diluted from the concentrated solution (1:50). Briefly, 50 µL of the sample was mixed with a 1.5 mL FRAP reagent. The FRAP solution was prepared according to the method defined by Karaçelik et al. (2015). The mixtures were incubated at 24 °C for 20 min and measured spectrophotometrically at 595 nm.

2,2-diphenyl-1-picrylhydrazyl radical scavenging activity

Radical scavenging activity was tested according to the method defined by Brand-Williams et al. (1995). The working interval was determined by pretesting all samples and standards (Trolox and BHT) prepared in different concentrations. Firstly, samples were mixed with an equal volume (750 µL) of 100 µM methanolic DPPH solution by vortexing and then they were incubated for 50 min at 24 °C. The maximum absorbances that DPPH gave were recorded at 517 nm. According to the SC_{50} values given as mg mL^{-1}, high radical scavenging potential is seen at a low SC_{50} value (Gülçin, 2006b; a; Ak and Gülçin, 2008).

RESULTS and DISCUSSION

Identification and quantification analysis of phenolic compounds by RP-HPLC-DAD

Previously some phenolic compounds in the bark of the pine species in Turkey were examined (İnce et al., 2009; Yeşil-Çeliktaş et al., 2009a; Yeşil-Çeliktaş et al., 2009b). However, these studies are very limited and local. Previously phenolic content of *P. pinaster* in Turkey has not been determined. There are also few studies on the phenolic content of other pine bark species (Kıvrak et al., 2013; Şeker et al., 2021). Normal phase and reverse phase liquid chromatographic methods were used in studies with pine bark.

Eleven phenolic compounds were analyzed in three different pine bark extracts with the RP-HPLC-DAD method. The chromatograms of three pine bark extracts are given in Figure 1. Catechin, protocatechuic acid, and taxifolin were found in three pine barks. Ferulic acid, gallic acid, and ellagic acid were not detected in any pine bark. Quantitative results of phenolic constitutes in pine barks are given in Table 3. Among the highest amounts, catechin and taxifolin (3.586 and 1.866 mg g^{-1}) in *P. pinea* and catechin in *P. pinaster* (1.231 mg g^{-1}) are noteworthy. Separation of all phenolic compounds was completed within 33 minutes.

Table 3. Quantitative results of phenolic compounds in pine barks
Çizelge 3. Çam kabuklarındaki fenolik bileşiklerin kantitatif sonuçları

Phenolics	mg g^{-1}		
	P. pinea	*P. sylvestris*	*P. pinaster*
Catechin	3.586±0.114	0.681±0.008	1.231±0.078
Epicatechin	0.022±0.001	n.d.	0.011±0.001
Protocatechuic acid	0.041±0.001	0.271±0.010	0.138±0.005
Protocatechuic aldehyde	n.d.	0.030±0.001	n.d.
Vanillic acid	n.d.	0.018±0.001	0.010±0.001
Vanillin	n.d.	0.024±0.001	n.d.
Caffeic acid	0.104±0.006	n.d.	n.d.
Taxifolin	1.866±0.096	0.500±0.006	0.360±0.004
Ferulic acid	n.d.	n.d.	n.d.
Gallic acid	n.d.	n.d.	n.d.
Ellagic acid	n.d.	n.d.	n.d.

n.d.: not detected.
Data are represented as means ±SD (standard deviation) of triple measurements.

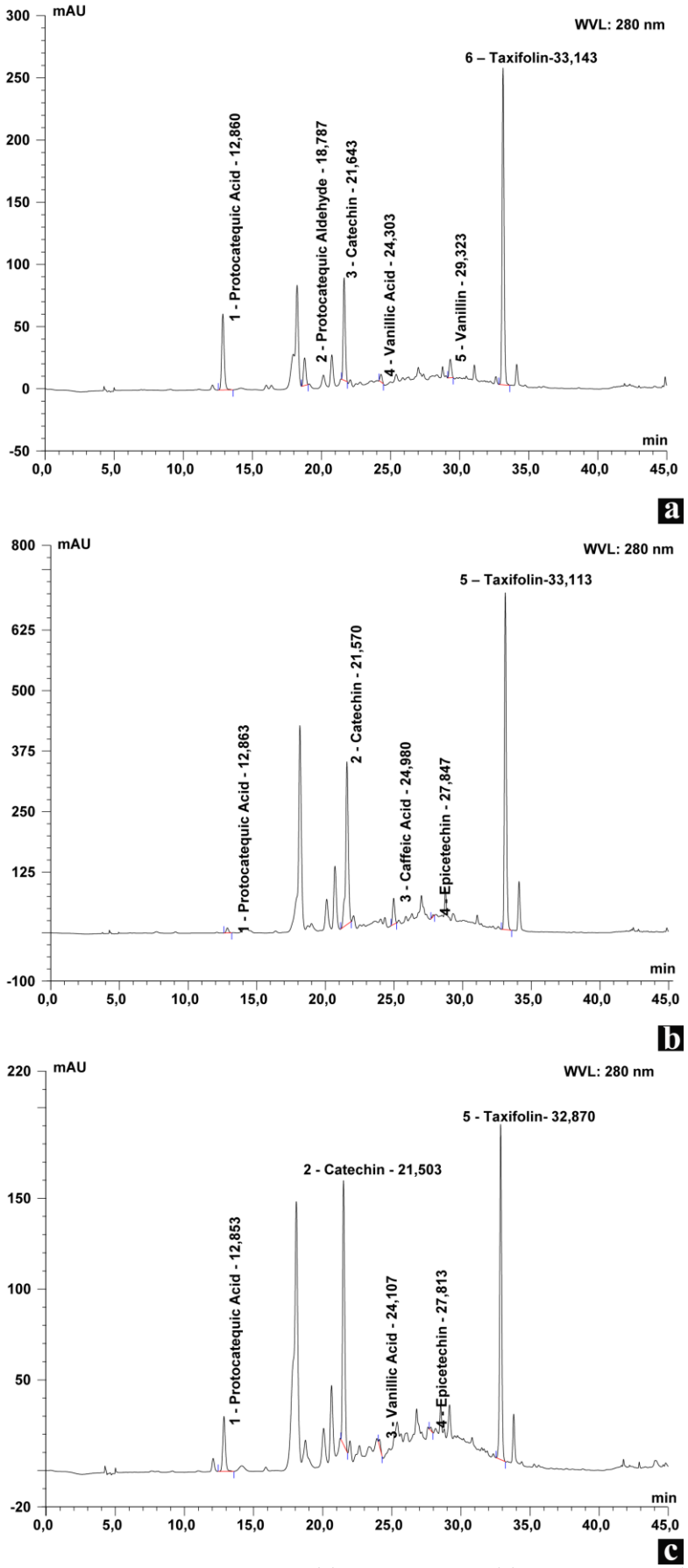

Figure 1. Chromatograms of (a) *P. sylvestris*, (b) *P. pinea*, and (c) *P. pinaster*
Şekil 1. (a) *P. sylvestris,* (b) *P. pinea ve* (c) *P. pinaster*'in kromatogramları

While the amount of catechin in *P. pinaster*, known as the French maritime bark, was found to be 7.92 mg g^{-1} (İnce et al., 2009), the amount of taxifolin ranged between 17-33 mg g^{-1} (İnce et al., 2009; Yeşil-Çeliktaş et al., 2009a). However, the amount of catechin and taxifolin in *P. pinaster* analyzed in the study was found to be far below these rates. This is probably because the tree does not grow in the area where it grows normally. Although these rates are quite low in *P. pinaster* obtained from the Espiye district, the amount of catechin in *P. pinea* is close to that in French *P. pinaster*. While the results given in the literature are the amount of gram phenolic per gram extract, it should be noted that the results given in Table 3 are the gram phenolic amount per gram pine bark. When evaluated in this way, it will be better understood that the amount of catechin in *P. pinea* is quite high. When compared with *P. pinea* and other *Pinus* species analyzed in other studies in the literature, the amount of catechin obtained from *P. pinea* barks collected from Espiye is quite high. Especially taxifolin appears to be 13.36 times higher than the values that were found in a study conducted by Yeşil-Çeliktaş et al. (2009b). In addition, the amount of taxifolin found in *P. sylvestris* collected from the Alucra region is also ten times higher when the other studies were examined. Compared to the same research, the catechin values obtained from *P. sylvestris* in Alucra are higher than the catechin values for *P. sylvestris* collected from Germany. However, compared to French maritime pine (*P. pinaster)*, the values obtained from the analysis are about fifty times lower, especially for epicatechin and taxifolin (İnce et al., 2009). When compared to *P. pinea*, which was collected from Bilecik and analyzed, although the amount of taxifolin in the *P. pinea* in Espiye was almost the same, the amount of catechin was found approximately twice. In both studies, the results are given as gram phenolic per gram pine bark (Şeker et al., 2021). When compared with *P. sylvestris* analyzed in the same study, the catechin and taxifolin contents of *P. sylvestris* collected from the Alucra region were found to be quite high.

On the other hand, the remarkable result is that protocatechuic acid was found in all three types. Unlike our benchmarking studies, protocatechuic acid was analyzed for the first time in pine barks. Also, caffeic acid was found in *P. pinea*. Catechin and taxifolin, which are found in high amounts in pine bark compared to others, are important compounds for human health. In experiments, it has been observed that taxifolin prevents enzyme increases due to inflammatory reactions similar to hydrocortisone (Gupta et al., 1971). In addition, taxifolin is recommended for the development of new drugs, as it has anti-tumors, anti-oxidants, anti-cardiovascular, and, more importantly, anti-cancer effects (Sunil and Xu, 2019). Likewise, anti-carcinogenic and antioxidant effects of catechin are known (Lotito and Fraga, 1998; Menon et al., 1999). As can be seen from the results obtained, pine bark is a rich source of catechin and taxifolin.

In addition, both normal phase and reversed-phase HPLC methods are used for pine bark analysis in the literature. Although it is stated that reverse phase analysis is more complicated and very successful chromatograms were obtained with the reverse phase method. For extraction, the boiling water method, which does not harm the environment, was used. Thus, only water-soluble species were obtained. Then they were easily taken to the ethyl acetate phase and the ethyl acetate phase was evaporated at 50°C. The remaining solid portion was easily dissolved in 25% methanol solution and injected into the device. Considering the studies made with pine bark in the literature, it is seen that the ratio of the same phenolic components found in the bark of the same type of pine can vary between 10 and 100 times. These rates vary according to the environment in which the tree grows.

Determination of total phenolic contents

In this study, the total phenolic contents of extracts were determined by Folin–Ciocâlteu method. Total phenolic content results were given in Table 4. The total phenolic content results were presented as µg gallic acid equivalent per mL sample (GAE, µg mL^{-1}) and µg catechin equivalent per mL sample (CE, µg mL^{-1}). The total amounts of phenolic contents ranged from 361.53±3.52 to 984.46±4.08 µg mL^{-1} in terms of GAE and ranged from 427.26±4.17 to 1163.33±4.04 µg mL^{-1} in terms of CE. The order of total phenolic contents of pine bark extracts was *P. pinea* > *P. pinaster* > *P. sylvestris*. Hamad et al. (2019) reported that the phenolic contents were about 88 µg mL^{-1} GAE for pine bark (*P. sylvestris*) extracts prepared by using a methanol-water mixture as solvent (Hamad et al., 2019). Skrypnik et al. (2019) prepared water extract by using whole bark and outer bark of *P. sylvestris* L. and found the total phenolic contents of extract between 4 mg GAE g^{-1} and 12 mg GAE g^{-1} (Skrypnik et al., 2019).

Antioxidant activity

Many antioxidant assays based on the methodological differences have been used for screening antioxidant activities of plant extracts in the literature. FRAP and DPPH• radical scavenging activities were used to test the antioxidant activities of pine bark extracts. The results of the DPPH• assay were expressed as SC_{50} (Table 4) means the effective concentration of test samples required for 50% antioxidant activity

under the experimental conditions. Lower SC_{50} values indicated higher radical scavenging activity. *P. pinea* bark extract showed higher values than the other two pine barks. *P. pinea* bark extract demonstrated the higher DPPH• radical scavenging activities (SC_{50}: 1.64310±0.00003 µg mL^{-1}), while *P. sylvestris* bark extract exhibited the lowest antioxidant activities (SC_{50}: 6.04765±0.09043 µg mL^{-1}). It was found that the DPPH scavenging activities of all pine bark extracts were higher than BHT standard antioxidant. In addition, the DPPH• scavenging activities of *P. pinea* and *P. pinaster* bark extracts were found to be higher than BHT and Trolox standard antioxidants. To compare study results with literature, the DPPH• scavenging activity of the extracts was also evaluated as %scavenging of DPPH• at 0.00300 mg mL^{-1} [*P. pinea* (91,7 %), *P. pinaster* (70,4 %), *P. sylvestris* (27,0 %)]. Results obtained are higher than those achieved in many studies (Yeşil-Çeliktaş et al., 2009a; Skrypnik et al., 2019).

The higher TEAC values in the FRAP test indicate higher antioxidant activity (Table 4). The results of three pine bark extracts were ranged from 549.37±8.59 to 1428.75±5.62 µM TEAC. While the highest FRAP value of pine bark extracts was found in *P. pinea* bark extract (1428.75±5.62 µM TEAC), the lowest value was in *P. sylvestris* bark extract (549.37±8.59 µM TEAC). According to all antioxidant assays, all extracts exhibited an antioxidant activity with the order of activity as *P. pinea* >*P. pinaster* >*P. sylvestris*. The extract of *P. pinea* bark showed strong DPPH• radical scavenging and FRAP activities possessing a high amount of total phenolic compounds was identified. It was also found that there was a good positive correlation between SC_{50} values of DPPH• radical scavenging and TEAC values (R^2: 0.9951).

In recent years, the number of publications for the qualitative and quantitative determination of biological activity potentials and active compounds of extracts obtained from forest residues has increased. In literature, there have been few studies on the biological activity research on the *P. pinea*, *P. pinaster*, and *P. sylvestris* bark extracts. *P. pinaster* and *P. sylvestris* bark extracts prepared with polar solvents such as ethanol or aqueous mixtures containing ethanol were found to have the highest phenolic compound contents and the highest antioxidant activity (Dróżdż and Pyrzynska, 2019; Skrypnik et al., 2019; Ferreira-Santos et al., 2020). The correlation coefficient between the results of the phenolic content and antioxidant assays (DPPH and FRAP) showed a good positive correlation with R^2 values of 0.9624 and 0.9844. These findings suggested that high antioxidant activity might be due to high phenolic contents.

Table 4. Antioxidant activity and total phenolic content of pine bark extracts and standards
Çizelge 4. Çam kabuğı ekstraktlarının ve standartların antioksidan aktivite ve toplam fenolik içeriği

Sample and standards	Total phenolic content (GAE, µg mL^{-1})	Total phenolic content (CE, µg mL^{-1})	DPPH radical scavenging (SC_{50}, µg mL^{-1})	FRAP (TEAC, µM)
P. pinea	984.46±4.08	1163.33±4.04	1.64310±0.00003	1428.75±5.62
P. sylvestris	361.53±3.52	427.26±4.17	6.04765±0.09043	549.37±8.59
P. pinaster	816.92±2.31	965.45±2.73	1.83300±0.01108	1279.68±3.97
BHT	n.d.	n.d.	8.52667±0.01528	n.d.
Trolox	n.d.	n.d.	5.04667±0.00577	n.d.

n.d.: not detected.
Data are represented as means ±SD (standard deviation) of triple measurements.

CONCLUSION

This is the first study to evaluate phenolic content of barks of pine grown in Giresun. In the study, the standard extraction method was cut short and chemical and time savings were achieved. According to the results of RP-HPLC-DAD system some of the high peaks in the chromatograms were not identified. Therefore, identification of these peaks can be identified in further studies. The results of this study will provide information to the literature about the content of pine bark. It may also form the basis for further research on alternative medical practices regarding the compounds it contains.

Author's Contributions

The contribution of the authors is equal.

Statement of Conflict of Interest

Authors have declared no conflict of interest.

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