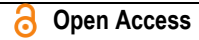




## Determination of Antioxidant Activity, Antimicrobial Effect and Fatty Acid Composition of Red Pine Bark (*Pinus brutia* Ten., Turkish Pine) Extracts

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### Research Article

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### Abstract

In this study, the antioxidant activity, antimicrobial effect, and fatty acid composition of the bark of the Turkish Pine (*Pinus brutia* Ten.) grown in Burdur Province (Türkiye) were investigated. Red pine bark extracts were analyzed using the Folin-Ciocalteu method for total phenolic content, the DPPH test for free radical scavenging activity, the FRAP test for reduction power, and the carotene-linoleic acid test to determine antioxidant activity. Red pine bark was found to have a significant antioxidant effect. The total phenomenon, expressed as gallic acid, was found to be 287.31 mg GAE/100 g, while the free radical inhibition rate was found to be 93.94%. The results were compared with the standard antioxidants BHA and BHT. The antimicrobial activity of the chloroform, methanol, and ethyl acetate extracts of red pine bark was examined against four bacteria strains using the microdilution method, and their fatty acid compositions were determined using a GC-FID device. Although it showed no activity against *Staphylococcus aureus*, it produced remarkable results against *Bacillus cereus* and *Escherichia coli* in particular. Therefore, it was found to have a good antibacteria effect and an important fatty acid profile for industry with long-chain (24-carbon) saturated and long-chain unsaturated fatty acids. While the SFA value was high (89.23%), C24:0 (lignoceric acid) was the predominant fatty acid with a proportion of 51.5%, followed by C22:0 at 28.38%.

**Keywords:** *Pinus brutia* Ten., Turkish pine, antioxidant, fatty acid, MIC

## Kızılçam Kabuğu (*Pinus brutia*, Türk Çamı) Ekstrelerinin Antioksidan Aktivitesi, Antimikrobiyal Etkisi ve Yağ Asidi Bileşiminin Belirlenmesi

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### Özet

Bu araştırmada, Burdur ilinde (Türkiye) yetiştirilen kızılçam kabuğunun (*Pinus brutia* Ten., Türk Çamı) antioksidan aktivitesi, antimikrobiyal etkisi ve yağ asidi bileşimi incelenmiştir. Kızılçam kabuğu ekstraktları, toplam fenolik madde içeriği için Folin-Ciocalteu yöntemi, serbest radikal temizleme aktivitesi için DPPH testi, güç azaltımı için FRAP testi ve antioksidan aktiviteyi belirlemek için β-Karoten -linoleik asit testi kullanılarak incelenmiştir. Kızılçam kabuğunun dikkate değer bir antioksidan etkisi görülmüştür. Gallik asit cinsinden toplam fenolik madde miktarı 287.31 mg GAE/100 g bulunmuşken serbest radikalleri inhibe etme oranı ise %93.94 olarak bulunmuştur. Sonuçlar, standart antioksidanlar BHA ve BHT ile karşılaştırılmıştır. Kızılçam kabuğunun kloroform, metanol ve etil asetat ekstraktlarının antimikrobiyal aktivitesi,

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dört bakteri suşuna karşı mikro seyreltme suyu yöntemi ile incelenmiş ve yağ asidi bileşimleri GC-FID cihazı kullanılarak belirlenmiştir. *Staphylococcus aureus*'a karşı bir etkinliği olmasa da özellikle *Bacillus cereus* ve *Escherichia coli* 'ye karşı dikkat çekici bir sonuç görülmüştür. Dolayısıyla iyi bir antibakteriyel etki göstermiş olmakla beraber uzun zincirli (24 karbonlu) doymuş ve uzun zincirli doymamış yağ asitleri ile endüstri açısından önemli bir yağ asidi profiline sahip olduğu bulunmuştur. SFA değeri yüksek (%8923) olmasına rağmen, C24:0 (lignoserik asit) %51.5 oranıyla baskın yağ asidiydi, bunu %28.38 ile C22:0 izlemiştir.

**Anahtar Kelimeler:** *Pinus brutia* Ten., Türk Çamı, antioksidan, yağ asidi, MİK

## Introduction

Free radicals play a significant role in both tissue repair processes and the progression of pathological conditions. Lipids containing polyunsaturated fatty acids (PUFA) readily react with molecular oxygen due to the double bonds in their structure, undergoing oxidation, which results in lipid peroxidation [1-3]. Lipid oxidation causes many diseases [1, 4-6]. An increase in the amount of lead also causes Parkinson's disease. Antioxidants neutralize free radicals. Free radicals, which are released in pollution, cigarettes, smoke, drugs, diseases and even some exercises, are reduced owing to antioxidants [7]. The importance of antioxidants increases as they reduce free radicals in living animals and extend the shelf life of foods. Many synthetic antioxidants are used for this. Also, many reports were performed recently for the production of antioxidants from natural sources [4, 8-13]. The bark of various pine species has been preferred for a long time to make food and medicine. Throughout history, people living in various parts of the world have met their food and medicine needs from pine bark [14-16]. *Pinus brutia* Ten., the Turkish pine, is a pine indigenous to the southern part of Türkiye. It is widely distributed across Türkiye, but it also extends to Greece, Cyprus, Azerbaijan, Georgia, northern Iraq, Israel, western Syria, Iran, and Lebanon. The variety of *Pinus* species in Türkiye is named as *P. halepensis* Miller (Aleppo pine), *P. brutia* Ten. (Turkish pine), *P. sylvestris* L. (Scots pine), *P. pinea* L. (stone pine, umbrella pine), and *P. nigra* J.F. Arnold (European black pine) [17]. *Pinus brutia* Ten. is widely used in afforestation and afforestation programs, as it is important in the timber industry. *Pinus* species are often utilized in the treatment, pharmaceutical and cosmetic industry. Pine bark extract, turpentine has been used in the treatment of abrasions and wounds and as an anti-aging cosmetic since ancient times [18-21]. The bioactive chemical compounds with the highest antioxidant effect compounds are phenolic acids, different flavonoid classes, carotenoids, vitamins A, E and C [22-25]. The most important source of antimicrobial compounds are aromatic compounds containing essential oils. Plants containing natural products have been consumed worldwide in traditional medicine since ancient ages and are a powerful source of medicine. The high stability of leaves as a reserve by new medicines remains mostly discovered, and very little of their biological or pharmacological properties have been studied. The therapeutic properties of plants are defined mainly in terms of their antimicrobial effects. Because plant-derived antimicrobial compounds reduce the likelihood that foodborne pathogenic bacteria can tolerate antibiotics, they can effectively reduce reliance on antibiotic powder [26]. The research aims to define the antioxidant activity, antimicrobial effect and fatty acid composition of red pine bark (*Pinus brutia* Ten. Turkish Pine).

## Material and Methods

### Red Pine Bark (*Pinus brutia* Ten., Turkish Pine)

Firstly, red pine bark provided from the forest area of Burdur province (Turkiye) was desiccated at room temperature and made pine mulch by using a mechanical mill. Next, the mulch samples were stored for further analysis in vacuum-sealed bags at room temperature in the dark.

### Materials

Fluka Chemical Co. (Buchs, Switzerland) was the supplier of gallic acid, Folin–Ciocalteu phenolic reagent, and butylated hydroxyanisole (BHA). Materials including DPPH, Tween 40, BHT, linoleic acid and  $\beta$ -carotene were sourced from Sigma–Aldrich. Merck provided dimethyl sulfoxide (DMSO), anhydrous sodium carbonate ( $\text{Na}_2\text{CO}_3$ ), potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ),  $\text{K}_2\text{HPO}_4$ , chloroform, and methanol. All reagents and solvents employed in the assays were of analytical grade purity.

### Total Phenolic Compounds (TPC)

Total phenolic content (TPC) was determined via new method described by Singleton et al. [27], together with the Folin–Ciocalteu technique. For this purpose, 0.01 g of red pine bark mulch was extracted in 10 mL methanol, and the solution was passed through Whatman No. 1 filter paper. From the obtained extract, 40  $\mu\text{L}$  was combined with 2.4 mL deionized water, followed by the addition of 200  $\mu\text{L}$  Folin–Ciocalteu reagent without dilution. After 3 minutes, 0.6 mL of 20%  $\text{Na}_2\text{CO}_3$  solution was incorporated. The product was sonicated in darkness at  $25^\circ\text{C}$  for 2h, and its absorbance was then recorded at 765 nm against a methyl alcohol blank via the UV–Vis device (Hitachi U-1900, Japan). Each measurement was repeated three times. A validation linear graphic was established with gallic acid ( $0\text{--}1000\text{ mg}\cdot\text{L}^{-1}$ ), and these results were shown as mg of GAE for each one hundred grams of red pine bark material.

### DPPH Determination Radical Gathering Behavior

The Brand-Williams et al. [28] approach was used to assess the red pine bark extract's capacity to scavenge radicals that are free of it. For this assay, 0.1 g of red pine bark mulch was extracted with 10 mL of methanol and passed through Whatman No. 1 filter paper. Aliquots of 0.2 mL from different concentrations of the extract ( $10\text{--}0.625\text{ mg/mL}$ ), as well as BHT and BHA standards in methanol, were mixed with 3.8 mL of a methanolic DPPH solution ( $6 \times 10^{-5}\text{ M}$ ). The mixtures were briefly vortexed and then incubated in darkness for 75 min at  $25^\circ\text{C}$ . Absorbance values were recorded at 517 nm against one methyl alcohol blank via the UV–Vis spectrophotometer (Hitachi U-1900, Japan). Every test was run in triplicate. Using the conventional formula, the samples' scavenging activity was represented as a percentage of inhibition.

$$\text{inhibition (\%)} = \frac{A_0 - A_s}{A_0} * 100 \quad (1)$$

### $\beta$ -Carotene Whitening Analysis

The  $\beta$ -carotene–linoleic acid model system was used to evaluate the antioxidant ability of the red pine bark methanolic acid extract [29]. First, chloroform was used to dissolve 0.2 milligrams per milliliter of  $\beta$ -carotene to create a stock  $\beta$ -carotene combination, from which, two milliliters was put into a flask measuring 100 milliliters with a round bottom. At  $40^\circ\text{C}$ , the solution evaporated. Following the addition of distilled water (50 mL), Tween 40 (200 mg), and linoleic acid (20 mg), the liquid was aggressively blended to create an emulsion. Test tubes holding 0.2 mL of the red pine bark extract were filled with portions of 4.8 mL of this emulsion. In order to initiate oxidation, the tubes were placed in a batch of

water at 50°C and exposed to air, heat, and light. Using a spectrophotometer, absorbance at 470 nm was measured every 15 minutes for a total of 120 minutes (Hitachi U-1900, Japan). The same procedure was conducted for BHA and BHT as reference antioxidants. The extent of  $\beta$ -carotene bleaching was measured according to prepared-standard equation.

$$\beta - \text{carotene whitening ratio values} = \ln (A_0/A_t) \cdot 1/t \quad (2)$$

where t is the duration in minutes, at is the absorbance at 120 minutes, and  $A_0$  is the emulsion's initial absorbance at time 0. The relationship between the oxidation of the linoleic acid emulsion system and the mean percent inhibition was used to calculate the antioxidant activity of  $\beta$ -carotene.

$$R_{\text{control}} - \frac{R_{\text{sample}}}{R_{\text{control}}} \cdot 100 \quad (3)$$

where  $R_{\text{sample}}$  and  $R_{\text{control}}$  are the whitening rates of  $\beta$ -carotene with *red pine bark* sample and in the emulsion without antioxidant, seriatim.

### Reducing Power (FRAP Assay)

The decreasing feature of red pine bark extract was observed via a modified Oyaizu method [30]. Five different methanolic solutions of red pine bark extract (10-0.625 mg/ml) were prepared. 0.2 M and 1.25 ml, 1.0%  $K_3[Fe(CN)_6]$  and 1.25 ml, pH 6.6 phosphate buffer were added to each concentration of 0.5 ml sample. The mixes were kept at 50°C for 30 minutes. One milliliter of 10% trichloroacetic acid was added, and the mixture was centrifuged for ten minutes at 3000 rpm. 2.5 ml of distilled water and 1.0 ml of 0.1% ferric chloride were mixed with 2.5 ml of supernatant. Then a spectrophotometer was used to measure the solutions' absorbances at 700 nm. BHA and BHT (1 mg. ml<sup>-1</sup>) were used as reference antioxidants.

### Antimicrobial Effects

#### Sample Preparation

The mulch of red pine bark samples were extracted by using Soxhlet device at fixed temperature for 24 hours. The obtained liquid solvents (chloroform, methanol and ethyl acetate) were evaporated completely by a Büchi Rotary evaporator under vacuum pressure. The crude extracts of samples were then dissolved in DMSO to get 2048  $\mu\text{g}.\text{ml}^{-1}$  concentration for each sample.

#### Antimicrobial Activity Test

Antimicrobial tests of the extracts were performed by microdilution method, modified according to Patel et al. [31]. The antimicrobial activity of mulch obtained from red pine bark was measured spectrophotometrically by using two Gram-positive (25923 *Bacillus cereus* RSKK 1122 and *Staphylococcus aureus* ATCC) and using the at least inhibitor concentration (MIC) increased dilution approach, two Gram-negative bacteria (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) were identified [29]. Antimicrobial tests were carried out with 96-well microtiter plates consisting of "U" shaped wells and shaken vigorously. All bacteria strains grown on nutrient agar for 24 hours briefly at 37°C were suspended in nutrient broth at a density fractionated to 0.5 McFarland standard. Last samples were  $5 \times 10^5$  CFU.L<sup>-1</sup> set as bacteria. All wells were filled with a hundred microliters Nutrient Broth and after that 100  $\mu\text{L}$  red pine bark samples added to the first wells and diluted (1/1) by using Socorex multi-channel pipette. In the assay, a hundred microliters of microbial suspension adjusted to a 0.5 McFarland system was dispensed into each well of the 96-well microplate, with the exception of the twelfth column. The eleventh column served as the positive control, containing both growth medium and microorganisms, while the final column functioned as the negative control with only medium. Initial absorbance (T0) was recorded at 630 nm by the ELISA analysis (BioTek,  $\mu\text{Quant}$ )

prior to incubation. After that, the plates were covered and incubated for 24 hours at 37°C. Following incubation, absorbance values (T24) were again measured at 630 nm. MIC values were measured by the BioTek GEN5 software by calculating the difference between T24 and T0 absorbance values for each well, and plotting the data against sample concentrations.

### GC-FID Analysis

The analyses were carried out using Hewlett Packard HP 6890 model GC, Headspace (Agilent 7694E Headspace sampler) and HP5-MS capillary column with GC-FID (Flame Ionization Detector and Agilent JW device). The flow rate and split value were set to be 0.6 mL/min and 1:40, respectively. The oven temperature was held isothermally at 100°C to 175°C at 5°C/min and then 175°C to 220°C at 8°C/min and held at 220°C for 10 [32].

### Results and Discussion

Red pine bark extract has a higher antioxidant activity because of its chemical makeup, particularly its flavonoids and polyphenolic chemicals. The sample's polyphenolic content was determined and is shown in Table 1. It is evident that the amount of GAE units each 100 grams of red pine bark was used to calculate the total phenolic content of the samples. Based on these findings, it was determined that a 100g sample of red pine bark contained 281.31 mg of phenolic mass. GAE. A common method for evaluating the in vitro free radical scavenging ability of different materials is to utilize DPPH, a free radical. The DPPH free radical, which is purple in color, has a significant absorption peak at 517 nm. BHA and BHT have the lowest I% value because they are synthetic polyphenolic antioxidant chemicals, according to the results of the DPPH test. Phenolic chemicals are primarily responsible for the red pine bark samples' excellent antioxidant quality. In accordance with the  $\beta$ -carotene-linoleic acid method BHA, The (+) checking group used was BHT. The antioxidant activity of the red pine bark sample was noted, and the sample was able to prevent the oxidation-induced discoloration of  $\beta$ -carotene. According to the FRAP assay results, the antioxidant activity followed the order BHA > BHT > red pine bark (Table 1). The results of the antioxidant tests are similar to one another.

**Table 1.** Antioxidant activity test results of red pine bark extracts

|                                        | Red pine bark | BHA    | BHT    |
|----------------------------------------|---------------|--------|--------|
| Total phenols (mg GAE/100 g red pine)  | 287.31        | ---    | ---    |
| DPPH assay (Inhibition %)              | 93.9400       | 97.57  | 92.22  |
| FRAP assay                             | 0.2931        | 1.1624 | 0.7672 |
| $\beta$ -carotene assay (Inhibition %) | 99.28         | 88.46  | 50.00  |

Ferric reducing power is a very common method of determining antioxidant activity in vitro. In these reactions, The hydroxyl radical, which is thought to be the most damaging radical to biomolecules, is created when  $\text{Fe}^{2+}$  combines with  $\text{H}_2\text{O}_2$ .



Many reductants have the ability to change oxidized iron ions into reduced form  $\text{Fe}^{2+}$ , which can enhance the production of hydroxyl radicals and cause more damage to DNA. In this method, after iron and hydrogen peroxide are added, they will react to form hydroxyl radicals, as shown in the following equations. The reducing power of the ginger peel (Red pine bark) was determined by a modified Oyaizu method. This result was compared with BHA and BHT as shown in Table 1. According to the FRAP values obtained through absorbance, BHA also clearly showed a stronger antioxidant effect than other extracts. Since BHA and BHT are synthetic antioxidant compounds, FRAP values are much higher than

lyophilized Red pine bark extracts. In this study, MIC was determined by spectrophotometric reading relative to an ELISA microplate reader, by averaging the lowest concentration of pine bark extract exhibiting growth of organisms in the wells. Environmental and economic problems have driven researchers to smaller sample sizes and the reduction of solvents needed. On the other hand, more achievable results are controlled with larger sample sizes. The MIC results obtained using the broth microdilution method are shown in Table 2. The chloroform extract of red pine bark was found to be effective on the antimicrobial potential of both Gram- negative bacteria and Gram-positive. The results showed that red pine bark extracts had generally similar antimicrobial capacity inhibitors except for *Klebsiella pneumoniae* microorganisms, but red pine bark was moderately sensitive to antimicrobial activity in this study.

**Table 2.** MIC values of red pine bark extracts ( $\mu\text{g.mL}^{-1}$ )

|                       | Gram positive                              |                                     | Gram negative                         |                                             |
|-----------------------|--------------------------------------------|-------------------------------------|---------------------------------------|---------------------------------------------|
|                       | <i>Staphylococcus aureus</i><br>ATCC 25922 | <i>Bacillus cereus</i> RSKK<br>1122 | <i>Escherichia coli</i><br>ATCC 25922 | <i>Pseudomonas aeruginosa</i><br>ATCC 27853 |
| Chloroform extract    | -                                          | 256                                 | 256                                   | 64                                          |
| Methanol extract      | -                                          | 256                                 | 256                                   | 256                                         |
| Ethyl acetate extract | -                                          | 256                                 | 128                                   | 64                                          |

Despite minor variations and the presence of several antioxidant components, including flavonoids, phenolics, and phenolic acids, all red pine bark samples generally demonstrated an antioxidant effect. These findings demonstrate that red pine bark is a naturally occurring substance with antibacteria and antioxidant qualities. Fatty acid compositions of red pine bark are given in Table 3. and Figure 1.

**Table 3.** Fatty acid composition of red pine bark (*P. brutia*)

| Fatty acid                        | %      | SFA   | 89.23  |
|-----------------------------------|--------|-------|--------|
| C 16:0                            | 2.63   | MUFA  | 6.32   |
| C 18:0                            | 1.15   | PUFA  | 4.45   |
| C 18:1 (trans - 9) (omega 9)      | 2.45   | TOTAL | 100.00 |
| C 18:2 (cis,cis - 9,12) (omega 6) | 4.45   |       |        |
| C 20:0                            | 5.57   |       |        |
| C 22:0                            | 28.38  |       |        |
| C 16:1 (cis - 9)                  | 3.87   |       |        |
| C 24:0                            | 51.5   |       |        |
| TOTAL                             | 100.00 |       |        |

As a result, eight fatty acids were determined. The ratio of fatty acids in the sample is (C 24:0) Lignoceric acid, 51.5%, and the ratio of another saturated fatty acid behenic acid is 28.38%. The total saturated fatty acid ratio is 89.23%. In addition, the ratios of monounsaturated fatty acids and polyunsaturated fatty acids were found to be quite low, 6.32% and 4.45%, respectively.

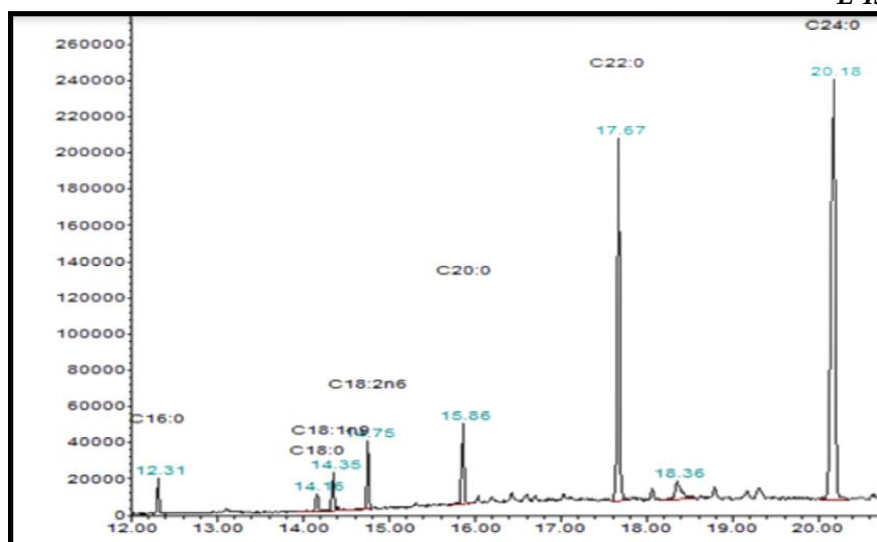


Figure 1. The GC-FID chromatogram of *P. brutia*

## Conclusion

The strong antioxidant potential of red pine bark extract can be attributed to its molecular composition, particularly the abundance of polyphenolic compounds and flavonoids. The phenolic content of the extract was quantified and is presented in Table 1. Results indicated that the total phenolic level of the red pine bark sample corresponded to 287.31 mg of gallic acid equivalents (GAE) per 100 g of material. DPPH, a chemically stable radical possessing a typical violet color and a maximum absorbance at 517 nm, was used to assess the activity of radical scavenging in vitro. Based on this assay, artificial antioxidants as BHA and BHT demonstrated the lowest inhibition values, in contrast to the high activity observed in red pine bark. *Pinus brutia* Ten., known as Turkish pine, is the most widespread pine species in Türkiye, generating large amounts of bark waste each year. Only limited quantities are utilized for fuel or agricultural purposes, while the majority remains unexploited. The present findings demonstrate that extracts derived from red pine bark exhibit biological activity and may hold promise for applications in alternative medicine, particularly in the management of bacteria infections. Moreover, the fatty acid composition of red pine bark is dominated by lignoceric acid (C24) and behenic acid (C22), accounting for nearly 80% of the total profile, both of which possess commercial significance. Owing to these valuable characteristics, further research is recommended to develop novel medicinal and industrial products from this abundant natural resource.

## Declarations

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