In Vitro Study on the Antioxidant Activity of a Polyphenol-Rich Extract from Pinus brutia Bark and Its Fractions

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ABSTRACT   A crude hydromethanolic extract from *Pinus brutia* bark and its fractions (diethyl ether, ethyl acetate, n-butanol, and aqueous fractions) were studied with regard to their phenolic content and antioxidant activities. The total phenolics and proanthocyanidins in each extract were quantified by spectrophotometric methods; the polyphenolic profile was analyzed by RP-HPLC-DAD-ESI-MS. All extracts were tested with regard to their ability to scavenge free radicals (ABTS radical cation, superoxide and hydroxyl radicals), reduce ferric ions, and inhibit 15-lipoxygenase. *P. brutia* bark extracts had high phenolic contents (303.79 ± 7.34–448.90 ± 1.39 mg/g). Except diethyl ether extract, all other extracts contained proanthocyanidins ranging from 225.79 ± 3.94 to 250.40 ± 1.44 mg/g. Several polyphenols were identified by RP-HPLC-DAD-ESI-MS: taxifolin in diethyl ether extract, a taxifolin-*O*-hexoside, catechin, procyanidin dimers, and trimers in ethyl acetate extract. Except diethyl ether extract, all other extracts were effective scavengers of superoxide and hydroxyl radicals ($EC_{50} = 33.5 \pm 1.1$–54.93 ± 2.85 μg/mL and 0.47 ± 0.06–0.6 ± 0.0 mg/mL, respectively). All extracts had noticeable 15-lipoxygenase inhibitory effects ($EC_{50} = 22.47 \pm 0.75$–34.43 ± 2.25 μg/mL). We conclude that *P. brutia* bark is very promising for the dietary supplements industry due to its high free radical scavenging and 15-lipoxygenase inhibitory effects.

KEY WORDS: ● *Calabrian pine* ● *hydroxyl radical* ● *15-lipoxygenase,* ● *proanthocyanidins* ● *RP-HPLC-DAD-ESI-MS* ● *superoxide anion radical* ● *taxifolin*

INTRODUCTION

BARKS OF DIFFERENT pine species have been used as food and medicine for more than 2000 years. In ancient times, pine bark was used to treat inflammatory conditions. European herbals of the fifteenth and sixteenth century mention the efficacy of pine bark in skin disorders, mainly wounds and ulcers. In North America, indigenous peoples used pine bark to prevent and treat scurvy. It is worthy to note that the older bark was often used for therapeutic purposes, while the younger bark was used as food. In addition, in northern Scandinavia, Sami people used pine inner bark as food.[1–4]

Nowadays, bark extracts of different *Pinus* species have been intensively investigated with regard to their chemical constituents and biological effects. The most studied one is Pycnogenol®—a standardized extract prepared from the bark of French maritime pine (*Pinus pinaster* Aiton, subspecies Atlantica des Villar syn. *P. maritima*). Pycnogenol contains procyanidins (65–75%), mainly oligomers of cat-echin and epicatechin, monomeric catechin and epicatechin, taxifolin, phenolic acids, and their glycosides.[1,3] It is widely used as a nutritional supplement and provides numerous health benefits due to its antioxidant, antiinflammatory, and enzyme-modulation effects.[1,4–6] Human studies have proved that Pycnogenol has beneficial effects in cardiovascular disorders, type 2 diabetes mellitus and its complications, asthma, osteoarthritis, muscular pain, cognitive decline in Alzheimer's disease, UV-induced skin inflammation, and sexual disorders.[3,4] Bark extracts from other *Pinus* species have been reported to possess potent antioxidant activity.[7–10] Extracts of *Pinus sylvestris*, *Pinus pinea*, and *Pinus massoniana* barks showed cytotoxic activity against human cancer cell lines.[8,9] In addition, *P. sylvestris* and *P. massoniana* bark extracts reduced the production of several inflammatory mediators (nitric oxide, prostaglandin E_2, and intercellular adhesion molecule-1).[8,11] *P. pinea* and *Pinus densiflora* bark extracts have been found very promising for diabetes treatment due to their inhibitory effects on glucose absorption and carbohydrate-hydrolysing enzymes, respectively.[12,13] Taken together, all these data show that bark extracts from different pine species have remarkable biological effects, thus making pine bark a valuable raw material for the food and pharmaceutical industries.

Pinus brutia Ten. (Pinaceae, Calabrian pine, Turkish pine) is naturally spread in the eastern Mediterranean region being one of the most important timber trees. The bark is the main waste from the industrial processing of the wood of mature trees that is still useless and discarded.[14–16] There are recent reports on the chemical composition and biological activities of the bark collected from pines growing in western Turkey (Izmir-Deliomer). Several low-molecular-weight compounds (taxifolin isomers, catechins, and procyanidins) have been identified in bark extracts. Bark extracts showed moderate cytotoxic effects against certain human cancer cell lines and antiinflammatory effects.[9,17] To the best of our knowledge, the only published data on the antioxidant activity of *P. brutia* bark extracts are those reporting radical scavenging effects assessed by the chemiluminescence and diphenylpicrylhydrazyl (DPPH) assays.[9,18]

Although *P. brutia* is the dominant tree species in Cyprus,[19] no investigations concerning the valorization of its bark, the main waste from the timber industry, have been carried out. As a part of an extensive study on the possible use of bark waste as raw material in the dietary supplements industry, the present work evaluated the antioxidant activity of *P. brutia* bark by several *in vitro* assays; in addition, the polyphenolic content and profile of bark extracts were studied.

MATERIALS AND METHODS

Plant material

P. brutia bark was generously supplied by the Cyprus Department of Forests of the Ministry of Agriculture, Natural Resources, and Environment in March 2008. The bark was dried in the dark at room temperature, milled, and then sieved to select particles smaller than 4 mm. The plant material was stored at $+4°C$ until use. A voucher specimen has been deposited in the Department of Pharmacognosy, the University of Medicine and Pharmacy ''Gr. T. Popa'' Iasi, Romania.

Extraction

An amount of 150 g of bark powder was extracted thrice (each time for 3 h) with 1.5 L of 80% aqueous methanol (v/v) at ambient temperature under constant stirring. The supernatants were filtered, combined, evaporated under reduced pressure at 40°C, and lyophilized, yielding 20.07 g of crude extract. A quantity of 18.36 g of the crude extract was suspended in ultrapure water (1:10) and successively partitioned with diethyl ether (7×190 mL), ethyl acetate (9×190 mL), and n-butanol (10×190 mL). The organic solvents were removed by low-pressure evaporation at 40°C. The remaining aqueous phase was concentrated under reduced pressure at 40°C and then lyophilized. All extracts were stored at $-20°C$ for further studies.

Total phenolic content

The total phenolic content was quantified spectrophotometrically using the Folin–Ciocalteu method as previously described.[20–22] The results were expressed as gallic acid equivalents (mg/g extract).

Proanthocyanidin content

The proanthocyanidin content was determined by the vanillin-hydrochloric acid method as previously described.[23,24] The results were expressed as (+)-catechin equivalents (mg/g extract).

RP-HPLC-DAD-ESI-MS analysis

Reversed-phase high-performance liquid chromatography coupled with diode array detection and electrospray ionization mass spectrometry (RP-HPLC-DAD-ESI-MS) analysis was performed in the negative ion mode.[25] The equipment consisted of a Perkin–Elmer HPLC-ESI-MS system (SCIEX API 365 LC/MS/MS mass spectrometer connected to a Series 200 HPLC system with UV/VIS detector and Analyst Software 1.1 data system). A Merck Superspher 100 RP-18 column (75 mm×4 mm×4 μm) was used. The mobile phase consisted of solvent A (acetonitrile) and solvent B (water and formic acid; 99:1, v/v). The elution profile was as follows: 0 min, 100% B; 3 min, 100% B; 30 min, 30% A in B; 33 min, 70% A in B; 42 min, 70% A in B; and 45 min, 100% B. All gradients were linear. The flow rate was set to 1 mL/min. Phenolic compounds were detected at 280 nm. Extracts were dissolved in ethanol–water mixtures at a concentration of 20 mg/mL and filtered through 0.45 μm PTFE filters; the injection volume was 20 μL. The spray needle voltage, dry temperature, and nebulizer gas were set to -4200 V, 320°C, and 10, respectively; a split ratio of 7:3 was used.[25]

Trolox equivalent antioxidant capacity assay

The Trolox equivalent antioxidant capacity (TEAC) assay is based on the ability of antioxidants to scavenge the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation.[26–28] The assay was performed as described by Re *et al.*[26] using quercetin as a positive control; quercetin was the positive control in all antioxidant assays. To calculate the TEAC values, a (R)-(+)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) curve was plotted. TEAC values (mM Trolox equivalent to 1 mg/mL extract or quercetin) were calculated as follows:

TEAC = gradient of the plot for test samples (extracts, quercetin)/gradient of the plot for Trolox.[26]

Reducing power assay

The assay was performed to evaluate the electron-donating ability of *P. brutia* bark extracts.[29,30] Within this assay, the absorbance at 700 nm is an indicator of the reducing power.

Superoxide anion radical scavenging assay

The assay was performed according to previously described procedures.[31,32] Superoxide anion radical, chemically generated in a beta-nicotinamide adenine dinucleotide-

reduced (NADH)-phenazine methosulfate system, was detected by its ability to reduce nitroblue tetrazolium to a formazan dye.[32] The percent of superoxide anion radical scavenging activity was calculated as $100 \times (C-S)/(C)$, where C is the absorbance of the control and S is the absorbance in the presence of the sample (extracts, quercetin).

Hydroxyl radical scavenging assay

The salicylate hydroxylation method was used to measure hydroxyl radicals generated via Fenton reaction.[33,34] The percent of hydroxyl radical scavenging activity was calculated using the following formula: $100 \times (C-S)/(C)$, where C is the absorbance of the control and S is the absorbance in the presence of extracts or quercetin.

15-Lipoxygenase inhibition assay

The assay is based on the property of 15-lipoxygenase (15-LO) to catalyze the peroxidation of polyunsaturated fatty acids (linoleic acid) to hydroperoxide derivatives that strongly absorb at 234 nm.[21] The assay was carried out using soybean 15-LO according to a procedure described in literature[21] except that the enzyme and the inhibitor were incubated for 10 min at 25°C. The percent inhibition of 15-LO activity was calculated using the formula: $100 \times [(C_{90} - C_{30}) - (S_{90} - S_{30})/(C_{90} - C_{30})]$, where C_{30}, C_{90} and S_{30}, S_{90} are the values of absorbance at 234 nm after 30 and 90 s reaction time for the control (mixtures without extracts or quercetin) and sample (mixtures with extracts or quercetin), respectively.

Statistics

All assays were carried out in triplicate, and the results were expressed as mean ± SD. The EC_{50} values were calculated by linear interpolation between values above and below 50% activity except the reducing power assay; in this assay, the EC_{50} values were the effective concentrations at which the absorbance was 0.5.[30] Statistical analyses were performed using the one-way analysis of variance and Duncan's multiple-range tests (SPSS version 17.0).

RESULTS

The extraction of *P. brutia* bark with 80% (v/v) methanol followed by fractionation of the crude extract (PbE; 18.36 g) by successive liquid–liquid partition led to four extractive fractions: diethyl ether (PbE1; 2.18 g), ethyl acetate (PbE2; 1.84 g), n-butanol (PbE3; 8.59 g), and aqueous (PbE4; 5.37 g) fractions. The results of the quantitative analyses showed that the extracts had high phenolic contents ranging from 303.79 ± 7.34 in PbE3 to 448.90 ± 1.39 mg/g in PbE2. Except PbE1, all other extracts contained proanthocyanidins in the range of 225.79 ± 3.94 to 250.40 ± 1.44 mg/g (Table 1).

The polyphenolic profile of *P. brutia* extracts was studied by RP-HPLC-DAD-ESI-MS. The RP-HPLC-UV traces (280 nm) are shown in Figure 1. Taxifolin and a taxifolin-*O*-hexoside were, by far, the dominant polyphenols in PbE1 and PbE2, respectively; in addition, PbE2 contained catechin and shorter procyanidin oligomers (dimers, trimers; Fig. 1B,

TABLE 1. TOTAL PHENOLIC AND PROANTHOCYANIDIN CONTENT
IN *PINUS BRUTIA* BARK EXTRACTS

	Total phenolic content (mg gallic acid/g extract)	Proanthocyanidin content (mg (+)-catechin/g extract)
PbE	412.42 ± 7.56[††††,‡‡‡‡,####,$]	225.79 ± 3.94[††††,$$$$]
PbE1	366.71 ± 5.63[****,‡‡‡‡,####,$$$]	10.05 ± 0.22[****,‡‡‡‡,####,$$$$]
PbE2	448.90 ± 1.39[****,††††,####,$$$$]	231.41 ± 1.24[††††,$$$$]
PbE3	303.79 ± 7.34[****,††††,‡‡‡‡,$$$$]	229.10 ± 1.13[††††,$$$$]
PbE4	393.49 ± 5.39[*,†††,‡‡‡‡,####]	250.40 ± 1.44[****,††††,‡‡‡‡,####]

Significant differences between extracts are indicated: *$P < .05$, ****$P < .001$ vs. PbE; †††$P < .002$, ††††$P < .001$ vs. PbE1; ‡‡‡‡$P < .001$ vs. PbE2; ####$P < .001$ vs. PbE3; $$P < .05$, $$$P < .002$, $$$$P < .001$ vs PbE4.
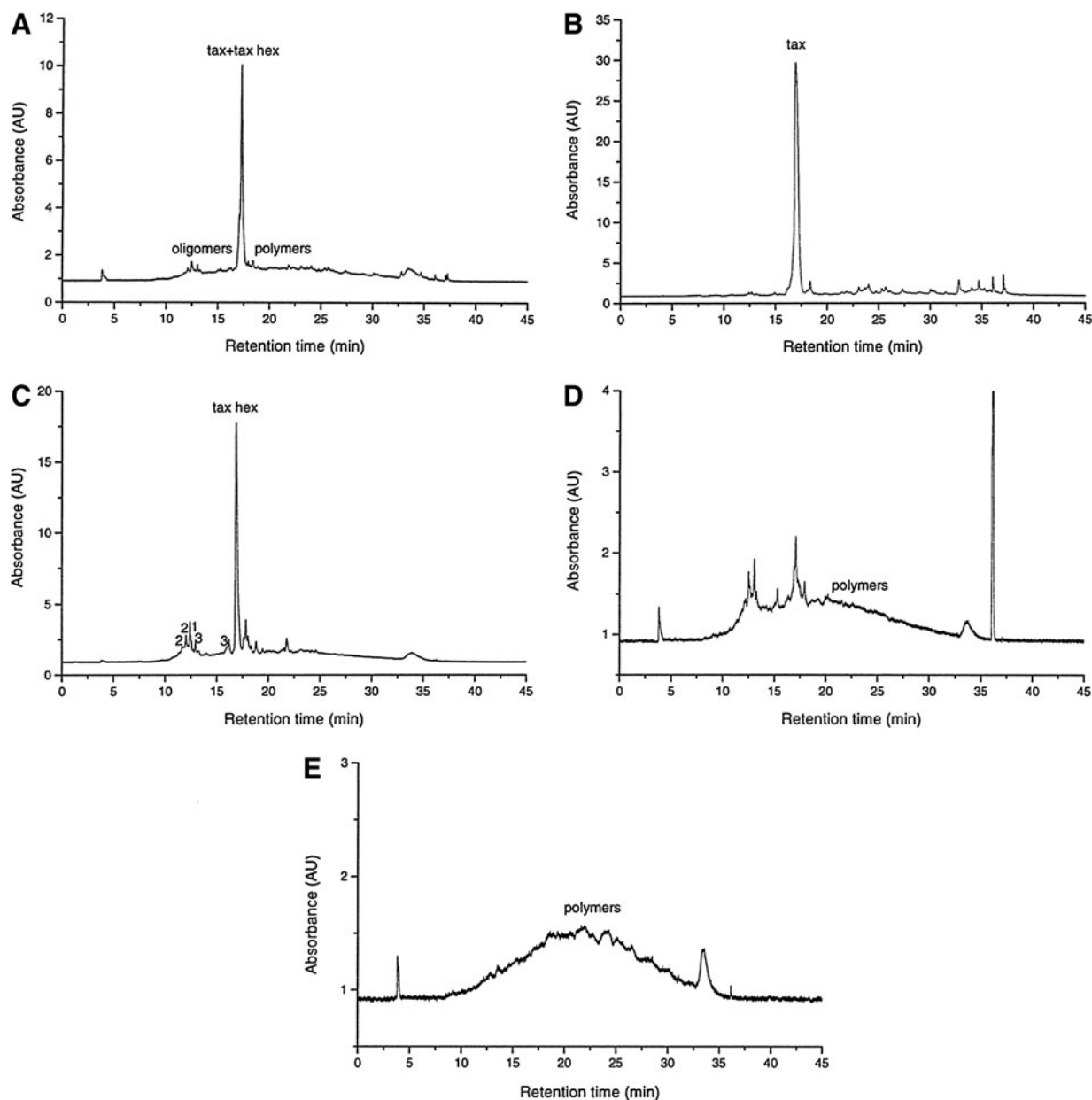
PbE, crude extract of *P. brutia* bark with 80% (v/v) methanol (18.36 g); PbE1, PbE fractionated with diethyl ether (2.18 g); PbE2, PbE fractionated with ethyl acetate (1.84 g); PbE3, PbE fractionated with n-butanol (8.59 g); PbE4, remaining aqueous phase (5.37 g).

C). Taxifolin and catechin were identified by comparing their UV spectra and MS fragmentation patterns with those of standard compounds analyzed in the same experimental conditions. On the basis of UV and MS data, the major polyphenol in PbE2 was tentatively identified as a *O*-hexoside of taxifolin. Its UV spectral characteristics ($\lambda_{max=}$ 226, 290, 340 sh nm) suggested a taxifolin-type structure.[35,36] The negative ion ESI-MS fragmentation pattern of taxifolin-*O*-hexoside showed the deprotonated molecules $[M-H]^-$ at m/z 465.1 and $[2M-H]^-$ at m/z 931.4 along with two fragment ions corresponding to the aglycone: $[A]^-$ at m/z 303.2, generated by the loss of a hexose moiety (162 amu) from the deprotonated molecule $[M-H]^-$ and $[A-H_2O]^-$ at m/z 284.6 (Table 2). These spectral data are in agreement with those reported in literature for taxifolin-*O*-glucosides.[11,37] Procyanidin oligomers (two dimers, two trimers) were tentatively identified on the basis of their MS spectral data (Table 2).[9,11,25] A broad, unresolved hump corresponding to high oligomeric and polymeric procyanidins is visible in PbE3 and PbE4 chromatograms (Fig. 1D, E).

All *P. brutia* extracts scavenged ABTS•+ in a concentration-dependent manner; at 2.5 mg/mL, all extracts were very effective in eliminating the radical, showing more than 94% scavenging activity (data not shown). All tested extracts (TEAC = 0.89 ± 0.01 − 1.54 ± 0.00) were less active than the positive control, quercetin (TEAC = 5.62 ± 0.04). On the basis of the TEAC values, the ABTS•+-scavenging effects of *P. brutia* extracts and quercetin can be ranked in the following order: quercetin > PbE4 > PbE > PbE2 > PbE1 > PbE3 (Table 3).

The reducing power assay showed no important differences among *P. brutia* extracts; the EC_{50} values ranged from 9.17 ± 0.13 to 11.38 ± 0.24 μg/mL. Quercetin ($EC_{50} = 3.40 ± 0.20$ μg/mL) was more active than *P. brutia* extracts. In terms of the EC_{50} values, the reducing power decreased in the following order: quercetin > PbE > PbE2 > PbE4 > PbE1 > PbE3 (Table 3).

The results of the superoxide scavenging assay showed significant differences in activity. According to the EC_{50} values, PbE4 (33.5 ± 1.1 μg/mL) and PbE (39.37 ± 0.85 μg/mL) were very efficient in scavenging the superoxide anion radical; their activity was slightly lower than that of

FIG. 1. RP-HPLC-UV traces (280 nm) of *Pinus brutia* extracts runs with a Merck Superspher 100 RP-18 column (75 mm × 4 mm × 4 μm) and a gradient consisting of solvent A (acetonitrile) and solvent B (water and formic acid; 99:1, v/v): 0 min, 100% B; 3 min, 100% B; 30 min, 30% A in B; 33 min, 70% A in B; 42 min, 70% A in B; 45 min, 100% B; flow rate: 1 mL/min. **(A)** PbE, **(B)** PbE1, **(C)** PbE2, **(D)** PbE3, **(E)** PbE4. Tax, taxifolin; tax hex, taxifolin-*O*-hexoside; 1, catechin; 2, dimer; 3, trimer.

quercetin (26.63 ± 0.75 μg/mL). The lowest scavenging activity was found for PbE1 (70.30 ± 2.65 μg/mL). With regard to the EC_{50} values, the superoxide scavenging effects might be ranked as follows: quercetin > PbE4 > PbE > PbE2 > PbE3 > PbE1 (Table 3).

In the hydroxyl radical scavenging assay, the activity decreased in the following order: quercetin > PbE4 > PbE > PbE2 > PbE3 > PbE1. According to the EC_{50} values, PbE4 (0.47 ± 0.06 mg/mL) was the most potent hydroxyl radical scavenger, while PbE1 (1.07 ± 0.06 mg/mL) showed the lowest scavenging activity. All extracts were less active than quercetin (0.2 ± 0.0 mg/mL; Table 3).

All *P. brutia* bark extracts exerted noticeable inhibitory effects on 15-LO. In terms of the EC_{50} values, the effects of PbE, PbE2, PbE3, and PbE4 (22.47 ± 0.75–24.9 ± 0.5 μg/mL) were slightly lower than that of quercetin (18.70 ± 0.85 μg/mL). The 15-LO inhibitory effects might be ranked as follows: quercetin > PbE > PbE4 > PbE2 > PbE3 > PbE1 (Table 3).

DISCUSSION

This study showed that a polyphenol-rich extract from *P. brutia* bark and its fractions possess strong free radical scavenging and 15-LO inhibitory effects. Since 15-LO and

TABLE 2. MAIN POLYPHENOLIC CONSTITUENTS IN *PINUS BRUTIA* BARK EXTRACTS

Polyphenolic constituent	Extractive fraction	Retention time (min)	MS data	Identification
Dimer 1	PbE2	11.78	$[M-H]^-$ at *m/z* 577.3 $[2M-H]^-$ at *m/z* 1155.7 fragment ions at *m/z* 286.7[a], *m/z* 289.2[a], *m/z* 407.6[b], *m/z* 425.3[c], *m/z* 451.2[d]	Lit.[e]
Dimer 2	PbE2	12.10	$[M-H]^-$ at *m/z* 577.3 $[2M-H]^-$ at *m/z* 1155.4 fragment ions at *m/z* 286.8[a], *m/z* 289.2[a], *m/z* 407.2[b], *m/z* 425.2[c], *m/z* 451.2[d]	Lit.[e]
Catechin[f]	PbE2	12.53	$[M-H]^-$ at *m/z* 289.0 $[2M-H]^-$ at *m/z* 579.1 fragment ion at *m/z* 244.7[h]	St.[g]
Trimer 1	PbE2	12.95	$[M-H]^-$ at *m/z* 865.5	Lit.[e]
Trimer 2	PbE2	15.89	$[M-H]^-$ at *m/z* 865.4	Lit.[e]
Taxifolin-*O*-hexoside	PbE2	16.83	$[M-H]^-$ at *m/z* 465.1 $[2M-H]^-$ at *m/z* 931.4 $[A]^-$ at *m/z* 303.2 $[A-H_2O]^-$ at *m/z* 284.6	Lit.[e]
Taxifolin	PbE1	16.92	$[M-H]^-$ at *m/z* 302.9 $[2M-H]^-$ at *m/z* 607.3 $[M-H_2O]^-$ at *m/z* 284.8	St.[g]

Fragment ions formed through [a]quinone methide cleavage of the interflavonoid bond; [b]loss of water from the ion at *m/z* 425; [c]retro–Diels–Alder fragmentation; [d]heterocyclic ring fission; [h]loss of $CH_2=CH-OH$ from $[M-H]^-$ at *m/z* 289.
[e]Comparison of UV and MS spectral data with literature data.[9,11,25,35–37]
[f]Co-elution with an unidentified compound.
[g]Comparison of UV and MS spectral data with those of the standard.

reactive oxygen species such as superoxide and hydroxyl radicals promote pathological conditions leading to the development of many diseases, these extracts are, undoubtedly, interesting for therapeutics.

Despite a low reactivity, superoxide anion radical is highly toxic due to its conversion into more aggressive reactive species (hydroxyl radical, peroxynitrite anion, and singlet oxygen).[38,39] There is scientific evidence for the involvement of superoxide anion in atherosclerosis, ischemia-reperfusion injury, high microvascular permeability, neurodegeneration, inflammation, and gout.[40,41] Quercetin, the positive control in our study, is an efficient scavenger of superoxide anion radical. In different enzymatic and non-enzymatic superoxide-generating systems, quercetin exerted stronger scavenging effects than other natural or synthetic antioxidants (quercitrin, cyanidol, rutin, myricetin, *Ginkgo biloba* extract, and butylated hydroxyanisole).[31,42,43] The EC_{50} values suggest high abilities to neutralize the superoxide anion radical for all *P. brutia* bark extracts, but to a lesser extent, for PbE1.

Hydroxyl radical is one of the most harmful reactive oxygen species damaging molecules (DNA, polyunsaturated fatty acids, proteins, and sugars) in or close to the generation site. Hydroxyl radical-induced oxidative damage is known to be involved in cancer, inflammation, neurodegeneration, and also in aging.[34,39] Since quercetin is a highly effective hydroxyl radical scavenger,[42,43] it is obvious that, except PbE1, all other *P. brutia* bark extracts, having EC_{50} values

about 2.3–3 times higher than that of quercetin, have good hydroxyl radical scavenging effects.

Mammalian 15-LO is involved in many processes that play important roles in the development of early atherosclerotic lesions such as the oxidation of low-density lipoproteins, the recruitment of circulating monocytes to the vessel wall, and the proliferation of vascular smooth muscle cells. On the other hand, 15-hydroperoxy eicosatetraenoic acid (15-HPETE), a 15-LO arachidonic acid metabolite, has been reported to have not only pro-thrombotic but also beneficial effects as a precursor of vasodilator and anti-inflammatory lipoxins.[44] Although the role of 15-LO in atherosclerosis remains controversial, there is emerging evidence for its involvement in diabetes, hypertension, renal disease, Alzheimer's disease, and Parkinson's disease.[45] Within this assay, *P. brutia* extracts were slightly less active than quercetin, a well-known 15-LO inhibitor.[46]

According to the TEAC and EC_{50} values, the crude extract (PbE) and its ethyl acetate (PbE2) and aqueous (PbE4) fractions were the most potent in all antioxidant assays. The fractionation of bark crude extracts by immiscible solvent–solvent partitioning has been reported to separate antioxidant-rich fractions.[7,47] On the contrary, in our study, the fractionation of the crude extract did not afford fractions with a considerably higher activity, supporting the idea that the phytocomplex in the crude extract can be as effective or even more effective than the fractions containing a part of its constituents. It is noteworthy that the use of the crude extract

TABLE 3. ANTIOXIDANT ACTIVITIES OF *PINUS BRUTIA* BARK EXTRACTS

	Trolox equivalent antioxidant capacity assay TEAC	Reducing power assay EC_{50} (µg/mL)	Superoxide anion radical scavenging assay EC_{50} (µg/mL)	Hydroxyl radical scavenging assay EC_{50} (mg/mL)[a]	15-LO inhibition assay EC_{50} (µg/mL)
PbE	1.47 ± 0.02†††‡,‡,###,$$$$,¶¶¶¶	9.17 ± 0.13††††,‡,###,$,¶¶¶¶	39.37 ± 0.85††††,‡‡‡,###,$$$,¶¶¶¶	0.5 ± 0.0††††	22.47 ± 0.75††††,‡,##,¶¶
PbE1	1.08 ± 0.04****‡‡‡,####,$$$$,¶¶¶¶	10.27 ± 0.11****,‡‡‡,###,$$,¶¶¶¶	70.30 ± 2.65****,‡‡‡,###,$$$$,¶¶¶¶	1.07 ± 0.06****,‡‡‡,###,¶¶¶¶	34.43 ± 2.25****,‡‡‡,###,$$$$,¶¶¶¶
PbE2	1.43 ± 0.01*,††††,####,$$$$,¶¶¶¶	9.49 ± 0.01*,††††,####,¶¶¶¶	51.10 ± 2.66***,††††,$$$$,¶¶¶¶	0.53 ± 0.06†††,¶¶¶¶	24.3 ± 0.4*,†††,$,¶¶¶¶
PbE3	0.89 ± 0.01****,††††,‡‡‡‡,$$$$,¶¶¶¶	11.38 ± 0.24****,†††,‡‡‡‡,$$$$,¶¶¶¶	54.93 ± 2.85****,†††,‡‡‡‡,$$$$,¶¶¶¶	0.6 ± 0.0†††,$	24.9 ± 0.5*,†††,$$$$,¶¶¶¶
PbE4	1.54 ± 0.00****,††††,‡‡‡‡,####,¶¶¶¶	9.54 ± 0.18*,††,####,¶¶¶¶	33.5 ± 1.1***,††††,‡‡‡,####,¶¶¶¶	0.47 ± 0.06†††,‡,¶¶¶¶	22.93 ± 0.55††††,‡,##,¶¶¶
Quercetin	5.62 ± 0.04****,††††,‡‡‡‡,####,$$$$	3.4 ± 0.2****,††††,‡‡‡‡,####,$$$$	26.63 ± 0.75****,††††,‡‡‡‡,####,$$$$	0.2 ± 0.0††††,‡‡‡,$$$$	18.70 ± 0.85**,††††,‡‡‡,####,$$$

[a]For the hydroxyl radical scavenging assay, statistical comparisons for PbE vs. PbE3, PbE vs, quercetin, and PbE3 vs. quercetin were not determined.
Significant differences between samples (extracts, quercetin) are indicated: *$P < .05$, **$P < .01$, ***$P < .002$, ****$P < .001$ vs. PbE; †$P < .05$, ††$P < .01$, †††$P < .002$, ††††$P < .001$ vs. PbE1; ‡$P < .05$, ‡‡$P < .01$, ‡‡‡$P < .002$, ‡‡‡‡$P < .001$ vs. PbE2; #$P < .05$, ##$P < .01$, ###$P < .002$, ####$P < .001$ vs. PbE3; $$P < .05$, $$$P < .01$, $$$$P < .002$, $$$$$P < .001$ vs. PbE4; ¶$P < .05$, ¶¶$P < .01$, ¶¶¶$P < .002$, ¶¶¶¶$P < .001$ vs. quercetin.

might have economic benefits, as it saves the fractionation costs.

Literature abounds in reports on the polyphenol content and antioxidant effects of plant extracts. In this regard, it needs to be mentioned that the antioxidant activity revealed for *P. brutia* bark extracts has both therapeutic and economic significance. The extracts showed strong antioxidant effects when compared with quercetin, a very potent antioxidant agent; these strong antioxidant effects represent a promising result for the valorization of a by-product of the wood industry. A literature survey showed no information regarding toxic constituents in *P. brutia* bark. Besides, extracts from *P. pinaster* and *P. radiata* barks were reported to have no toxic effects in human studies being considered safe.[4,48] *P. brutia* bark is, undoubtedly, a valuable source of antioxidants and can be used as raw material in the development of antioxidant dietary supplements. Further *in vivo* studies are needed to assess the efficacy and toxicity of *P. brutia* bark extracts.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist for any of the authors.

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