Article

Chemical Characterisation, Antioxidant and Antibacterial Activities of *Pinus pinaster* Ait. and *Pinus pinea* L. Bark Polar Extracts: Prospecting Forestry By-Products as Renewable Sources of Bioactive Compounds

Patrícia A. B. Ramos [1,2] , Carla Pereira [3] , Ana Peixoto Gomes [3] , Rodrigo T. Neto [1] , Adelaide Almeida [3] , Sónia A. O. Santos [1,*] , Artur M. S. Silva [2] and Armando J. D. Silvestre [1]

[1] CICECO—Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal; patriciaaramos@ua.pt (P.A.B.R.); rtneto@ua.pt (R.T.N.); armsil@ua.pt (A.J.D.S.)
[2] LAQV-REQUIMTE, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal; artur.silva@ua.pt
[3] CESAM—Centre for Environmental and Marine Studies, Department of Biology, University of Aveiro, 3810-193 Aveiro, Portugal; csgp@ua.pt (C.P.); ana.peixoto@ua.pt (A.P.G.); aalmeida@ua.pt (A.A.)
*   Correspondence: santos.sonia@ua.pt; Tel.: +351-234-370-711

Abstract: Agroforestry by-products have gained rising attention in recent years as they represent inexpensive and abundant raw materials that are a source of added-value chemicals, e.g., for food and pharmaceutical applications, as well as for bioenergy generation. *Pinus pinaster* Ait. bark extracts are consumed worldwide for their cardiovascular benefits, whilst the health potential of *Pinus pinea* L. bark has not yet been deeply exploited. Therefore, this study highlights the chemical characterisation of Portuguese *P. pinaster* Ait. and *P. pinea* L. bark polar extracts, via ultra-high performance liquid chromatography-diode array detection-tandem mass spectrometry (UHPLC-DAD-MS^n) analysis, and their antioxidant and antibacterial activities. Quinic acid, an A-type procyanidin dimer isomer, protocatechuic acid, and quercetin were identified for the first time as *P. pinea* L. bark components. Moreover, this bark demonstrated a higher total content of identified polar compounds than *P. pinaster* Ait. bark, with quinic acid being the most abundant compound identified. Regarding antioxidant activity, the pine bark polar extracts exhibited strong reducing power and 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) radical scavenging effects compared to natural antioxidants. Moreover, the bactericidal actions of pine bark extracts were shown against *Staphylococcus aureus* and *Escherichia coli* at a 3.13–25 mg mL^{-1} range. Globally, these promising insights can boost the sustainable exploitation of *P. pinea* L. bark, as already occurs with *P. pinaster* Ait. bark, for the food and biomedical fields.

Keywords: *Pinus pinaster* Ait.; *Pinus pinea* L.; pine bark polar extracts; UHPLC-DAD-MS^n analysis; quinic acid; phenolic compounds; antioxidant activity; antibacterial activity; biorefinery

1. Introduction

In the scope of the 2030 Agenda for Sustainable Development, countries and stakeholders are committed to contributing to society and to world prosperity, tackling economic, social, and environmental challenges [1]. In particular, the management of forests affects not only human livelihood, health, and wellbeing but also food production and biodiversity preservation [2]. Therefore, Goal 15 of the 2030 Agenda is devoted to avoiding deforestation, restoring degraded forests, and promoting afforestation and reforestation [1]. Indeed, forests are scattered in ca. 31% of the global land area, representing 4.06×10^9 ha in 2020 [2]. In Portugal, the agroforest activity is very important for the national economy and ecosystem balance, occupying 36% of the continental soil (ca. 3.2×10^6 ha), according to data from 2015 [3]. *Pinus* ssp. represents the second major tree family in Portuguese

forests (*ca.* 9.6×10^5 ha of continental soil), after the *Quercus* spp. family. The Portuguese pine forest mainly consists of *Pinus pinaster* Ait. and *P. pinea* L., which, in 2015, accounted for ca. 7.1×10^5 and 1.9×10^5 ha of the continental soil, respectively [3]. Given their good adaptation to edaphoclimatic conditions, notably frost resistance, adaptation to summer drought, and tolerance to poor soils, *P. pinaster* Ait. has been planted for coastal sand dunes stabilisation, soil conservation, and agricultural culture protection against salt spray.

Additionally, *P. pinaster* Ait. wood plays a crucial role in the national economy as it is used for construction, furniture, panels, pulp fibers, and energy generation, whereas the resin is primarily intended for manufacturing oils, varnishes, and adhesives [4,5]. In a similar way, *P. pinea* L. is also well-adapted to the coastal Mediterranean climate and thrives preferentially in siliceous and sandy soils. Thus, *P. pinea* L. has been also cultivated in sand dunes for coastal area preservation. Furthermore, the edible seeds or nuts are considered to be the most economically valuable product of the *P. pinea* L. forest due to their unique taste and also their interesting nutritional composition in terms of proteins, fats, ascorbic acid, thiamine, and riboflavin [6].

Both the pine wood and the seed industries generate considerable quantities of by-products, mainly of the *P. pinaster* Ait. and *P. pinea* L. barks, accounting, in 2015, for 5000 and 600 t in Portugal, respectively [3]. *Pinus* spp. bark has been used for energy generation, but it can be valorised as a renewable and low-cost source of biologically active compounds for food, nutraceutical, and pharmaceutical applications [7]. Actually, Pycnogenol®, Flavangenol®, and Oligopin® are commercially available extracts of *P. pinaster* Ait. bark, which contains phenolic compounds, namely procyanidins, phenolic acids (e.g., protocatechuic, gallic, ferulic, and caffeic acids), and flavanones (e.g., taxifolin and taxifolin-3'-β-D-glucoside) [7–9]. These commercial extracts have been associated with diverse health benefits. Pycnogenol® is generally used to treat circulation and cardiovascular disorders, whereas Flavangenol® has exhibited antihypertensive and arteriosclerosis-suppressing potential [7]. Interestingly, *P. pinaster* Ait. bark phenolic-rich extracts have also demonstrated effective antioxidant and antimicrobial actions, whilst the biological potential of *P. pinea* L. bark polar fractions has not yet been deeply exploited [10–14]. Additionally, food and cosmetic stakeholders are more and more interested in sustainably exploiting natural antioxidant and antibacterial compounds, such as phenolic compounds, rather than using synthetic additives. Given these bioactivities, phenolic compounds have shown potential for the improvement of food shelf life and nutritional value, as well as for skin care and treatment [15,16].

In this vein, *Pinus* spp. bark, which, as mentioned above, is available in large quantities, can be prospected as a promising source of bioactive compounds for food and nutraceutical applications, contributing to its sustainable valorisation within the bioeconomy concept. Nonetheless, the detailed chemical composition of *P. pinea* L. bark polar fractions and their related antioxidant and antibacterial activities are still poorly explored [14,17,18] in comparison to *P. pinaster* Ait. bark [10,13,19,20].

Considering our interest in the valorisation of agroforest by-products [21–23], the present work aims to deepen knowledge regarding the chemical composition, as well as the antioxidant and antibacterial activities, of *P. pinaster* Ait. and *P. pinea* L. bark polar extracts. Thus, the total phenolic content and proanthocyanidin (PAC) content were determined by using spectrophotometric assays, whilst the phenolic compounds were identified and quantified via ultra-high performance liquid chromatography-diode array detection-tandem mass spectrometry (UHPLC-DAD-MS^n). The antioxidant activity was in chemico analysed by DPPH and ABTS scavenging effects as well as, for the first time, through reducing power assay. Moreover, the in vitro antibacterial effect was approached against the growth of Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* pathogenic bacteria, which were selected considering that *S. aureus* is one of the leading causes of bacteremia and the second leading pathogen causing sepsis in industrialized countries, being responsible for skin, soft tissue, and bone infections and gastrointestinal problems [24], while E. *coli* is a major cause of diarrhea and is commonly associated with

extraintestinal infections in humans and animals [25]. To the best of our knowledge, the antibacterial activity of the *P. pinea* L. bark polar extract against *E. coli* is reported here for the first time.

2. Materials and Methods

2.1. Chemicals

Dichloromethane (p.a., $\geq$99%), methanol (p.a., $\geq$99.8%), HPLC-grade methanol, and acetonitrile were supplied by Fisher Scientific (Pittsburgh, PA, USA). Before UHPLC analysis, mobile phase solvents were previously filtered through a Solvent Filtration Apparatus 58061 from Supelco (Bellefonte, PA, USA). Acetic acid glacial (p.a., $\geq$99.5%) was purchased from Labkem (Madrid, Spain). Sodium carbonate (p.a., $\geq$99.9%) was obtained from Panreac AppliChem ITW Reagents (Barcelona, Spain). Gallic acid ($\geq$97.5%), Folin–Ciocalteu's phenol reagent (2 N), HPLC-grade water, formic acid ($\geq$98%), protocatechuic acid (>97%), catechin hydrate (>99%), quercetin (>98%), taxifolin ($\geq$85%), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^\bullet$), 2,2-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) diammonium salt, ascorbic acid ($\geq$99.5%), and ($\pm$)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (hereinafter named as Trolox) (97%) were supplied by Sigma-Aldrich (St. Louis, MO, USA). Sodium dihydrogen phosphate dihydrate was purchased from Panreac (pa., 99.0–101.0%), and disodium hydrogen phosphate dihydrate (pa., 98%) was supplied by Fluka. Potassium ferrocyanide (pa., $\geq$99.0%) was obtained from Carlo Erba. Trichloroacetic acid (pa., 99%) and iron (III) chloride (pa., 98%) were purchased from Alfa Aesar.

2.2. Pinus spp. Bark Samples

The *P. pinaster* Ait. and *P. pinea* L. barks were sampled in January 2017 from the nearby Aveiro region, Portugal. After 2 weeks of air-drying, these pine bark samples were milled using a coffee milling machine, and then, a fraction with particle size lower than 1 mm was selected. The samples were kept at room temperature and in the dark before analysis.

2.3. Preparation of Pinus spp. Bark Polar Extracts

Lipophilic compounds were previously extracted from the milled bark of *Pinus* spp. bark, as mentioned elsewhere [22]. Thereafter, 2 g of dry bark residue was submitted to a methanol/water/acetic acid (49.5:49.5:1, $v/v/v$) extraction for 24 h, at constant stirring (900 r.p.m.), in the dark. After separation of the extract from the biomass via vacuum filtration, the methanol was evaporated at low pressure using a rotative evaporator, and water was removed by freeze-drying. *Pinus* spp. bark polar extracts were obtained in triplicate, and the respective extractive yields (EYs) were expressed as the percentage of dry bark. The extracts were left in the dark, at room temperature, until their analyses were carried out.

2.4. Total Phenolic Content

The total phenolic content (TPC) of the *Pinus* spp. barks was evaluated using the Folin–Ciocalteu reagent, following a previously described procedure [23,26], with some adaptations [21]. The TPC was determined through a standard curve of gallic acid (5–100 μg mL^{-1}) and expressed as mg of gallic acid equivalents (GAE) per g of extract and g GAE per kg of dw. Two aliquots of each pine bark extract were tested in triplicate.

2.5. Proanthocyanidins Content

The proanthocyanidins content (PC) was determined through the acid butanol assay, according to the method described elsewhere [27]. Three bark polar extracts of each pine species were dissolved in methanol/water (50:50, v/v) at 1 mg mL^{-1}. The absorbance of the cynanidin chromophore was recorded at 520 nm using the Thermo Scientific Multiskan$^{\text{TM}}$ FC microplate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA). The PC was calculated via the linear regression equation of PACs purified from

grape pomace (100–500 µg mL^{-1}) and expressed as mg of PAC per g of extract and g of PAC per kg of bark dw. Two aliquots of each pine bark extract were tested in triplicate.

2.6. Qualitative Analysis of Polar Extracts by UHPLC-DAD-MS^n Analysis

UHPLC-UV-MS^n analysis of the *Pinus* spp. bark extracts was carried out in a UHPLC system equipped with an Accela 600 LC pump, an Accela autosampler (set at 16 °C), and an Accela 80 Hz photo diode array detector (DAD) (Thermo Fisher Scientific, San Jose, CA, USA). The extracts were prepared in methanol/water (50:50, *v/v*) at 10 mg mL^{-1} and the filtration process was carried out by tetrafluoroethylene filter having pore size 0.2 µm. The injection volume was 10 µL. The column used for separation was a Hypersil Gold RP C18 (100 × 2.1 mm; 1.9 µm packing) provided by Thermo Fisher Scientific (San Jose, CA, USA), with a C18 pre-column (2.1 mm i.d.) supplied by Thermo Fisher Scientific (San Jose, CA, USA); both were kept at 45 °C. The mobile phase composed of water:acetonitrile (99:1, *v/v*) (A) and acetonitrile (B), each containing HCOOH (0.1% (*v/v*)) . Gradient elution was carried out at a flow rate of 0.45 mL min^{-1} for 32 min, as follows: 1% B kept from 0 to 3 min; 1–31% B over 27 min; and 31–100% B over 2 min, followed by reequilibration at 1% B for 4 min. The chromatograms were recorded at 280 and 370 nm, and UV spectra collected from 210 to 600 nm.

The UHPLC system was coupled to an LCQ Fleet ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA) with an electrospray ionization (ESI) source, conducted in the negative ionization mode, according to previously described conditions [21].

2.7. Quantitative Analysis of Polar Extracts by UHPLC-UV Analysis

Standard solutions of gallic and protocatechuic acids, catechin, naringenin, and quercetin were injected in the UHPLC to obtain standard curves. The solutions were prepared in HPLC grade methanol, with six concentrations ranging from 0.45 to 41.04 µg mL^{-1}. The compounds were quantified by using the linear regression equation (Table 1) of the most similar standard compound. The pine bark extracts were injected in triplicate.

Table 1. UHPLC-UV standard data used for the quantitative analysis of the identified compounds in *Pinus* spp. bark polar extracts.

Standard	λ (nm) [1]	Concentration Range (µg mL^{-1})	Linear Regression Equation [2]	R^2	LOD [3] (µg mL^{-1})	LOQ [4] (µg mL^{-1})
Catechin	280	0.50–30.29	$y = 30528x - 19621$	0.9973	2.08	6.93
Gallic acid	280	0.52–31.20	$y = 104717x - 38562$	0.9986	1.56	5.19
Naringenin	280	0.51–41.04	$y = 394428x + 172880$	0.9999	0.44	1.48
Protocatechuic acid	280	0.50–30.26	$y = 112312x - 18937$	0.9997	0.66	2.20
Quercetin	370	0.45–27.05	$y = 364286x - 66014$	0.9949	2.55	8.51

[1] Wavelength used in the HPLC-UV quantification. [2] *y* and *x* represent peak area and concentration in µg mL^{-1}, respectively. [3] LOD, limit of detection. [4] LOQ, limit of quantification.
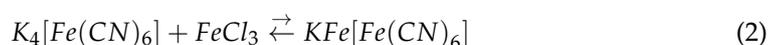
2.8. Antioxidant Activity

2.8.1. Reducing Power

The reducing power was assayed according to the procedure described [28], with some modifications, and adapted to the 96-well microplate scale. Trolox was considered as the antioxidant reference. The stock solution of Trolox was prepared in methanol, whilst three bark polar extracts of each *Pinus* spp. were dissolved in methanol/water (50:50, *v/v*) at 1 mg mL^{-1}. Different volumes of extract and standard stock solutions were added to deionised water up to 1000 µL and then mixed with 1000 µL of 0.2 M phosphate buffer solution (pH 6.6) and 1000 µL of 1% (*w/v*) potassium ferrocyanide solution, obtaining the concentration ranges of 3.4–49.5 µg mL^{-1} and 3.6–71.1 µg mL^{-1} for the extracts and Trolox, respectively. The reactional mixture was kept at 50 °C for 20 min. Thereafter, 1000 µL of 10% (*w/v*) trichloroacetic acid solution was added to stop the redox reaction, and the mixture was left to cool to room temperature for 10 min. An aliquot of 125 µL of

the mixture was transferred to the microplate well, and 125 µL of deionised water and 25 µL of 0.1% (*w/v*) ferric (III) chloride solution were added. Then, the absorbance was recorded against the blank at 690 nm in a Thermo Scientific Multiskan™ FC microplate reader. An increased absorbance means a higher reducing power of sample. The half-maximal inhibitory concentration (IC_{50}) was considered as the sample concentration at which the absorbance was measured as 0.500, being calculated through the respective linear regression equations from the graph plotting absorbance at 690 nm against the sample concentration. Each concentration was tested in triplicate.

The reducing power represents a ferric-ion-based total antioxidant capacity assay, in which the potassium ferricyanide ($K_3[Fe(CN)_6]$) is the oxidant species and reacts with the antioxidant, forming potassium ferrocyanide ($K_4[Fe(CN)_6]$) and the oxidized antioxidant (Equation (1)). Then, potassium ferrocyanide combines with ferric chloride ($FeCl_3$), yielding the Prussian blue chromophore ($KFe[Fe(CN)_6]$) (Equation (2)). The reducing power of the tested extract or compound increases as much as the absorbance of Prussian blue [29].

$$K_3[Fe(CN)_6] + antioxidant \rightleftarrows K_4[Fe(CN)_6] + oxidized\ antioxidant \tag{1}$$

$$K_4[Fe(CN)_6] + FeCl_3 \rightleftarrows KFe[Fe(CN)_6] \tag{2}$$

2.8.2. DPPH Free Radical Scavenging Effect

The antioxidant activity against DPPH of *Pinus* spp. bark extracts was approached as explained before [21,30] and adapted to the 96-well plate scale. Ascorbic acid was used as the antioxidant reference, being dissolved in methanol/water (50:50, *v/v*) at 1 mg mL^{-1}. Stock solutions (1 mg mL^{-1}) of three polar extracts from the *P. pinaster* Ait. and *P. pinea* L. barks were also prepared in methanol/water (50:50, *v/v*). Briefly, in each well, 30 µL of 1 mM DPPH methanolic solution was mixed to 75 µL of sample and 195 µL of methanol, obtaining the concentration ranges of 1.0–43.2 µg mL^{-1} for the extracts and 0.5–15.9 µg mL^{-1} for ascorbic acid. The blank was composed of 30 µL of DPPH (1 mM) in methanol and 270 µL of methanol. After mixing, the microplate was protected from the light for 30 min. The absorbance of the resulting solution was measured at 520 nm in a microplate reader (Thermo Scientific Multiskan™ FC). The DPPH scavenging effect percentage was determined according to Equation (3).

$$DPPH\ scavenging\ effect\ (\%) = [\frac{A_{control} - A_{sample}}{A_{control}}] \times 100\% \tag{3}$$

The IC_{50} was determined by plotting the percentage of scavenged DPPH free radicals against the concentration logarithm. For comparison reasons, the AAI was calculated according to Equation (4) [31].

$$AAI = \frac{DPPH_{final\ concentration}(\mu g\ mL^{-1})}{IC_{50}(\mu g\ mL^{-1})} \tag{4}$$

with the DPPH final concentration equal to 61.874 µg mL^{-1}. Each concentration was assayed in triplicate.

2.8.3. ABTS Radical Cation Scavenging Effect

The ABTS radical cation (ABTS$^{\bullet+}$) scavenging activity of the *Pinus* spp. bark extracts was measured according to a described procedure [23,32] and adapted to the 96-well plate scale. Firstly, stock solutions (1 mg mL^{-1}) of three bark polar extracts from each *Pinus* species were prepared in methanol/water (50:50, *v/v*). Moreover, stock solutions (1 mg mL^{-1}) of ascorbic acid and trolox (antioxidant standards) were prepared in methanol/water (50:50, *v/v*) and methanol, respectively. The ABTS radical cation was prepared by reacting 7 mM ABTS with 2.45 mM potassium persulfate. The reactional mixture was kept in the dark at room temperature for 16 h and then diluted with methanol, to give

an absorbance value of 0.700 at 750 nm. In each microwell, 250 μL of ABTS$^{\bullet+}$ solution was mixed with 50 μL of sample, obtaining the 0.5–21.2, 0.5–15.9 , and 0.5–19.4 μg mL^{-1} concentration ranges for the extracts, ascorbic acid, and trolox, respectively. The control was constituted by 250 μL of ABTS$^{\bullet+}$ solution and 50 μL of methanol. After 30 min, protected from the light, the absorbance was recorded at 750 nm by a Thermo Scientific MultiskanTM FC microplate reader.

The IC$_{50}$ was calculated by plotting the percentage of scavenged ABTS radical cation as a function of the sample concentration logarithm. Each concentration was tested in triplicate.

2.9. Antibacterial Activity

The antibacterial effect of the *Pinus* spp. bark polar extracts was tested against the growth of *S. aureus* (ATCC$^{\circledR}$ 6538) and *E. coli* (ATCC$^{\circledR}$ 25922).

Before the antibacterial tests, a bacterial colony was inoculated in 30 mL of tryptic soy broth at 37 °C and 170 r.p.m. for 18 h. Later, the bacterial culture was diluted in phosphate buffered saline (PBS) solution (pH 7.4), to be adjusted to 0.5 McFarland standard, which corresponds to 10^8–10^9 colony forming units (CFUs) per mL. The bacterial inoculum was added to the *Pinus* spp. bark polar extracts, obtaining the final concentrations of 0.39, 0.78, 1.56, 3.13, 6.25, and 12.5 e 25 mg mL^{-1}. Two bacterial controls were included, namely a bacterial growth control with only bacterial inoculum and a solvent control containing bacterial inoculum with 12.5% (*v/v*) ethanol, representing the maximum amount of ethanol added to the bacterial culture. The bacterial suspensions were incubated at 37 °C and 120 r.p.m. for 24 h. At 24 h of incubation, the aliquots of bacterial suspensions were sampled, serially diluted in PBS and plated in triplicate in tryptic soy agar. After the incubation at 37 °C for 18 h, the CFUs were calculated, and the viable bacterial density was determined as log CFU mL^{-1} and compared with the solvent control. The bacteriostatic and bactericidal effects were considered as the decrease of <3-log and ≥3-log in CFU mL^{-1}, respectively. The assays were performed as three independent experiments, with three replicates for each concentration.

2.10. Statistical Analysis

The statistical analysis was performed using the IBM$^{\circledR}$ SPSS$^{\circledR}$ Statistics Version 25 (IBM Corporation, New York, NY, USA). Tukey's Honestly Significant Difference (HSD) and Duncan Post Hoc Tests were used to determine the significance of difference ($p < 0.05$) for antioxidant and antibacterial activity data, respectively.

3. Results and Discussion

3.1. Extraction Yield, Total Phenolic, and Proanthocyanidins Contents

The polar extracts of the *P. pinaster* Ait. and *P. pinea* L. barks were obtained by solid liquid extraction with a mixture of methanol/water/acetic acid (49.5:49.5:1, *v/v/v*), which has been shown to be suitable for the efficient extraction of phenolic compounds from biomass, e.g., cultivated cardoon and willow bark [21,33]. The obtained extraction yields (EYs) were 18.3 ± 1.1% and 21.3 ± 0.7% *w/w* dry weight (dw) for the *P. pinea* L. and *P. pinaster* Ait. barks, respectively. These values were higher than those previously reported using ethanol (4.6–13.2% *w/w* dry *P. pinaster* Ait. bark and 12.1% *w/w* dry *P. pinea* L. bark) and water (2.4–3.2% *w/w* dry *P. pinaster* Ait. bark and 4.9% *w/w* dry *P. pinea* L. bark) [10,34–36]. In addition to the extraction media, the geographical origin, edaphoclimatic conditions, pine tree age and cultivar, and sample conservation and extraction techniques can strongly affect EYs.

The *P. pinaster* Ait. and *P. pinea* L. bark polar extracts were evaluated regarding their TPC and PC, which are summarized in Table 2. The TPC of the studied *Pinus* spp. barks accounted for 103 g of gallic acid equivalents (GAE) kg^{-1} dw in the *P. pinea* L. bark and 116 g of GAE kg^{-1} dw in the *P. pinaster* Ait. bark. The TPC values reported here for the *P. pinaster* Ait. and the *P. pinea* L. bark extracts are within the described range for

the aqueous and hydroethanolic extracts of *P. pinaster* Ait. bark collected in the north of Portugal (5.30–163.64 g GAE kg^{-1} bark) [10,37]. When the TPC was expressed as mg GAE per g of extract (Table 2), it ranged between 539 mg g^{-1} and 568 mg g^{-1} of the extracts derived from the *P. pinaster* Ait. and *P. pinea* L. barks, respectively. These values were in agreement with those described in the literature, regarding the ethanol, water, and alkaline extracts of *P. pinaster* Ait. bark (260–738.0 mg GAE g^{-1} of extract) [38–40], including Pycnogenol® (572.28 mg GAE g^{-1} of extract) [41]. Furthermore, the TPCs of the studied extracts were concordant with those reported for the hot water extracts (HWE), derived from other *Pinus* spp. barks, ranging from a 111 mg g^{-1} extract of *P. rigida* x *taeda* to an 862 mg g^{-1} extract of *P. koraiensis* [42], as well as for the 80% (*v*/*v*) methanol extract of *P. brutia* bark (412.42 mg GAE g^{-1} extract) [43].

Table 2. Total phenolic content (TPC) and proanthocyanidin content (PC) of *Pinus pinaster* Ait. and *Pinus pinea* L. bark polar extracts.

Pinus spp. by-Product	TPC (g GAE kg^{-1} dw)	TPC (mg GAE g^{-1} of Extract)	PC (g PAC kg^{-1} dw)	PC (mg PAC g^{-1} of Extract)
P. pinaster Ait. bark	116 ± 11	539 ± 26	174 ± 12	814 ± 58
P. pinea L. bark	103 ± 9	568 ± 12	157 ± 18	857 ± 50

The values represent the mean ± standard deviation of two aliquots of each extract tested in triplicate. Abbreviations: GAE, gallic acid equivalents; PAC, proanthocyanidins purified from grape pomace; PC, proanthocyanidin content; TPC, total phenolic content.

Both *Pinus* spp. barks showed considerably high PCs (Table 2), accounting for 174 and 157 g PACs kg^{-1} dw in the *P. pinaster* Ait. and *P. pinea* L. barks, respectively. Interestingly, the PC of the studied pine bark extracts was much higher than those of the alkaline extracts from the *P. pinaster* Ait. bark (1.20–5.43 g cyanidin equivalents kg^{-1} bark) [44], despite the use of a different standard. Actually, the PC determined through the cyanidin standard curve has been considered as underestimated as not all the PCs are converted into anthocyanidins [45]. When the PCs of the *Pinus* spp. bark polar extracts were expressed as mg PAC g^{-1} of the extract (Table 2), it varied between 814 mg PAC g^{-1} of the *P. pinaster* Ait. bark extract and 857 mg g^{-1} of the *P. pinea* L. bark extract. These values are ~22% higher than the value reported for Pycnogenol® (700 mg g^{-1} extract) [8] and up to 6.7-fold higher than the values achieved for phloem extracts from *P. pinaster* and *P. pinea* [46].

3.2. Chemical Characterisation of Pinus spp. Bark Polar Extracts

3.2.1. Qualitative Analysis

The compounds present in the *P. pinaster* Ait. and *P. pinea* L. bark polar extracts were identified by UHPLC-DAD-MS^n.

Table 3 lists the retention time, maxima UV wavelengths, the [M−H]$^-$ ions, and the MS^n product ions of the 15 compounds (Figure 1) identified in the studied polar extracts. In addition to quinic acid (**1**), 14 phenolic compounds were found in the studied *Pinus* spp. barks, such as protocatechuic acid (**2**), 10 flavan-3-ols (**3–12**), 2 flavanonols (**13–14**), and a flavonol, i.e., quercetin (**15**). The identification of these compounds was performed by comparing the obtained spectroscopic results with the literature data and with those of the available commercial standards injected under the same experimental conditions, as discussed below.

Table 3. UHPLC-DAD-MS^n data of compounds identified in *Pinus pinaster* Ait. and *Pinus pinea* L. bark polar extracts.

No.	RT (min)	Compound	λ_{max} (nm)	[M−H]$^-$ (m/z)	MS^n Product Ions (m/z and Relative Intensity (%)) [2]	Reference
1	0.66	Quinic acid	244, 279, 300 (sh) [1]	191	MS^2: 173 (15), 127 (0.4), 111 (100), 93 (1), 85 (2)	[13]
2	2.36	Protocatechuic acid	277, 259, 283	153	MS^2: 153 (3), 135 (1), 121 (1), 109 (100), 95 (0.1)	Co [3]
3	3.22	B-type (epi)catechin trimer isomer 1	230, 279	865	MS^2: 822 (25), 739 (100), 713 (29), 695 (15), 659 (23), 575 (50), 533 (40), 406 (26), 353 (47)	[47,48]
4	5.78	B-type (epi)catechin dimer isomer 1	232, 278	577	MS^2: 559 (36), 451 (100), 425 (99.8), 407 (23), 289 (25), 287 (8), 245 (3), 203 (1) MS^3: 271 (13), 245 (100), 205 (29), 203 (13), 179 (6)	[21,47,48]
5	6.20	B-type (epi)catechin dimer isomer 2	232, 278	577	MS^2: 559 (31), 451 (98), 425 (100), 407 (37), 289 (28), 287 (8), 245 (3), 203 (1) MS^3: 245 (100), 205 (35), 203 (6), 179 (23)	[21,47,48]
6	6.70	Catechin	230, 278	289	MS^2: 271 (5), 245 (100), 205 (33), 203 (17), 179 (10)	Co [3]
7	6.75	B-type (epi)catechin dimer isomer 3	232, 278	577	MS^2: 559 (1), 451 (100), 425 (90), 407 (26), 289 (25), 287 (9), 245 (5), 205 (0.3), 203 (0.5), 179 (0.2) MS^3: 271 (5), 245 (100), 205 (44), 203 (11), 179 (13)	[47–49]
8	7.15	B-type (epi)catechin trimer isomer 2	231, 278	865	MS^2: 847 (75), 739 (100), 695 (61), 577 (29), 575 (21), 425 (17)	[47,48]
9	7.51	A-type (epi)gallocatechin-(epi)catechin dimer	232, 281	591	MS^2: 573 (18), 563 (2), 555 (5), 489 (2), 465 (5), 439 (100), 433 (2), 301 (3), 287 (3)	[50]
10	8.50	B-type (epi)catechin trimer isomer 3	232, 278	865	MS^2: 847 (28), 739 (97), 713 (63), 695 (93), 677 (32), 577 (100), 575 (60), 451 (14), 425 (9), 413 (23), 407 (21), 287 (16)	[47,48]
11	11.02	B-type (epi)catechin trimer isomer 4	233, 279	865	MS^2: 739 (77), 713 (41); 695 (86), 587 (34), 575 (100), 451 (51), 449 (53), 287 (32)	[47,48]
12	11.98	B-type (epi)catechin dimer isomer 4	233, 279	577	MS^2: 559 (58), 451 (100), 425 (89), 407 (69), 289 (38), 287 (3), 245 (8), 205 (5), 179 (3)	[21,47,48]
13	12.32	Taxifolin-O-hexoside	234, 279, 300 (sh)	465	MS^2: 447 (18), 437 (100), 285 (61), 259 (32)	[13,51,52]
14	12.82	Taxifolin	237, 287, 320 (sh) [2]	303	MS^2: 285 (100), 177 (10), 125 (5) MS^3: 267 (2), 257 (10), 243 (18), 241 (100), 217 (16), 199 (19), 175 (59), 161 (4)	[13,53]
15	20.52	Quercetin	237, 279, 361	301	MS^2: 273 (33), 257 (26), 229 (7), 193 (10), 179 (1), 151 (100), 107 (3)	Co [3]

[1] *sh* is used to represent a shoulder detected in the UV spectrum. [2] *m/z* underlined was subjected to MS^3 analysis. [3] Co, Co-injection of a commercial standard.
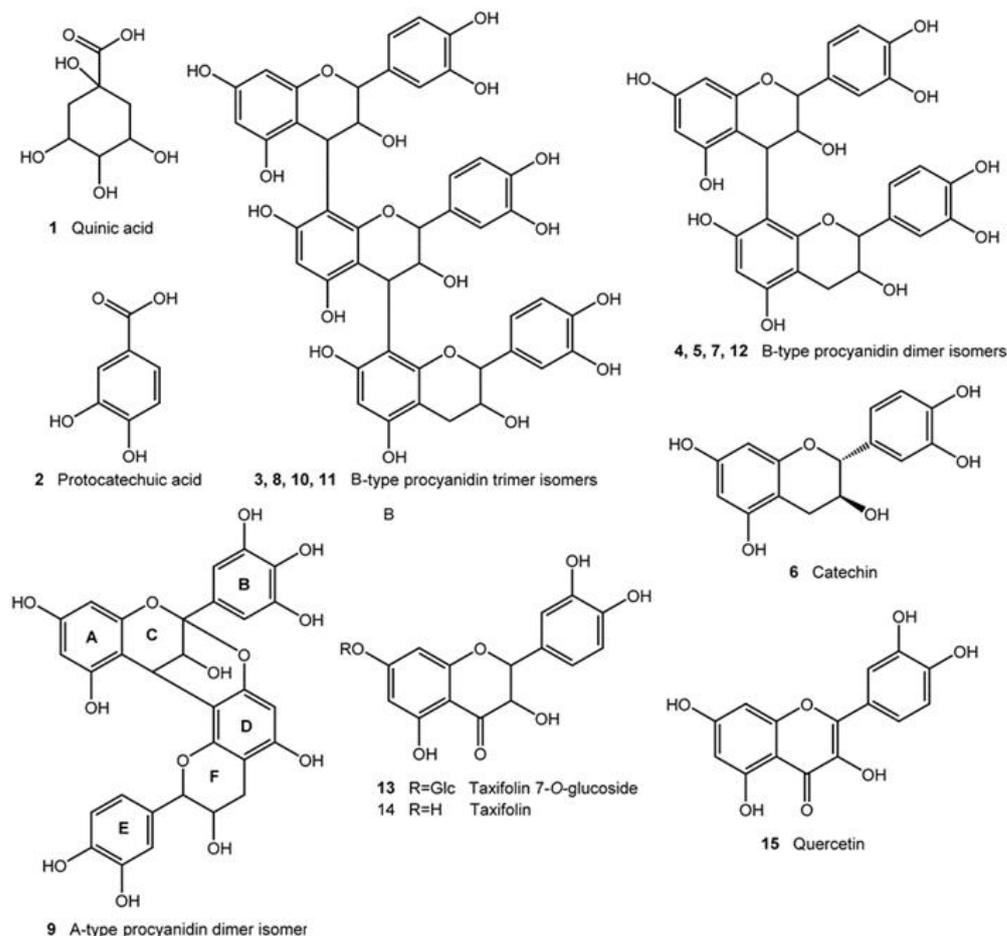
Figure 1. Chemical structures of the compounds identified in *Pinus pinaster* Ait. and *Pinus pinea* L. bark polar extracts.

- Flavan-3-ols

Based on the characteristic UV absorption spectra, the corresponding [M−H]$^-$ ions and respective MS^n fragmentations (Table 3), catechin (**6**), 4 B-type (epi)catechin dimers (**4**, **5**, **7**, and **12**), 4 B-type (epi)catechin trimers (**3**, **8**, **10**, and **11**), and an A-type (epi)gallocatechin-(epi)catechin dimer (**9**) were identified in the analysed *P. pinaster* Ait. and *P. pinea* L. barks, as thoroughly described below.

Catechin (**6**) (Figure 2) was identified via the UV spectral data and the detection of the [M−H]$^-$ ion at *m/z* 289, as well as its MS^2 fragmentation [21,54], which agreed with those obtained for a commercial standard injected under the same experimental conditions. (+)-Catechin has been previously identified in the hydroethanolic extracts of *P. pinaster* Ait. bark [10,37], as well as in the commercial pine bark extracts [13,55,56]. This compound has also been detected in the ethyl acetate fraction of the aqueous extract of the *P. pinea* L. bark from Turkey [17].

Four B-type (epi)catechin dimers (**4**, **5**, **7**, and **12**) (Figure 1) were identified according to the UV absorption data and the [M−H]$^-$ ion detected at *m/z* 577, as well as the characteristic product ions resulting from the MS^2 fragmentation of the ion at *m/z* 577 [21,47,48]. Furthermore, the (epi)catechin units present in compounds **4**, **5**, **7**, and **12** can be bonded by C4→C6 or C4→C8 (B-type), but the obtained mass spectrometry data did not allow us to unequivocally distinguish between them (Figure 2) [48,56]. Considering that the procyanidin dimers identified so far in nature range from B1 to B16 [48], the identity of compounds **4**, **5**, **7**, and **12** may be attributed to four of those procyanidins, with the exception of either procyanidins B10, B12, B13, and B16, as these include an epigallocatechin monomer, or procyanidin B11, due to the presence of a galloylcatechin upper subunit. Moreover,

procyanidins B1, B3, B6, and B7 have been already identified in Pycnogenol® [8] and three B-type procyanidin isomers in *P. pinea* L. bark [17].
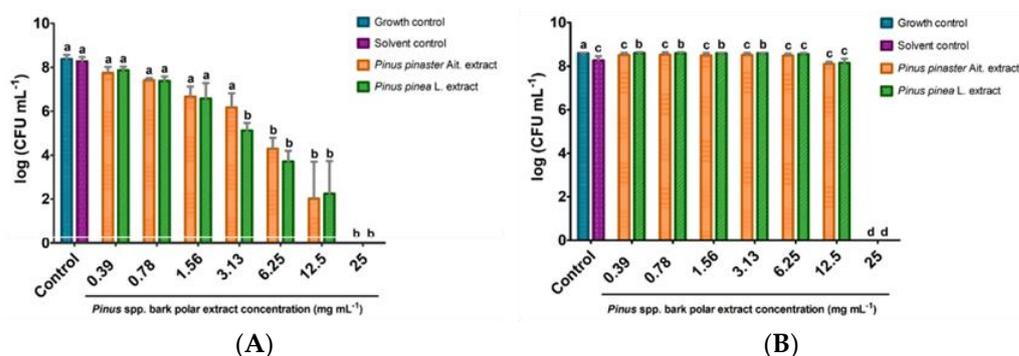

Figure 2. Inhibitory effect of *Pinus pinaster* Ait. and *Pinus pinea* L. bark polar extracts against the growth of (**A**) *S. aureus* (ATCC® 6538) and (**B**) *E. coli* (ATCC® 25922). The solvent control represents the bacterial growth under 12.5% (*v/v*) ethanol. The columns and bars depict, respectively, the mean and the mean-standard of the error of three independent experiments performed in triplicate. Columns with different letters are statistically different from the solvent control (unidirectional ANOVA followed by Duncan's Test; $p < 0.05$). CFU, colony forming units.

Compounds **3**, **8**, **10**, and **11** were tentatively identified as B-type (epi)catechin trimers as their molecular absorption spectra, the detection of the $[M-H]^-$ ion at *m/z* 865, and the MS^2 fragmentation of the $[M-H]^-$ ion (Table 3) were in agreement with the literature data [48,57]. Indeed, the MS^2 spectrum of the $[M-H]^-$ ion at *m/z* 865 yielded the product ions at *m/z* 577 ($[M-H-288]^-$) and *m/z* 287 ($[(epi)catechin-3H]^-$), resulting from the quinone-methide fission between the C and D rings, with the loss of the extension unit [48,57]. Furthermore, the retro Diels–Alder fission in the C ring of the $[M-H]^-$ ion at *m/z* 865 originated the product ion at *m/z* 713, which yielded the product ion at *m/z* 695, after the loss of a water molecule [48]. Additionally, the product ions at *m/z* 847 ($[M-H-H_2O]^-$) and *m/z* 739 (heterocyclic C ring fission) were detected. The product ion at *m/z* 587 yielded from the product ion at *m/z* 739 after the retro Diels–Alder fission in the F ring. In the MS^2 [865] spectrum, the characteristic product ions at *m/z* 451 (heterocyclic F ring fission), *m/z* 425 (retro Diels–Alder fission in F ring), *m/z* 407 (loss of a water molecule from the product ion at *m/z* 425), and *m/z* 287 (trimer upper subunit after the quinone-methide fission between the C and D rings) were also found [47,48]. Interestingly, only one B-type procyanidin trimer isomer has already been found in commercial *P. pinaster* Ait. bark extracts [56,58], and two others in *P. pinea* L. bark [17].

Compound **9** was tentatively identified as an A-type procyanidin dimer (Figure 2) as its UV molecular absorption spectrum, the detection of the $[M-H]^-$ ion at *m/z* 591, and the corresponding MS^2 spectrum (Table 3) were all concordant with the literature data [50,57,59]. The chemical structure of this compound can be envisaged as one of four hypotheses, as follows: (epi)gallocatechin-(2→O7, 4→8)-(epi)catechin; (epi)gallocatechin-(2→O5, 4→6)-(epi)catechin; (epi)catechin-(2→O7, 4→8)-(epi)gallocatechin; or (epi)catechin-(2→O5, 4→6)-(epi)gallocatechin [50,59]. Considering the detection of the product ions at *m/z* 301 and *m/z* 289 in the MS^2 spectrum of the ion at *m/z* 591, the former two hypotheses are more likely than the remaining two as these product ions represent, respectively, $[(epi)gallocatechin-5H]^-$ and $[(epi)catechin-H]^-$ ions formed by the methide fission between the C ring of the (epi)gallocatechin unit (upper unit) and the D ring of the (epi)catechin (terminal unit) [59] (Figure S1). Furthermore, the MS^2 spectrum of the ion at *m/z* 591 presented a base peak at *m/z* 439, which resulted from the retro-Diels–Alder fission in the F-ring of the (epi)catechin unit, with the loss of 152 mass units (Figure S1), strengthening the hypothesis of (epi)catechin being the terminal unit of compound **9** [59]. Nonetheless, it is not possible to distinguish the linkages of the units solely by mass spectrometry [59]. Thus, the chemical structure of compound **9** may be an isomer of the

(epi)gallocatechin-(epi)catechin dimer. To the best of our knowledge, this procyanidin isomer is identified here for the first time in the *P. pinaster* Ait. and *P. pinea* L. barks.

- Flavanonols

Compound **13** was assigned as taxifolin-*O*-hexoside (Figure 2), based on the UV molecular absorption spectrum, the $[M-H]^-$ ion at *m/z* 465, and the corresponding MS^2 spectrum [13,49]. Actually, this MS^2 spectrum presented the product ion at *m/z* 285 due to the loss of a water molecule from the product ion at *m/z* 303 ([taxifolin$-H]^-$) [13,51,52]. Additionally, taxifolin-*O*-hexoside was eluted before taxifolin (**14**), as reported elsewhere [13].

Compound **14** was identified as taxifolin (Figure 2) as its retention time, the UV spectrum, the detection of the $[M-H]^-$ ion at *m/z* 303, and the respective MS^2 and MS^3 [303→285] data matched with those of the commercial standard injected under the same experimental conditions. Regarding the fragmentation data profile of this compound, the MS^2 spectrum of the $[M-H]^-$ ion at *m/z* 303 depicted the base peak at *m/z* 285 ($[M-H-H_2O]^-$), whilst the MS^3 [303→285] fragmentation yielded the product ions at *m/z* 243 (loss of C_2H_2O) and *m/z* 217 (loss of C_3O_2), due to the presence of 4'-hydroxyl groups at C4' in the B ring and at C5 or C7 in the A ring, respectively [60]. Indeed, taxifolin has also been identified in commercial [13,55] and hydroethanolic [10,37] extracts of *P. pinaster* Ait. bark. Two taxifolin isomers have also been described in *P. pinea* L. bark [17].

- Other compounds

The identification of quinic acid (**1**) (Figure 2) was based on the characteristic UV molecular absorption spectrum, the detection of the $[M-H]^-$ at *m/z* 191, and the corresponding MS^2 spectrum, which showed a base peak at *m/z* 111 and the characteristic product ions at *m/z* 173 ($[M-H-H_2O]^-$) and *m/z* 93 [13]. This compound has already been found in Pycnogenol® [13], whereas it was identified for the first time, in this work, in *P. pinea* L. bark.

After confirmed by authentic standard, compound **2** was assigned as protocatechuic acid (Figure 2). In fact, this phenolic compound had been previously identified in *P. pinaster* Ait. [10] and *P. pinaster* Ait. var. *moghrebiana* barks [61], as well as in commercial pine bark extracts [13,55]. In contrast, protocatechuic acid was identified for the first time in *P. pinea* L. bark, to the best of our knowledge.

Compound **15** was identified quercetin, whose retention time, molecular absorption spectrum, $[M-H]^-$ at *m/z* 301, and MS^2 spectrum were concordant with those of the authentic standard. This flavonol has been found in a commercial concentrated extract [56] and hydroethanolic extracts [10,37], derived from *P. pinaster* Ait. bark, but it has been reported here for the first time as a *P. pinea* L. component.

3.2.2. Quantitative UHPLC-UV Analysis of *Pinus* spp. Bark Polar Extracts

The fifteen compounds detected in the *Pinus* spp. bark extracts were quantified by HPLC-UV, and the individual contents of each component in the *P. pinaster* Ait. and *P. pinea* L. polar extracts are depicted in Table 4.

The total content of identified compounds varied between 14.81 g kg^{-1} of dry bark of *P. pinaster* Ait. and 18.56 g kg^{-1} of dry bark of *P. pinea* L. Quinic acid (**1**) represented the major compound identified in both *Pinus* spp. barks, accounting for 40% and 42% (*w/w*) of the total content of the identified compounds in the *P. pinaster* Ait. and *P. pinea* L. barks, respectively.

Table 4. Abundance of compounds identified in the *Pinus pinaster* Ait. and *Pinus pinea* L. bark polar extracts.

No.	Compound	λ (nm)	g kg⁻¹ of dw		mg g⁻¹ of Extract	
			Pinus pinaster **Ait.**	*Pinus pinea* **L.**	*Pinus pinaster* **Ait.**	*Pinus pinea* **L.**
1	Quinic acid [A]	280	5.97 ± 0.32	7.86 ± 0.25	27.98 ± 1.12	43.17 ± 4.13
2	Protocatechuic acid [B]	280	0.35 ± 0.01	0.39 ± 0.06	1.66 ± 0.10	2.13 ± 0.29
	∑ Phenolic acids		**0.35 ± 0.01**	**0.39 ± 0.06**	**1.66 ± 0.10**	**2.13 ± 0.29**
3	B-type (epi)catechin trimer isomer 1 [C]	280	<LOQ [F]	<LOQ [F]	<LOQ [F]	<LOQ [F]
4	B-type (epi)catechin dimer isomer 1 [C]	280	0.37 ± 0.03	0.53 ± 0.02	1.75 ± 0.20	2.89 ± 0.24
5	B-type (epi)catechin dimer isomer 2 [C]	280	1.43 ± 0.13	1.63 ± 0.09	6.71 ± 0.82	8.91 ± 0.46
6	Catechin [C]	280	2.29 ± 0.30 [G (6+7)]	2.80 ± 0.17 [G (6+7)]	10.78 ± 1.72 [G (6+7)]	15.30 ± 0.84 [G (6+7)]
7	B-type (epi)catechin dimer isomer 3 [C]	280	[G (6+7)]	[G (6+7)]	[G (6+7)]	[G (6+7)]
8	B-type (epi)catechin trimer isomer 2 [C]	280	0.78 ± 0.09	0.60 ± 0.02	3.66 ± 0.41	3.30 ± 0.34
9	A-type (epi)gallocatechin-(epi)catechin dimer [C]	280	0.90 ± 0.05	1.16 ± 0.10	4.20 ± 0.09	6.36 ± 0.63
10	B-type (epi)catechin trimer isomer 3 [C]	280	0.85 ± 0.04	1.26 ± 0.06	3.99 ± 0.30	6.90 ± 0.61
11	B-type (epi)catechin trimer isomer 4 [C]	280	0.24 ± 0.03	0.79 ± 0.07	1.12 ± 0.16	4.29 ± 0.10
12	B-type (epi)catechin dimer isomer 4 [C]	280	0.17 ± 0.01	0.13 ± 0.01	0.82 ± 0.07	0.69 ± 0.01
	∑ Flavan-3-ols		**7.03 ± 0.52**	**8.89 ± 0.33**	**33.03 ± 3.34**	**48.66 ± 2.46**
13	Taxifolin-*O*-hexoside [D]	280	<LOQ [F]	0.03 ± 0.00	<LOQ [F]	0.18 ± 0.01
14	Taxifolin [D]	280	1.46 ± 0.00	1.40 ± 0.03	6.83 ± 0.24	7.66 ± 0.57
	∑ Flavanonols		**1.46 ± 0.00**	**1.43 ± 0.03**	**6.83 ± 0.24**	**7.83 ± 0.58**
15	Quercetin [E]	370	<LOQ [F]	<LOQ [F]	<LOQ [F]	<LOQ [F]
	∑ Flavonols		**<LOQ [F]**	**<LOQ [F]**	**<LOQ [F]**	**<LOQ [F]**
	TOTAL		**14.81 ± 0.20**	**18.56 ± 0.34**	**69.49 ± 3.18**	**101.79 ± 6.95**

The values represent the mean ± standard deviation obtained from *Pinus* spp. bark extracts injected in triplicate. Standard curves used for the quantification of the identified compounds: [A] gallic acid; [B] protocatechuic acid; [C] catechin; [D] naringenin; [E] quercetin. [F] The abundance is lower than the limit of quantification (LOQ). [G] The abundance of compounds 6 and 7 was determined at 280 nm, using the standard curve of catechin.

Flavan-3-ols constituted the major phenolic subclass detected in the studied pine bark polar extracts (Table 4), accounting for 47% and 48% (w/w) of the total identified content in the *P. pinaster* Ait. and *P. pinea* L. barks, respectively. Considering the abundance of flavan-3-ols expressed as mg of compound per g of extract, it was higher in the *P. pinea* L. bark extract (48.66 mg g^{-1} of extract) than in the *P. pinaster* Ait. Bark extract (33.03 mg g^{-1} of extract) (Table 4). Indeed, the flavan-3-ols contents of the *Pinus* spp. bark extracts analysed in the present work are concordant with that detected in Pycnogenol® [13] but lower than that reported by Romani and colleagues [55] in a commercial pine bark extract (776.6 mg g^{-1} powder).

Moreover, catechin (**6**) and a B-type procyanidin dimer (**7**) were the main flava-3-ols present in both pine bark samples, representing 15% (w/w) of the total content of the identified compounds (Table 4). In particular, catechin and B-type procyanidin dimers accounted for 27.79 mg g^{-1} of the *P. pinea* L. extract and 20.06 mg g^{-1} of the *P. pinaster* Ait. extract, being slightly higher than that earlier evidenced for Pycnogenol® (15.49 mg g^{-1} of extract) [13]. Catechin (**6**) has also been detected in *P. pinea* L. bark extracts, namely in an ethyl acetate fraction of HWE (35.8 mg g^{-1} of extract) [17]. B-type procyanidin trimers (8, 10, and 11) were also abundant in both pine barks, accounting for 14.2% (w/w) and 12.6% (w/w) of the total identified content in the *P. pinea* L. and *P. pinaster* Ait. barks, respectively. Their abundances agreed with those described for a commercial extract of *P. maritima* L. bark (21.1% (w/w) of total content) [55]. To the best of our knowledge, the abundances of procyanidins in *P. pinea* L. bark are exhibited, for the first time, in this study.

Flavanonols were also detected at considerable abundances in both pine barks, representing 9.8% (w/w) and 7.7% (w/w) of the total identified contents of the *P. pinaster* Ait. and *P. pinea* L. barks, respectively (Table 4). Taxifolin (**14**) was the major flavanonol, accounting for 7.66 mg g^{-1} of the *P. pinea* L. bark extract and 6.83 mg g^{-1} of the *P. pinaster* Ait. bark extract (Table 4). Taxifolin abundance in the *P. pinaster* Ait. bark extract is within the range reported for commercial *P. pinaster* Ait. bark extracts (1.20 mg g^{-1} extract-33.1 mg g^{-1} powder) [13,55]. However, the taxifolin abundance in the *P. pinea* L. bark polar extract was 4.2-fold lower than that reported for the ethyl fraction of HWE (28.7 mg g^{-1} fraction) [17]. It is noteworthy that both pine barks contained much higher taxifolin abundances than the respective Spanish species (up to 12.5-fold) [18]. Regarding the taxifolin-*O*-hexoside (13) content, the *P. pinea* L. bark demonstrated a much lower content than that of Pycnogenol® (16.5-fold) [13].

Protocatechuic acid (**2**) was found in the *P. pinaster* Ait. and *P. pinea* L. barks, representing 2.4% (w/w) and 2.1% (w/w) of the respective biomasses (Table 4). Considering its concentration, expressed as mg per g of extract, the studied polar extracts evidenced similar values to the published data for the commercial pine bark extracts (1.81 mg g^{-1} of Pycnogenol®-9.1 mg g^{-1} of *P. maritima* L. bark extract). Furthermore, the flavonol quercetin (**15**) was detected in both pine bark polar extracts, however in contents lower than the limit of quantification (LOQ) of the respective standard curve (Table 4).

Taking into account the antioxidant and antibacterial activities attributed to the quinic acid and phenolic compounds identified in the *P. pinaster* Ait. and *P. pinea* L. bark [62–65], the polar extracts were herein tested regarding their antioxidant activity, via reducing power, DPPH, and ABTS scavenging effects, as well as their antibacterial activities against *E. coli* and *S. aureus*.

3.3. Antioxidant Activity

P. pinaster Ait. and *P. pinea* L. bark polar extracts were evaluated regarding their antioxidant activities by three different methods, namely reducing power, DPPH and ABTS scavenging assays. Table 5 highlights the IC$_{50}$ values obtained for each extract.

Table 5. Antioxidant activity of *Pinus pinaster* Ait. and *Pinus pinea* L. bark polar extracts, through reducing power, DPPH, and ABTS scavenging effects.

| Extract/Standard | Reducing Power | DPPH Scavenging Effect | | ABTS Scavenging Effect | |
	IC_{50} ($\mu g\ mL^{-1}$)	IC_{50} ($\mu g\ mL^{-1}$)	IC_{50} (mg AAE g^{-1} of Dry Bark)	IC_{50} ($\mu g\ mL^{-1}$)	IC_{50} (mg TE g^{-1} of Dry Bark)
Pinus pinaster Ait. bark polar extract	22.53 ± 0.46 b	6.79 ± 0.48 b	125.48 ± 12.66	3.95 ± 0.05 a	255.24 ± 10.24
Pinus pinea L. bark polar extract	21.21 ± 1.04 b	6.46 ± 0.36 b	112.68 ± 9.04	3.89 ± 0.40 a	223.69 ± 10.35
Ascorbic acid	-	3.96 ± 0.18 a	-	3.55 ± 0.03 a	-
Trolox	28.82 ± 0.60 a	-	-	4.73 ± 0.07 b	-

The values represent the mean $\pm$ standard deviation. Means with different minor case letters (a, b) within the same column are significantly different (one-way ANOVA, followed by Tukey's HSD test, $p < 0.05$). Abbreviations: AAE, Ascorbic acid equivalents; IC_{50}, inhibitory concentration at 50%. TE, Trolox equivalents.

To the best of our current knowledge, the reducing power of the *P. pinaster* Ait. and *P. pinea* L. bark polar extracts was determined for the first time in the present study. The IC_{50} of these extracts ranged between 21.21 $\mu g\ mL^{-1}$ of *P. pinea* L. extract and 22.53 $\mu g\ mL^{-1}$ of *P. pinaster* Ait. extract. Both pine bark polar extracts were slightly more efficient than the trolox (up to 1.4-fold) ($p < 0.05$). Although the Ferric Reducing Antioxidant Power (FRAP) of phenolic-containing extracts, derived from the *P. pinaster* Ait. bark, was previously addressed, the mechanisms underlying this assay differ from the reducing power approach used here. To the best of our current knowledge, only pine bark extracts from *P. brutia* (80% (*v/v*) methanol extracts) were assessed through the reducing power, evidencing a IC_{50} range of 9.17–11.38 $\mu g\ mL^{-1}$ [43]. Thereby, both the *P. pinaster* Ait. and the *P. pinea* L. bark polar extracts were slightly less effective as reducing agents than those of the aforementioned *Pinus* species (2.3-fold).

The *P. pinea* L. and *P. pinaster* Ait. bark polar extracts demonstrated strong DPPH scavenging effects (Table 5), whose IC_{50} values were, respectively, 6.46 and 6.79 $\mu g\ mL^{-1}$, which are slightly lower than that of ascorbic acid (3.96 $\mu g\ mL^{-1}$). Considering the Antioxidant Activity Index (AAI), both pine bark polar extracts have shown much higher values (9.58 and 9.11 for the *P. pinea* L. and *P. pinaster* Ait. bark extracts, respectively) than those of the *P. pinaster* Ait. bark aqueous (AAI = 0.53) and hydroethanolic (30–90% (*v/v*) ethanol) extracts (0.53–1.06 AAI range). Actually, the AAI of the analysed *P. pinea* L. bark polar extract corresponds to up to 18.1-fold compared to the aqueous and 90% (*v/v*) ethanol extracts of another Portuguese *P. pinaster* Ait. bark [10].

Moreover, the pine extracts tested herein have shown to be more active in scavenging the DPPH free radicals than the *P. pinaster* Ait. bark aqueous, 50% (*v/v*) ethanol, and ethanol extracts (82.24–108.74 mg AAE g^{-1} dry bark), achieving up to 1.5-fold [37]. Even considering the bark extracts of other pine species, the *P. pinaster* Ait. and *P. pinea* L. bark polar extracts characterised in the present work have shown better DPPH scavenging action than the extracts obtained by Soxhlet acetone/water (7:3, *v/v*) and microwave-assisted extraction of *P. radiata* bark, with AAI ranges of 1.71–1.81 and 2.27–3.37, respectively [66].

The ABTS scavenging effect of *P. pinea* L. bark extract was close to that of the *P. pinaster* Ait. bark extract (Table 6), showing promising IC_{50} values of 3.89 and 3.95 $\mu g\ mL^{-1}$, respectively. Compared with the antioxidant standards, both extracts were as active as ascorbic acid in scavenging $ABTS^{\bullet+}$ but less active than Trolox in this assay ($p < 0.05$). Considering the IC_{50} values expressed as mmol Trolox equivalents (TE) per g of extract, the data represented here are concordant with those described in the literature for *P. pinaster* Ait. bark water (4.35 mmol TE g^{-1} of extract) [19] and alkaline extracts (4.70–5.38 mmol TE per g^{-1} of extract) [40], but much stronger than ethanol extracts (28.8-fold) [10]. Moreover, both the studied extracts revealed similar ABTS scavenging actions compared to those of 80% methanol extracts, derived from *P. brutia* bark (0.65–1.12 mmol TE g^{-1} dw) [43].

Table 6. Pearson correlation coefficients between the concentration of compounds and the IC_{50} values of reducing power, DPPH, and ABTS scavenging effects exhibited by *Pinus pinaster* Ait. and *Pinus pinea* L. bark polar extracts.

Concentration	Pearson Correlation Coefficients (r)		
	Reducing Power	DPPH Scavenging Effect	ABTS Scavenging Effect
Total phenolic content	−0.403	−0.146	0.228
Proanthocyanidin content	0.289	−0.146	0.625
Total identified compounds	−0.757	−0.257	−0.325
Total identified phenolic compounds	−0.642	−0.203	−0.211
Flavan-3-ols	−0.627	−0.226	−0.196
Flavanonols	−0.828 [1]	−0.071	−0.562
Quinic acid	−0.856 [1]	−0.305	−0.441
Protocatechuic acid	−0.363	0.221	0.226
B-type (epi)catechin dimer isomer 1	−0.732	−0.122	−0.308
B-type (epi)catechin dimer isomer 2	−0.502	−0.098	−0.108
Catechin + B-type (epi)catechin dimer isomer 3	−0.484	−0.119	−0.093
B-type (epi)catechin trimer isomer 2	0.013	−0.114	−0.392
A-type (epigallocatechin-(epi)catechin dimer	−0.770	−0.179	−0.212
B-type (epi)catechin trimer isomer 3	−0.721	−0.416	−0.361
B-type (epi)catechin trimer isomer 4	−0.579	−0.334	−0.071
B-type (epi)catechin dimer isomer 4	0.721	0.680	0.217
Taxifolin	−0.823 [1]	−0.029	−0.605

[1] The correlation is statistically significant at $p < 0.05$.

Pearson correlations were determined between the total phenolic content, the total proanthocyanidins, and the abundances of identified compounds present in the studied pine bark polar extracts and the IC_{50} values of the three antioxidant activity tests, as indicated in Table 6.

The highest Pearson correlation coefficient was observed between the quinic acid content and the IC_{50} of the pine bark polar extracts in the reducing power assay ($r = −0.856$). There is an increasing attention on the antioxidant activity of cyclic polyols, as quinic acid, in addition to other health-promoting effects, such as the treatment of diabetic diseases [62], paving the way for plentiful applications for this compound. Strong correlations have been also found between the flavanonols and taxifolin contents and the IC_{50} values of the reducing power (r values of −0.828 and −0.823, respectively). Although the remaining linear correlations presented in Table 6 did not have statistical significance ($p > 0.05$), the linear correlations between the taxifolin and flavanonols contents and the IC_{50} of extracts in the ABTS scavenging effect assay (r values of −0.605 and −0.562, respectively), as well as the one between the C-type procyanidin trimer isomer **3** abundance and the IC_{50} in the DPPH scavenging assay ($r = −0.416$), were moderate. Nevertheless, it is important to underline that other non-identified components in the analysed pine bark polar extracts may have interacted with the phenolic compounds.

3.4. Antibacterial Activity

The antibacterial activity of the *P. pinaster* Ait. and *P. pinea* L. bark polar extracts was assessed against two infectious agents of public health relevance, namely the Gram-positive *S. aureus* (ATCC® 6538) and the Gram-negative *E. coli* (ATCC® 25922), as illustrated in Figure 2.

The *P. pinaster* Ait. bark polar extracts exhibited bactericidal action in comparison with the solvent control, at a 6.25–25 mg mL^{-1} range (Figure 2A), causing 8.3-, 6.3-, and 4.0-log (CFU mL^{-1}) statistically significant decreases at 6.25, 12.5, and 25 mg mL^{-1}, respectively ($p < 0.05$) against the Gram-positive bacterium *S. aureus*. Furthermore, the *P. pinea* L. bark polar extracts showed a bactericidal effect at the same concentration range (Figure 2A), revealing 8.3-, 6.0-, and 4.6-log (CFU mL^{-1}) reductions in relation to the solvent control, at

6.25, 12.5, and 25 mg mL^{-1}, respectively ($p < 0.05$). On the one hand, *P. pinea* L. bark polar extracts even significantly suppressed *S. aureus* growth at 3.13 mg mL^{-1} compared to the solvent control ($p < 0.05$), via bactericidal action, resulting in a 3.15-log (CFU mL^{-1}) loss; on the other hand, *P. pinaster* Ait. bark polar extracts decreased *S. aureus* growth, at that concentration, with no statistical difference in comparison to the solvent control ($p > 0.05$), leading to a 2.10-log (CFU mL^{-1}) decrease.

Regarding the inhibitory effects of *Pinus* spp. bark polar extracts against the Gram-negative bacterium *E. coli* growth (Figure 2B), with the 24 h incubation with 12.5 mg mL^{-1}, no statistical difference ($p > 0.05$) was observed between the extract and the solvent control. Surprisingly, when tested at 25 mg mL^{-1}, both extracts acted as strong bactericidal agents, with a significant 8.26-log (CFU mL^{-1}) loss in relation to the solvent control ($p < 0.05$). The essay was repeated several times, corroborating the results reported here. In fact, a previous study has already pointed out that Gram-positive bacteria are more easily inactivated by pine extracts than Gram-negative ones [67]. To the best of our knowledge, the inhibitory effect of *P. pinea* L. bark polar extract against *E. coli* is revealed here for the first time.

Some studies have highlighted the antibacterial action of *P. pinaster* Ait. and *P. pinea* L. bark phenolic-rich extracts against *S. aureus* and *E. coli*. Recently, Ferreira-Santos et al. [10] have demonstrated the inhibitory action of 50 mg mL^{-1} aqueous and ethanolic extracts derived from *P. pinaster* Ait. bark against *S. aureus* (ATCC® 6538), under the 24 h treatment, but they have not prevented *E. coli* (ATCC® 9337) growth, whilst Douglas et al. [13] have also proved the suppressing capacity of Pycnogenol® against methicillin-resistant *S. aureus* viability. Conversely, Torras et al. [20] evidenced bacteriostatic activity of 20 μg mL^{-1} Pycnogenol® for *S. aureus* (ATCC® 6538) and *E. coli* (ATCC® 9337) but not bactericidal effect. All these research groups have suggested that phenolic compounds may be involved in the antibacterial action of pine bark polar extracts, with particular attention to catechin and proanthocyanidins [68,69]. Additionally, the hypothesis of complexation between phenolic compounds and bacterial proteins, through hydrogen bond and hydrophobic connections [20] and/or synergic effects between phenolic compounds and other extract components, should be posed. Furthermore, quinic acid present in the studied pine bark polar extracts may also have an important role in their antibacterial effect; Bai et al. [70] have recently demonstrated the antibacterial effect of quinic acid against ten foodborne pathogens, including *S. aureus* ATCC 6538 (minimum inhibitory concentration of 2.5 mg mL^{-1}), with an ability to damage the normal function of the *S. aureus* cell membrane. This compound has also shown an inhibitory effect against *E. coli* ATCC® 11229 growth (minimum inhibitory concentration of 5 mg mL^{-1}) [70].

Considering the promising data revealed in the present study, polar fractions from Portuguese *P. pinaster* and *P. pinea* barks exhibit interesting antioxidant and antibacterial actions, which may be ascribed to quinic acid and phenolic compounds. Therefore, these forest by-products can be regarded as renewable raw materials of polar added-value bioactive compounds for food and nutraceutical and biomedical applications, such as skin care, in addition to the current energy generation, in accordance with the biorefinery concept.

4. Conclusions

The present study highlights promising insights on the chemical composition and related antioxidant and antibacterial activities of *P. pinaster* Ait. and *P. pinea* L. bark polar extracts. Indeed, the *P. pinea* L. bark polar fraction has demonstrated higher abundances of total phenolic and proanthocyanidins, as well as of quinic acid and phenolic compounds identified by UHPLC-DAD-MS^n analysis, in comparison to that of *P. pinaster* Ait. Moreover, quinic acid (**1**) and three phenolic compounds, namely an isomer of (epi)gallocatechin-(epi)catechin dimer (**9**), protocatechuic acid (**2**), and quercetin (**15**), have been described herein, for the first time, as *P. pinea* L. bark components. Interestingly, both studied pine bark extracts have exhibited similarly effective reducing power and scavenging effects against DPPH and ABTS free radicals, being comparable to Trolox and ascorbic acid. Considering the current emergence in searching for alternative antibacterial agents, *P. pinaster* Ait. and

P. pinea L. bark polar extracts have revealed bactericidal effects, at the 3.13–25 mg mL^{-1} concentration range and at 25 mg mL^{-1}, against *S. aureus* and *E. coli* growth, respectively. Synergisms between quinic acid and the phenolic compounds, or even with the components of the other extracts, may have promoted the bioactivities of the pine bark polar extracts. Although there are several commercial *P. pinaster* Ait. bark extracts with nutraceutical usage, this knowledge can provide prospective innovative food and cosmetic applications for the studied agroforest by-products, especially with regard to the *P. pinea* L. bark. Nevertheless, the use of alternative solvents, such as natural deep eutectic solvents, for the extraction of quinic acid and phenolic compounds from *P. pinaster* Ait. and *P. pinea* L. barks is a further important step forward for their sustainable exploitation.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/app12020784/s1, Figure S1: mass fragmentation of (epi)gallocatechin-(2→O7, 4→8)-(epi) catechin in the negative ionisation mode, Table S1: *S. aureus* counts after application of *Pinus pinaster* Ait. and *Pinus pinea* L. bark polar extracts, Table S2: *E. coli* counts after application of *Pinus pinaster* Ait. and *Pinus pinea* L. bark polar extracts.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. UN. Transforming Our World: The 2030 Agenda for Sustainable Development. Available online: https://sdgs.un.org/2030agenda (accessed on 30 June 2021).
2. FAO; UNEP. The State of the World's Forests 2020—Forests, Biodiversity and People. Available online: https://doi.org/10.4060/ca8642en (accessed on 30 June 2021).
3. ICNF. IFN 6—6° Inventário Florestal Nacional. Available online: https://www.agroportal.pt/6o-inventario-florestal-nacional/ (accessed on 30 June 2021).
4. Viñas, R.A.; Caudullo, G.; Oliveira, S.; de Rigo, D. Pinus pinea in Europe: Distribution, habitat, usage and threats. In *European Atlas of Forest Tree Species*; San-Miguel-Ayanz, J., de Rigo, D., Caudullo, G., Houston Durrant, T., Mauri, A., Eds.; Publications Office of the European Union: Luxembourg, 2016; p. 130.
5. Centro PINUS. A Fileira Do Pinho Em 2019—Indicadores Da Fileira Do Pinho. Available online: https://www.centropinus.org/files/upload/indicadores/indicadores-centro-pinus-2020-1.pdf (accessed on 30 June 2021).
6. Nergiz, C.; Dönmez, İ. Chemical Composition and Nutritive Value of *Pinus pinea* L. Seeds. *Food Chem.* **2004**, *86*, 365–368. [CrossRef]
7. Mármol, I.; Quero, J.; Jiménez-Moreno, N.; Rodríguez-Yoldi, M.J.; Ancín-Azpilicueta, C. A Systematic Review of the Potential Uses of Pine Bark in Food Industry and Health Care. *Trends Food Sci. Technol.* **2019**, *88*, 558–566. [CrossRef]
8. Rohdewald, P. A Review of the French Maritime Pine Bark Extract (Pycnogenol), a Herbal Medication with a Diverse Clinical Pharmacology. *Int. J. Clin. Pharmacol. Ther.* **2002**, *40*, 158–168. [CrossRef]
9. Ferreira-Santos, P.; Zanuso, E.; Genisheva, Z.; Rocha, C.M.R.; Teixeira, J.A. Green and Sustainable Valorization of Bioactive Phenolic Compounds from *Pinus* By-Products. *Molecules* **2020**, *25*, 2931. [CrossRef]
10. Ferreira-Santos, P.; Genisheva, Z.; Botelho, C.; Santos, J.; Ramos, C.; Teixeira, J.A.; Rocha, C.M.R. Unravelling the Biological Potential of *Pinus pinaster* Bark Extracts. *Antioxidants* **2020**, *9*, 334. [CrossRef]
11. Venditti, A.; Maggi, F.; Saab, A.M.; Bramucci, M.; Quassinti, L.; Petrelli, D.; Vitali, L.A.; Lupidi, G.; el Samrani, A.; Borgatti, M.; et al. Antiproliferative, Antimicrobial and Antioxidant Properties of *Cedrus Libani* and *Pinus pinea* Wood Oils and Juniperus Excelsa Berry Oil. *Plant Biosyst.* **2020**, 1–12. [CrossRef]

12. Fekih, N.; Allali, H.; Merghache, S.; Bouchentouf, S.; Costa, J. In Vitro Assessment of Two Species of the Genus *Pinus* Growing in Algeria for Their Antimicrobial and Antioxidant Activity. *Agric. Conspec. Sci.* **2019**, *84*, 103–114.

13. Douglas, T.E.L.; Lopez-Heredia, M.A.; Pulczynska, A.; Łapa, A.; Pietryga, K.; Schaubroeck, D.; Santos, S.; Pais, A.; Brackman, G.; de Schamphelaere, K.; et al. Phenolic Plant Extract Enrichment of Enzymatically Mineralized Hydrogels. *Eng. Biomater.* **2019**, *149*, 2–9.

14. Hameş-Kocabaş, E.E.; Yeşil-Çeliktaş, Ö.; İşleten, M.; Vardar-Sukan, F. Antimicrobial Activity of Pine Bark Extract and Assessment of Potential Application in Cooked Red Meat. *Gida* **2008**, *33*, 123–127.

15. Moreirinha, C.; Vilela, C.; Silva, N.H.C.S.; Pinto, R.J.B.; Almeida, A.; Rocha, M.A.M.; Coelho, E.; Coimbra, M.A.; Silvestre, A.J.D.; Freire, C.S.R. Antioxidant and Antimicrobial Films Based on Brewers Spent Grain Arabinoxylans, Nanocellulose and Feruloylated Compounds for Active Packaging. *Food Hydrocoll.* **2020**, *108*, 105836. [CrossRef]

16. Morais, E.S.; Silva, N.H.C.S.; Sintra, T.E.; Santos, S.A.O.; Neves, B.M.; Almeida, I.F.; Costa, P.C.; Correia-Sá, I.; Ventura, S.P.M.; Silvestre, A.J.D.; et al. Anti-Inflammatory and Antioxidant Nanostructured Cellulose Membranes Loaded with Phenolic-Based Ionic Liquids for Cutaneous Application. *Carbohydr. Polym.* **2019**, *206*, 187–197. [CrossRef]

17. Yesil-Celiktas, O.; Ganzera, M.; Akgun, I.; Sevimli, C.; Korkmaz, K.S.; Bedir, E. Determination of Polyphenolic Constituents and Biological Activities of Bark Extracts from Different *Pinus* Species. *J. Sci. Food Agric.* **2009**, *89*, 1339–1345. [CrossRef]

18. Gascón, S.; Jiménez-Moreno, N.; Jiménez, S.; Quero, J.; Rodríguez-Yoldi, M.J.; Ancín-Azpilicueta, C. Nutraceutical Composition of Three Pine Bark Extracts and Their Antiproliferative Effect on Caco-2 Cells. *J. Funct.* **2018**, *48*, 420–429. [CrossRef]

19. Touriño, S.; Selga, A.; Jiménez, A.; Juliá, L.; Lozano, C.; Lizárraga, D.; Cascante, M.; Torres, J.L. Procyanidin Fractions from Pine (*Pinus pinaster*) Bark: Radical Scavenging Power in Solution, Antioxidant Activity in Emulsion, and Antiproliferative Effect in Melanoma Cells. *J. Agric. Food Chem.* **2005**, *53*, 4728–4735. [CrossRef] [PubMed]

20. Torras, M.A.C.; Faura, C.A.; Schönlau, F.; Rohdewald, P. Antimicrobial Activity of Pycnogenol®. *Phytother. Res.* **2005**, *19*, 647–648. [CrossRef]

21. Ramos, P.A.B.; Moreirinha, C.; Silva, S.; Costa, E.M.; Veiga, M.; Coscueta, E.; Santos, S.A.O.; Almeida, A.; Pintado, M.M.; Freire, C.S.R.; et al. The Health-Promoting Potential of *Salix* spp. Bark Polar Extracts: Key Insights on Phenolic Composition and in Vitro Bioactivity and Biocompatibility. *Antioxidants* **2019**, *8*, 609. [CrossRef]

22. Sousa, J.L.C.; Ramos, P.A.B.; Freire, C.S.R.; Silva, A.M.S.; Silvestre, A.J.D. Chemical Composition of Lipophilic Bark Extracts from *Pinus pinaster* and *Pinus pinea* Cultivated in Portugal. *Appl. Sci.* **2018**, *8*, 2575. [CrossRef]

23. Santos, S.A.O.; Vilela, C.; Domingues, R.M.A.; Oliveira, C.S.D.; Villaverde, J.J.; Freire, C.S.R.; Neto, C.P.; Silvestre, A.J.D. Secondary Metabolites from *Eucalyptus Grandis* Wood Cultivated in Portugal, Brazil and South Africa. *Ind. Crops Prod.* **2017**, *95*, 357–364. [CrossRef]

24. Vogel, M.; Schmitz, R.P.H.; Hagel, S.; Pletz, M.W.; Gagelmann, N.; Scherag, A.; Schlattmann, P.; Brunkhorst, F.M. Infections Disease Consultation for *Staphylococcus aureus* Bacteremia—A Systematic Review and Meta-Analysis. *J. Infect.* **2016**, *72*, 19–28. [CrossRef]

25. Vila, J.; Sáez-López, E.; Johnson, J.R.; Römling, U.; Dobrindt, U.; Cantón, R.; Giske, C.G.; Naas, T.; Carattoli, A.; Martínez-Medina, M.; et al. *Escherichia coli*: An Old Friend with New Tidings. *FEMS Microbiol. Rev.* **2016**, *40*, 437–463. [CrossRef]

26. Singleton, V.L.; Rossi, J.A. Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144–158.

27. Neto, R.T.; Santos, S.A.O.; Oliveira, J.; Silvestre, A.J.D. Tuning of Proanthocyanidin Extract's Composition through Quaternary Eutectic Solvents Extraction. *Antioxidants* **2020**, *9*, 1124. [CrossRef]

28. Oyaizu, M. Studies on Products of Browning Reaction. *Jpn. J. Nutr. Diet.* **1986**, *44*, 307–315. [CrossRef]

29. Berker, K.I.; Güçlü, K.; Tor, İ.; Apak, R. Comparative Evaluation of Fe(III) Reducing Power-Based Antioxidant Capacity Assays in the Presence of Phenanthroline, Batho-Phenanthroline, Tripyridyltriazine (FRAP), and Ferricyanide Reagents. *Talanta* **2007**, *72*, 1157–1165. [CrossRef]

30. Pais, A.C.S.; Pinto, C.A.; Ramos, P.A.B.; Pinto, R.J.B.; Rosa, D.; Duarte, M.F.; Abreu, M.H.; Rocha, S.M.; Saraiva, J.A.; Silvestre, A.J.D.; et al. High pressure extraction of bioactive diterpenes from the macroalgae *Bifurcaria bifurcata*: An efficient and environmentally friendly approach. *RSC Adv.* **2019**, *9*, 39893–39903. [CrossRef]

31. Scherer, R.; Godoy, H.T. Antioxidant Activity Index (AAI) by the 2,2-Diphenyl-1-Picrylhydrazyl Method. *Food Chem.* **2009**, *112*, 654–658. [CrossRef]

32. Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant Activity Applying an Improved ABTS Radical Cation Decolorization Assay. *Free Radic. Biol. Med.* **1999**, *26*, 1231–1237. [CrossRef]

33. Ramos, P.A.B.; Santos, S.A.O.; Guerra, Â.R.; Guerreiro, O.; Freire, C.S.R.; Rocha, S.M.; Duarte, M.F.; Silvestre, A.J.D. Phenolic Composition and Antioxidant Activity of Different Morphological Parts of *Cynara cardunculus* L. var. Altilis (DC). *Ind. Crops Prod.* **2014**, *61*, 460–471. [CrossRef]

34. Nunes, E.; Quilhó, T.; Pereira, H. Anatomy and Chemical Composition of *Pinus pinaster* Bark. *IAWA J.* **1996**, *17*, 141–150. [CrossRef]

35. Fradinho, D.M.; Neto, C.P.; Evtuguin, D.; Jorge, F.C.; Irle, M.A.; Gil, M.H.; de Jesus, J.P. Chemical Characterisation of Bark and of Alkaline Bark Extracts from Maritime Pine Grown in Portugal. *Ind. Crops Prod.* **2002**, *16*, 23–32. [CrossRef]

36. Nunes, E.; Quilhó, T.; Pereira, H. Anatomy and Chemical Composition of *Pinus pinea* L. Bark. *Ann. For. Sci.* **1999**, *56*, 479–484. [CrossRef]

37. Vieito, C.; Pires, P.; Fernandes, É.; Velho, M. Chemical Characterization of Pine Bark (*Pinus pinaster* Aiton Subsp. *Atlantica*), Antioxidant Properties and Phenolic Profile of Its Extracts. *Millenium* **2019**, *8*, 79–87. [CrossRef]

38. Seabra, I.J.; Dias, A.M.A.; Braga, M.E.M.; de Sousa, H.C. High Pressure Solvent Extraction of Maritime Pine Bark: Study of Fractionation, Solvent Flow Rate and Solvent Composition. *J. Supercrit. Fluids* **2012**, *62*, 135–148. [CrossRef]

39. Dudonné, S.; Vitrac, X.; Coutière, P.; Woillez, M.; Mérillon, J.-M. Comparative Study of Antioxidant Properties and Total Phenolic Content of 30 Plant Extracts of Industrial Interest Using DPPH, ABTS, FRAP, SOD, and ORAC Assays. *J. Agric. Food Chem.* **2009**, *57*, 1768–1774. [CrossRef] [PubMed]

40. Galiñanes, C.; Freire, M.S.; González-Álvarez, J. Antioxidant Activity of Phenolic Extracts from Chestnut Fruit and Forest Industries Residues. *Eur. J. Wood Wood Prod.* **2015**, *73*, 651–659. [CrossRef]

41. Ustun, O.; Senol, F.S.; Kurkcuoglu, M.; Orhan, I.E.; Kartal, M.; Baser, K.H.C. Investigation on Chemical Composition, Anti-cholinesterase and Antioxidant Activities of Extracts and Essential Oils of Turkish *Pinus* Species and Pycnogenol. *Ind. Crops Prod.* **2012**, *38*, 115–123. [CrossRef]

42. Ku, C.S.; Jang, J.P.; Mun, S.P. Exploitation of Polyphenol-Rich Pine Barks for Potent Antioxidant Activity. *J. Wood Sci.* **2007**, *53*, 524–528. [CrossRef]

43. Cretu, E.; Karonen, M.; Salminen, J.-P.; Mircea, C.; Trifan, A.; Charalambous, C.; Constantinou, A.I.; Miron, A. In Vitro Study on the Antioxidant Activity of a Polyphenol-Rich Extract from *Pinus Brutia* Bark and Its Fractions. *J. Med. Food* **2013**, *16*, 984–991. [CrossRef]

44. Chupin, L.; Motillon, C.; Bouhtoury, F.C.-E.; Pizzi, A.; Charrier, B. Characterisation of Maritime Pine (*Pinus pinaster*) Bark Tannins Extracted under Different Conditions by Spectroscopic Methods, FTIR and HPLC. *Ind. Crops Prod.* **2013**, *49*, 897–903. [CrossRef]

45. Scalbert, A.; Monties, B.; Janin, G. Tannins in Wood: Comparison of Different Estimation Methods. *J. Agric. Food Chem.* **1989**, *37*, 1324–1329. [CrossRef]

46. Simões, R.; Pimentel, C.; Ferreira-Dias, S.; Miranda, I.; Pereira, H. Phytochemical characterization of phloem in maritime pine and stone pine in three sites in Portugal. *Heliyon* **2021**, *7*, e06718. [CrossRef]

47. Santos, S.A.O.; Vilela, C.; Camacho, J.F.; Cordeiro, N.; Gouveia, M.; Freire, C.S.R.; Silvestre, A.J.D. Profiling of Lipophilic and Phenolic Phytochemicals of Four Cultivars from Cherimoya (*Annona cherimola* Mill.). *Food Chem.* **2016**, *211*, 845–852. [CrossRef]

48. Teixeira, N.; Azevedo, J.; Mateus, N.; de Freitas, V. Proanthocyanidin Screening by LC–ESI-MS of Portuguese Red Wines Made with Teinturier Grapes. *Food Chem.* **2016**, *190*, 300–307. [CrossRef]

49. Ramos, P.A.B.; Moreirinha, C.; Santos, S.A.O.; Almeida, A.; Freire, C.S.R.; Silva, A.M.S.; Silvestre, A.J.D. Valorisation of Bark Lipophilic Fractions from Three Portuguese *Salix* Species: A Systematic Study of the Chemical Composition and Inhibitory Activity on *Escherichia coli*. *Ind. Crops Prod.* **2019**, *132*, 245–252. [CrossRef]

50. Hamed, A.I.; Al-Ayed, A.S.; Moldoch, J.; Piacente, S.; Oleszek, W.; Stochmal, A. Profiles Analysis of Proanthocyanidins in the Argun Nut (*Medemia argun*—An Ancient Egyptian Palm) by LC–ESI–MS/MS. *J. Mass Spectrom.* **2014**, *49*, 306–315. [CrossRef] [PubMed]

51. Svensson, L.; Sekwati-Monang, B.; Lutz, D.L.; Schieber, A.; Gänzle, M.G. Phenolic Acids and Flavonoids in Nonfermented and Fermented Red Sorghum (*Sorghum bicolor* (L.) Moench). *J. Agric. Food Chem.* **2010**, *58*, 9214–9220. [CrossRef] [PubMed]

52. Kang, J.; Price, W.E.; Ashton, J.; Tapsell, L.C.; Johnson, S. Identification and Characterization of Phenolic Compounds in Hydromethanolic Extracts of Sorghum Wholegrains by LC-ESI-MSn. *Food Chem.* **2016**, *211*, 215–226. [CrossRef] [PubMed]

53. Touati, R.; Santos, S.A.O.; Rocha, S.M.; Belhamel, K.; Silvestre, A.J.D. Phenolic Composition and Biological Prospecting of Grains and Stems of *Retama sphaerocarpa*. *Ind. Crops Prod.* **2017**, *95*, 244–255. [CrossRef]

54. Santos, S.A.O.; Vilela, C.; Freire, C.S.R.; Neto, C.P.; Silvestre, A.J.D. Ultra-High Performance Liquid Chromatography Coupled to Mass Spectrometry Applied to the Identification of Valuable Phenolic Compounds from *Eucalyptus* Wood. *J. Chromatogr. B* **2013**, *938*, 65–74. [CrossRef]

55. Romani, A.; Ieri, F.; Turchetti, B.; Mulinacci, N.; Vincieri, F.F.; Buzzini, P. Analysis of Condensed and Hydrolysable Tannins from Commercial Plant Extracts. *J. Pharm. Biomed.* **2006**, *41*, 415–420. [CrossRef] [PubMed]

56. de la Luz Cadiz-Gurrea, M.; Fernandez-Arroyo, S.; Segura-Carretero, A. Pine Bark and Green Tea Concentrated Extracts: Antioxidant Activity and Comprehensive Characterization of Bioactive Compounds by HPLC-ESI-QTOF-MS. *Int. J. Mol. Sci.* **2014**, *15*, 20382–20402. [CrossRef]

57. Lin, L.-Z.; Sun, J.; Chen, P.; Monagas, M.J.; Harnly, J.M. UHPLC-PDA-ESI/HRMSn Profiling Method To Identify and Quantify Oligomeric Proanthocyanidins in Plant Products. *J. Agric. Food Chem.* **2014**, *62*, 9387–9400. [CrossRef]

58. Weber, H.A.; Hodges, A.E.; Guthrie, J.R.; O'Brien, B.M.; Robaugh, D.; Clark, A.P.; Harris, R.K.; Algaier, J.W.; Smith, C.S. Comparison of Proanthocyanidins in Commercial Antioxidants: Grape Seed and Pine Bark Extracts. *J. Agric. Food Chem.* **2007**, *55*, 148–156. [CrossRef]

59. Esatbeyoglu, T.; Wray, V.; Winterhalter, P. Identification of Two Novel Prodelphinidin A-Type Dimers from Roasted Hazelnut Skins (*Corylus avellana* L.). *J. Agric. Food Chem.* **2013**, *61*, 12640–12645. [CrossRef]

60. Ye, M.; Yang, W.-Z.; Liu, K.-D.; Qiao, X.; Li, B.-J.; Cheng, J.; Feng, J.; Guo, D.-A.; Zhao, Y.-Y. Characterization of Flavonoids in *Millettia nitida* var. *Hirsutissima* by HPLC/DAD/ESI-MSn. *J. Pharm. Anal.* **2012**, *2*, 35–42. [CrossRef]

61. Benlarbi, R.; Fechtal, M.; Meziane, D.; Istambouli, A. Carboxylic Acids of Moroccan *Pinus pinaster* Bark Extract. *Holzforschung* **1999**, *53*, 580–584. [CrossRef]

62. Karaman, M.; Tesanovic, K.; Gorjanovic, S.; Pastor, F.T.; Simonovic, M.; Glumac, M.; Pejin, B. Polarography as a Technique of Choice for the Evaluation of Total Antioxidant Activity: The Case Study of Selected *Coprinus comatus* Extracts and Quinic Acid, Their Antidiabetic Ingredient. *Nat. Prod. Res.* **2021**, *35*, 1711–1716. [CrossRef]

63. Jerez, M.; Touriño, S.; Sineiro, J.; Torres, J.L.; Núñez, M.J. Procyanidins from Pine Bark: Relationships between Structure, Composition and Antiradical Activity. *Food Chem.* **2007**, *104*, 518–527. [CrossRef]

64. Artem'eva, O.A.; Pereselkova, D.A.; Fomichev, Y.P. Dihydroquercetin, the Bioactive Substance, to Be Used against Pathogenic Microorganisms as an Alternative to Antibiotics. *Selskokhoziaistvennaia Biol.* **2015**, *50*, 513–519. [CrossRef]

65. Cueva, C.; Moreno-Arribas, M.V.; Martín-Álvarez, P.J.; Bills, G.; Vicente, M.F.; Basilio, A.; Rivas, C.L.; Requena, T.; Rodríguez, J.M.; Bartolomé, B. Antimicrobial Activity of Phenolic Acids against Commensal, Probiotic and Pathogenic Bacteria. *Res. Microbiol.* **2010**, *161*, 372–382. [CrossRef]

66. Aspé, E.; Fernández, K. The Effect of Different Extraction Techniques on Extraction Yield, Total Phenolic, and Anti-Radical Capacity of Extracts from *Pinus radiata* Bark. *Ind. Crops Prod.* **2011**, *34*, 838–844. [CrossRef]

67. Nisca, A.; Ștefănescu, R.; Stegăruș, D.I.; Mare, A.D.; Farczadi, L.; Tanase, C. Comparative study regarding the chemical composition and biological activity of Pine (*Pinus nigra* and *P. sylvestris*) bark extracts. *Antioxidants* **2021**, *10*, 327. [CrossRef]

68. Ajiboye, T.O.; Aliyu, M.; Isiaka, I.; Haliru, F.Z.; Ibitoye, O.B.; Uwazie, J.N.; Muritala, H.F.; Bello, S.A.; Yusuf, I.I.; Mohammed, A.O. Contribution of Reactive Oxygen Species to (+)-Catechin-Mediated Bacterial Lethality. *Chem. Biol. Interact.* **2016**, *258*, 276–287. [CrossRef]

69. Mayer, R.; Stecher, G.; Wuerzner, R.; Silva, R.C.; Sultana, T.; Trojer, L.; Feuerstein, I.; Krieg, C.; Abel, G.; Popp, M.; et al. Proanthocyanidins: Target Compounds as Antibacterial Agents. *J. Agric. Food Chem.* **2008**, *56*, 6959–6966. [CrossRef]

70. Bai, J.; Wu, Y.; Wang, X.; Liu, X.; Zhong, K.; Huang, Y.; Chen, Y.; Gao, H. In Vitro and in Vivo Characterization of the Antibacterial Activity and Membrane Damage Mechanism of Quinic Acid against *Staphylococcus aureus*. *J. Food Saf.* **2018**, *38*, e12416. [CrossRef]