European Food Research and Technology
https://doi.org/10.1007/s00217-021-03796-w

ORIGINAL PAPER

In vitro antioxidant and antibacterial activities with polyphenolic profiling of wild cherry, the European larch and sweet chestnut tree bark

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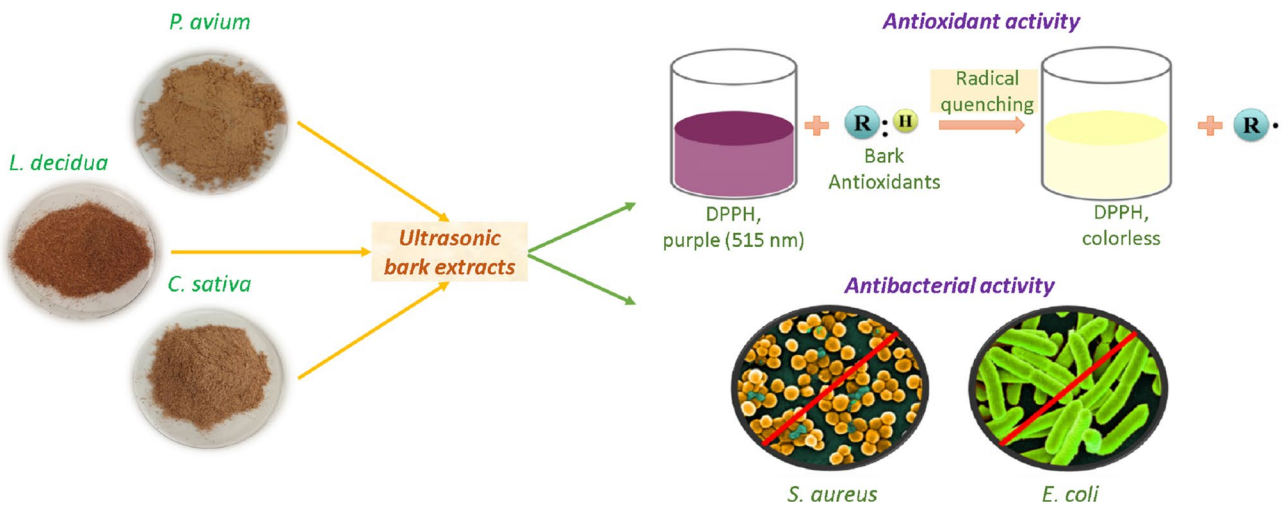
Received: 8 March 2021 / Revised: 31 May 2021 / Accepted: 5 June 2021

Abstract

This study is a comparative investigation of antioxidant and antibacterial properties of tree bark extracts of three common European species, *Prunus avium* L., *Larix decidua* Mill. and C*astanea sativa* Mill. The bioactive compounds present in the bark were recovered in 80% aqueous ethanol using ultrasound as the green extraction method. The antioxidant potential of the extracts was assessed with multiple biochemical assays: total phenol content (TPC) expressed in gallic acid equivalent (GAE), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) expressed in trolox equivalent (TE), and ferric reducing ability of plasma (FRAP) expressed in ascorbic acid equivalent (AAE). Sweet chestnut bark extract showed the highest antioxidant activity with TPC of 174.25 ± 16.95 mg GAE/g dry weight, DPPH (IC_{50}) of 2.69 ± 0.03 µg/mL, ABTS of 739.65 ± 24.41 mg TE/g dry weight and FRAP of 207.49 ± 3.62 mg AAE/g dry weight. The antibacterial activity of the extracts was evaluated by disk diffusion test, minimal inhibitory concentration (MIC) assay and bacterial growth curves. Sweet chestnut bark extract gave IC_{50} values of 0.25 mg/mL and 1.00 mg/mL against *E. coli* and *S. aureus*, respectively. The polyphenolic profiling of the bark extracts was performed to identify the major compounds responsible for the bioactivities using high-performance liquid chromatography/tandem mass spectrometry (HPLC–MS/MS). The bark extracts were rich in natural antioxidants, thus holding tremendous potential for use as natural additives in food industry.

Graphic abstract



Keywords Antioxidant capacity · Antibacterial activity · Bark polyphenols · Food bioactives · Liquid chromatography · Ultrasonic extraction

Extended author information available on the last page of the article

Published online: 12 June 2021

Introduction

In Europe, forests cover 215 million ha, i.e., one-third of the land area, and the forest-sector constitutes about 0.8% of its GDP giving a livelihood to over 3 million people [1]. Wood is the primary forest resource spanning over 150 million ha, which is used in furniture, construction, paper-making, and as a source of renewable energy. The processing of wood generates tons of bark that is mostly ineffectively dumped as landfill or incinerated. Bark protects the tree against external or functional damage, and prevents water loss. Recent studies have identified bark as a source of bioactive phenolic compounds for its valorization [2, 3].

The polyphenols in plants are involved in their internal defense, regulation of growth, hormonal activity, pH and metabolism [4]. Furthermore, they exhibit numerous physiological properties such as antioxidant, antimicrobial, anti-inflammatory, anti-carcinogenic, and therefore, have tremendous potential to be used in making dietary supplements, functional food additives, pharmaceutical products and cosmetics [5]. The extraction process is made challenging due to the different chemical structures of the compounds, their ability to degrade or hydrolyze and process selectivity towards the target analyte. The choice of extraction method and solvent depends on the plant matrix properties, physicochemical properties of analyte, analyte-solvent compatibility, process efficiency in terms of yield and purity, environmental impact, as well as the overall cost [3]. Ultrasound has proven to be very effective to enhance the yield and rate of mass transfer of extraction processes [6]. The principle of ultrasonic extraction is based on cavitation, i.e., the formation, growth and violent collapse of bubbles in the solvent medium giving rise to local hotspots. The resulting physical effects cause the fragmentation and erosion of the plant cells, increased solvent penetration (sonocapillary effect) and subsequent washing out of the cell contents (sonoporation) [7].

Limited literature is available on the extraction, characterization and biological effects of the bioactive compounds from tree bark. The bark extracts of *Quercus robur, Alnus glutinosa, Larix decidua* and *Picea abies* showed high free radical scavenging activity, elastase and collagenase inhibitory activities for use in the dermo-cosmetic industry [8]. Another study tested the antifungal activity of bark extracts of several European trees including Norway maple (*Acer platanoides* L.), which significantly inhibited the growth of *Fomitopsis pinicola* (brown rot fungi) and *Heterobasidion parviporum* (white rot fungi) [9]. In this study, three commercially relevant tree species viz., wild cherry (*Prunus avium* L.), the European larch (*Larix decidua* Mill.) and sweet chestnut (*Castanea sativa* Mill.), which are common in Hungarian forests were chosen. Earlier studies have shown that these species have exceptionally high antioxidant capacity in their bark as well as antimicrobial effects [10–12]. Moreover, in Europe, these trees are abundant and easily accessible (Fig. 1). The wild cherry is a fast growing and short-lived deciduous tree with edible sweet fruits and dense wood that is widely used in veneer production, cabinet-making and paneling [1]. Although the wild cherry fruits and other vegetal parts have been widely investigated [13, 14], only a few studies are available on its bark [8]. The European larch is a large deciduous, coniferous tree, typically adapted to continental alpine climates [1]. Its wood is durable due to high tannin content and is used in carpentry, outdoor and naval construction, as well as for pulping. Few recent works have examined the European larch bark for its active constituents and bioactivity [15, 16]. The sweet chestnut, a long-living deciduous variety adapted to warm-temperate climate, is a multi-purpose tree cultivated for its timber and nuts [1]. An earlier study has linked sweet chestnut bark with neuroprotective effects against oxidative stress [17].

The aim of the present work was to extract and identify bioactive compounds, especially polyphenols from these species using ultrasound followed by a comparative analysis of their bioactivities. The polyphenols were extracted in aqueous ethanol due to its low toxicity and ecological impact. The antioxidant properties of bark extracts were evaluated using Folin–Ciocâlteu total phenol content (TPC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay, and the ferric reducing ability of plasma (FRAP); while the antibacterial activities against *E. coli* and *S. aureus* were determined using disk diffusion test (DDT), minimal inhibitory concentration (MIC) assay and bacterial growth curves. The bioactive compounds were characterized by high-performance liquid chromatography (HPLC)–electrospray ionization (ESI)/tandem mass spectrometry (MS/MS) technique. The study provides a comprehensive investigation and comparative assessment of the polyphenolic constituents and their correlation with the bioactivities of the tree bark. It is expected to provide useful insights towards the development of natural additives for food.

Materials and methods

Materials

Ethanol and methanol were purchased from Molar Chemicals Ltd., Hungary. Folin–Ciocâlteu phenol reagent (2N), sodium carbonate, 2,2-diphenyl-1-picrylhydrazyl, 2,4,6-tri(2-pyridyl)-1,3,5-triazine, ascorbic acid, gallic acid, ferric chloride, acetic acid, hydrochloric acid and sodium acetate were obtained from Sigma-Aldrich, Hungary. 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), potassium persulfate, trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) and LC/MS grade acetonitrile were procured from

Merck, Germany. Dimethyl sulfoxide (DMSO) of 99.90% purity was obtained from Sigma-Aldrich, Germany; while Amoxicillin and Penicillin were purchased from Thermo Fisher Scientific, USA. All the chemicals were of analytical grade unless otherwise stated, and used without further purification. Deionized water was used for preparing the reagents and solutions, carrying out extractions and chromatographic analyses.

Standard strains of *Staphylococcus aureus* (CCM 4223) and *Escherichia coli* (CCM 3954) were obtained from the Czech collection of microorganisms (Brno, Czech Republic). The strains were cultivated over night at 37 °C on 5% Columbia blood agar (Sigma-Aldrich, Germany).

Sampling of tree bark

Whole bark samples were collected from the tree trunks of *Prunus avium* L., *Larix decidua* Mill. and C*astanea sativa* Mill. originating from the forests of TAEG (Tanulmányi Erdőgazdaság) Forestry Company, Sopron (Hungary) during December 2019. The samples were dried in air at room temperature for 3 days in the dark. The dried samples were subsequently ground, sieved and stored in plastic bags at −20 °C. The meshed fraction in the range of 0.2–0.63 mm was used for extraction. The moisture content of the samples was found to be 6–10% using an infrared moisture analyzer (Sartorius MA35).

Extraction of bioactive compounds assisted by ultrasound

The extraction was done using a horn sonicator (Tesla 150 WS) consisting of a titanium horn (18 mm in diameter) operating at an ultrasonic frequency of 20 kHz with a maximum power output of 150 W. Bark specimens were extracted in 80% aqueous ethanol for 15 min at full amplitude, and a solid to solvent ratio of 1:100 (g/mL) with 34.9% ultrasonic horn energy efficiency, based on our earlier work [18]. The filtered extracts were stored in amber-colored glass bottles under refrigeration at −20 °C for antioxidant and liquid chromatographic analyses. The extract solutions were evaporated to obtain dry powder for antibacterial testing.

Estimation of in vitro antioxidant potential

Determination of TPC

The TPC assay was run according to the procedure of Singleton and Rossi [19]. In a typical experiment, 500 µL of the extract and 2.5 mL of Folin–Ciocâlteu reagent were thoroughly mixed together, followed by the addition of 2 mL of Na_2CO_3 solution (0.7 M) after 1 min. The reaction mixture was incubated in a water bath at 50 °C for 5 min. The absorbance was measured at 760 nm against the blank solution on a UV–VIS spectrophotometer (Hitachi U-1500). The mean values of three replicates were expressed in mg equivalents of gallic acid/g dry weight of specimen (mg GAE/g dw).

Determination of DPPH scavenging activity

The DPPH radical scavenging assay was performed using the method of Sharma and Bhat with some modifications [20]. The assay involved dilution of 10 µL of extract with 2090 µL of unbuffered methanol and the subsequent addition of 900 µL of DPPH. It was incubated at ambient temperature away from light for 30 min and the drop in absorbance was measured at 515 nm. The calibration curve was plotted by measuring absorbance at different dilutions of a standard methanolic solution of DPPH (2×10^{-4} M). The mean values of three replicates were expressed as IC_{50} (50% inhibition concentration) in µg extractives/mL.

Determination of ABTS scavenging activity

The ABTS radical scavenging assay was done according to the protocol of Stratil et al. [21]. A solution of ABTS radical (7 mM) and potassium persulfate (12.5 mM) was prepared to have an absorbance of 0.70 ± 0.02 at 734 nm. For the assay, 1960 µL of the radical solution was mixed well with 40 µL of the extract followed by incubation at ambient temperature for 10 min. The absorbance was measured at 734 nm in dim light. The calibration curve was plotted using serial dilutions of a standard trolox solution (1 mM). The mean values of three replicates were expressed in mg equivalents of trolox/g dry weight of specimen (mg TE/g dw).

Determination of FRAP

The FRAP was performed in accordance with the procedure of Benzie and Strain [22]. In a typical run, 50 µL of the extract was mixed with 1500 µL of FRAP reagent, allowing the reaction to occur in the dark at room temperature. After 5 min, the absorbance was measured at 593 nm against the blank solution, while standard solutions of ascorbic acid were used for the calibration curve. The mean values of three replicates were expressed in mg equivalents of ascorbic acid/g dry weight of specimen (mg AAE/g dw).

Estimation of extractive content

To estimate the extractive content (yield), aliquots of the extracts were dried in an oven at 40 °C. The residual solids were weighed on a digital scale (Sartorius MSA225P) and the yield was expressed in mg extractives/mL extract units.

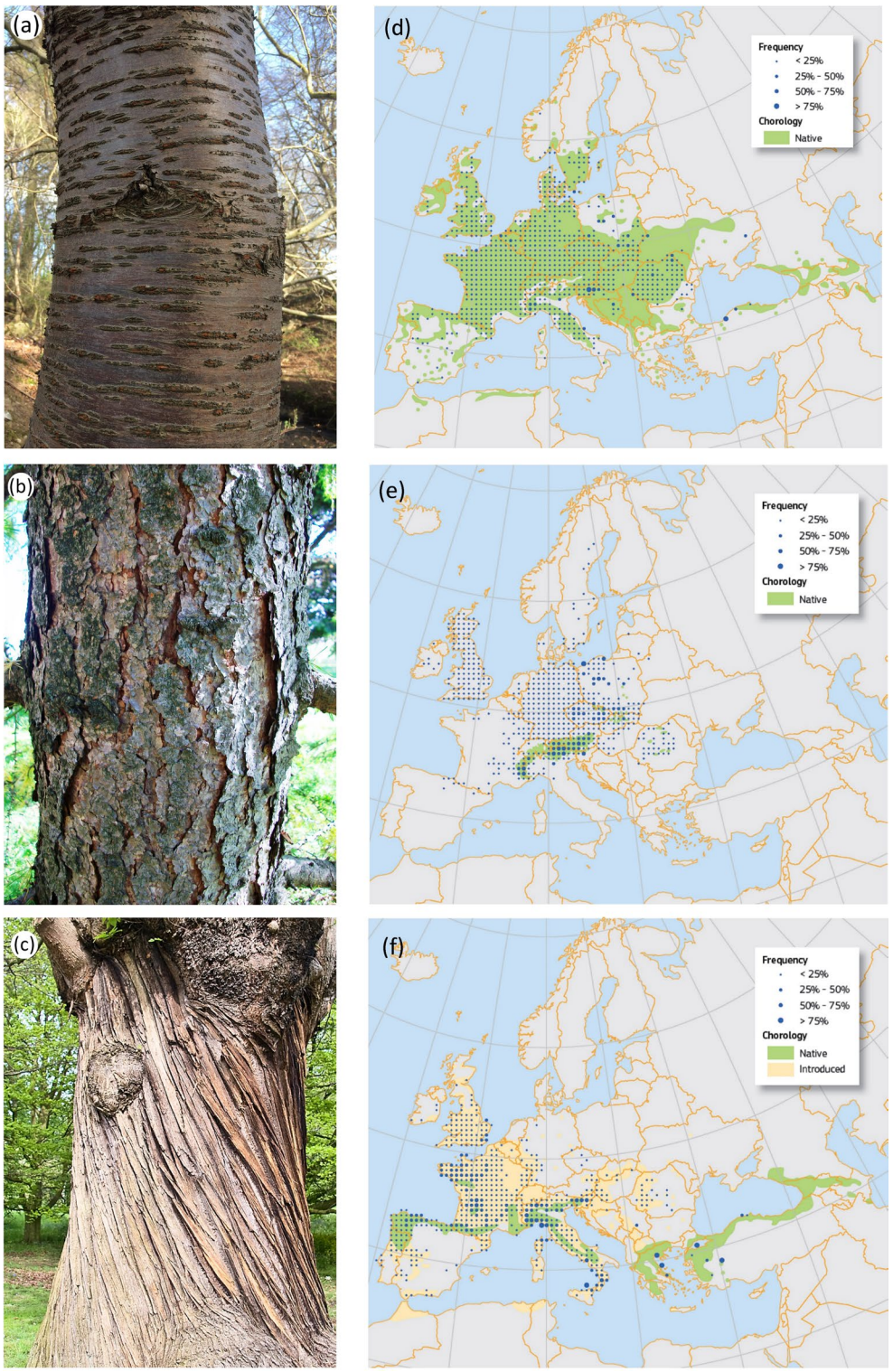


Fig. 1 **a** *Prunus avium* bark—smooth purplish-brown with horizontal fissuring (© Gus Routledge, www.flickr.com), **b** *Larix decidua* bark—corky, brownish gray with fissured plates (© Kate Field, www.flickr.com), **c** *Castanea sativa* bark—grayish brown with deep furrows (© Robert Silverwood, www.flickr.com); maps showing distribution and simplified chorology across Europe in (**d**), (**e**) and (**f**) of the respective species (© European Union 2016 [1])

The results were taken into account for the determination of DPPH IC_{50} values.

Estimation of in vitro antibacterial activity

Sample preparation

The dried extracts of wild cherry, the European larch, and sweet chestnut were dissolved in 1% DMSO to give a final concentration of 1 mg/mL. The prepared extracts were evaluated for their antibacterial activity against Gram-positive bacteria, *Staphylococcus aureus* (CCM 4223) and Gram-negative bacteria, *Escherichia coli* (CCM 3954). For reproducibility, the disk diffusion test and the growth curve assay were performed in duplicate, while the minimal inhibitory concentration assay was performed in tetraplicate.

Disk diffusion test (DDT)

The antibacterial activity of the extracts was determined by DDT performed according to the EUCAST 2019 guidelines [23]. Inoculum (10 µL) of each standard strain was prepared from a suspension adjusted to 0.5 McFarland density using deionized water. Amoxicillin and Penicillin were used as positive controls for Gram-negative and Gram-positive bacterial strains, respectively. The inoculated plates were incubated at 37 °C for 12 h.

Minimal inhibitory concentration (MIC) assay

The MIC assay was performed according to EUCAST 2020 guidelines in 96-well microtiter plates [24]. Extracts were diluted in Mueller Hinton Broth (Sigma-Aldrich, Germany) to achieve a two-fold dilution from 1.000 mg/mL to 0.016 mg/mL. Standard strain of 0.5 McFarland density was diluted 100× using deionized water to give cell density of 1–2 × 10^6 CFU/mL. Cultures were incubated at 37 °C on plate shaker at 120 rpm. The absorbance at 620 nm was monitored at time zero, and then after 15 h of incubation. The results were expressed as IC_{50} values (mg/mL), which is the concentration of extracts that caused a 50% inhibition of the tested bacteria.

Bacterial growth curves

Extract concentrations from 1.000 mg/mL to 0.031 mg/mL were obtained by the broth dilution method. The procedure for sample preparation and concentration range were same as for the MIC assay. The growth curve of bacteria in the presence of the extract was measured by a Bioscreen C MBR (Dynex, Czech Republic) in 100-well microtiter plates. The absorbance at 620 nm was monitored at time zero, and then at 30 min intervals for 24 h, while the culture was incubated at 37 °C.

The HPLC–PDA–ESI–MS/MS characterization of the extracts

Separation of bark extract constituents was achieved using a Shimadzu LC-20 type high-performance liquid chromatograph coupled with a Shimadzu SPD-M20A photodiode array detector (PDA) (Shimadzu Corporation, Kyoto, Japan) and an AB Sciex 3200 QTrap triple quadrupole/linear ion trap mass spectrometer (MS) (AB Sciex, Framingham, USA). The stationary phase used a Phenomenex Synergy Fusion-RP 80A, 250 mm × 4.6 mm, 4 µm column with a Phenomenex SecurityGuard ULTRA LC type guard column (Phenomenex Inc., Torrance, USA) at 40 °C. The injection volume was 8 µL. Gradient elution was run using A (H_2O + 0.1% HCOOH) and B (CH_3CN + 0.1% HCOOH) solvents with 1.2 mL/min flow-rate with the following schedule: 3% B (0–4 min), 6% B (10 min), 20% B (34 min), 57% B (73 min), 100% B (90–98 min), 3% B (99–106 min). The PDA signal (250–380 nm) was recorded to monitor the separation of peaks. A negative electrospray ionization mode was set for the MS detector by allowing 0.6 mL/min flow to enter the MS ion source using a split valve. Polyphenol structures were analyzed and identified with the information dependent analysis (IDA) function of the mass spectrometer using survey (Q1) scans between 150 and 1300 m/z and dependent (Q3) product ion scans between 80 and 1300 *m/z*. Ion source settings were as follows: spray voltage was −4500 V, source temperature was 500 °C; curtain gas, spray gas and drying gas (N_2) pressures were 40 psi, 30 psi, and 30 psi, respectively. Chromatographic data were evaluated using the Analyst 1.6.3 software. Mass spectra evaluation and compound identification was achieved using tandem mass spectral databases and data found in the literature.

Statistical analysis

All the antioxidant assays were done in triplicate and the results were expressed as mean ± standard deviations of the three values. The experimental data on the various bark species were compared by analysis of variance (ANOVA) on Statistica 11 (StatSoft Inc., Tulsa, USA) software applying the Tukey Post Hoc test at a 95% level of confidence.

Results and discussion

Antioxidant activity of bark extracts

Plants produce a wide range of phenolic antioxidants including flavonoids, proanthocyanidins, cinnamic acids, benzoic acids, coumarins, stilbenes, lignans and lignins. The antioxidant activity of polyphenols is mainly influenced by their chemical structure, ability to form hydrogen bonds, the capability of metal ions for chelation and reduction, kinetic solvents effect, adduct formation, and reduction potential [4]. The TPC assay is based on the reaction of phenolic compounds in the extract with a colorimetric reagent due to the transfer of electrons between them in an alkaline medium. Table 1 shows the results of the various antioxidant assays for the three bark species. Among the investigated species, the highest TPC was obtained for the chestnut bark (174.25 ± 16.95 mg GAE/g dw), while the lowest TPC was obtained for the cherry bark (112.88 ± 17.27 mg GAE/g dw). Many studies have explored the sweet chestnut tree for its phenolic content, particularly the shells, burs and leaves [25, 26]; but only a few can be found on the bark [27]. Interestingly, chestnut leaves and burs showed lower phenolic contents of 115.4 ± 1.8 and 93.3 ± 2.4 mg GAE/g dw, respectively, compared to the bark [28]; while cherry fruits showed TPC of 284.48 ± 3.07 GAE mg/100 g of fresh cherries [14]. One of the earlier studies evaluated the phenolic content of the bark extracts after separating the outer bark from the inner bark [10]; although for practical reasons and from a commercialization point of view, we chose to study the whole bark of the tree trunks. Other studies have evaluated the TPC values of bark of *Quercus rubra* (276.50 ± 3.23 mg GAE/g dw extract), *Betula celtiberica* (432.02 ± 3.00 mg GAE/g dw extract) [27], *Larix laricina* (34 ± 2 g GAE/100 g extract) [29], as well as *Fagus sylvatica* L. (65.22 ± 5.57 mg GAE/g dw) [30]. Reports on bark extraction using methods such as solid–liquid extraction with aqueous methanol [28], and subcritical water extraction [31] showed a lesser yield of phenolics.

The DPPH assay is commonly used to evaluate the scavenging potential of extract constituents against the DPPH free radicals. Although the assay does not reflect the overall in vivo scavenging potential, it gives rapid and reproducible results [32]. Very limited literature is available on the antioxidant properties of bark extracts, DPPH being the most widely employed method and commonly expressed in µg/mL as IC_{50} values or in mg equivalent of a standard/g dw. The phenolic compounds act as excellent oxygen radical scavengers due to their lower electron reduction potential; thus, a high phenolic content can be correlated to a high radical scavenging capacity [32]. Accordingly, as indicated by the IC_{50} values in Table 1, the bark extracts of larch and chestnut showed high DPPH scavenging capacities of 2.54 ± 0.15 and 2.69 ± 0.03 µg/mL, respectively, that were not significantly different from each other. The IC_{50} value of larch bark was found to be lower than that of its cones (13.73 ± 1.30 µg/mL) [33]. Similarly, the IC_{50} value of chestnut bark was lower than that of its leaves (7.05 ± 0.66 µg/mL) [34]. On the other hand, the cherry bark exhibited the least IC_{50} value of 4.31 ± 0.18 µg/mL, which was lower than the IC_{50} value of cherry leaves (27.29 ± 0.77 µg/mL) and flowers (61.59 ± 0.71 µg/mL) [13]. These IC_{50} values are comparable to DPPH scavenging activity of bark extracts of *Juniperus oxycedrus* (1.1 µg/mL) [35], and *Fagus sylvatica* L. (7.45 ± 0.07 µg/mL) [30]. A similar trend on DPPH activity was observed in a study on bark extracts of temperate trees including wild cherry and the European larch using magnetic stirring in ambient conditions and methanol as the solvent [8]. Another study used pressurized hot water as an extraction medium for larch industrial waste and found the bark had a higher IC_{50} value (0.3 mg/mL) than the branches and the sapwood, but not the heartwood [36]. Notably, these values were considerably lower than those in this work, which may be primarily attributed to the extraction intensification using ultrasonic horn that enhanced the yield of polyphenols.

Table 1 Values (mean ± standard deviation) of TPC, DPPH, ABTS and FRAP for tree bark extracts in 80% aqueous ethanol

	TPC[a] (mg GAE/g dw)	DPPH[b] (IC_{50}) (µg extractives/mL)	ABTS[c] (mg TE/g dw)	FRAP[d] (mg AAE/g dw)
	$p < 0.0001$	$p < 0.001$	$p < 0.01$	$p < 0.001$
Wild cherry	112.88 ± 17.27^{a}	4.31 ± 0.18^{a}	424.90 ± 18.30^{a}	72.26 ± 1.08^{a}
European larch	145.22 ± 6.11^{ab}	2.54 ± 0.15^{b}	432.25 ± 45.23^{a}	84.08 ± 3.15^{b}
Sweet chestnut	174.25 ± 16.95^{b}	2.69 ± 0.03^{b}	739.65 ± 24.41^{b}	207.49 ± 3.62^{c}

The small letters in superscript indicate significant differences between bark extracts at the given *p* value

[a]total phenol content (mg GAE/g dw)

[b]2,2-diphenyl-1-picrylhydrazyl (IC_{50}, µg extractives/mL)

[c]2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (mg TE/g dw)

[d]ferric reducing ability of plasma (mg AAE/g dw)

The ABTS assay gives the ABTS radical scavenging capacity expressed in trolox equivalents, hence it is also known as trolox equivalent antioxidant capacity (TEAC). It has been widely used due to its simplicity for screening of hydrophilic and lipophilic compounds, as the radical is soluble in both water and organic solvents. However, like DPPH, ABTS does not occur naturally, inviting possible criticism that the assay is not directly relevant to real biological environments [30]. As evident from Table 1, the sweet chestnut bark had the highest ABTS scavenging activity of 739.65 ± 24.41 mg TE/g dw. This value was much higher than that of chestnut leaves (323.63 ± 16.00 mg TE/g dw) [34], as well as chestnut shells (156.59 mg AAE/g dw) [37]; thus indicating higher quenching capacity of the chestnut bark. In contrast, bark extracts of the European larch and wild cherry showed lower scavenging activities of 432.25 ± 45.23 and 424.90 ± 18.30 mg TE/g dw, respectively, not differing significantly from each other. It is interesting to note that while the DPPH scavenging activity of the European larch was similar to that of sweet chestnut, its ABTS scavenging activity was only a little over half that of the latter. This may possibly be explained by the diverse selectivity of methods to different compounds and various modes of action of the antioxidants [32]. It should be pointed out that both inner bark and outer bark of the tree trunk influence the antioxidant properties of the bark. A study found that the inner bark showed higher antioxidant capacity compared to the outer bark for most species [10]. The variations in genetic factors (cultivars) or geographic locations may also affect the metabolite profile [38]. The results obtained in this study were comparable to those of bark extracts of *Fraxinus angustifolia* (4.5 µM TE/mg extract) [39], and *Goniothalamus velutinus* (78.88 ± 0.56 mg TE/g extract) [40]. Nevertheless, the reported values for radical scavenging assays in the literature may vary due to differences in the experimental conditions, thus making the comparison difficult. Another drawback is that the antioxidant-free radical reaction may not reach completion within the designated time span, resulting in an underestimation of its actual value [41].

The FRAP assay is based on low-pH reduction of a colorless ferric complex to a blue-colored ferrous complex by antioxidants acting as electron-donors. It uses inexpensive reagents and is straightforward to perform, giving highly reproducible results [22]. A downside of the assay is that it not only measures antioxidants but also other compounds having a redox potential lesser than 0.77 V and thus are capable of reducing the ferric ion [30]. The FRAP antioxidant capacity in Table 1 is consistent with the other antioxidant assays, with sweet chestnut bark having the highest antioxidant capacity of 207.49 ± 3.62 mg AAE/g dw. The European larch bark showed FRAP value of 84.08 ± 3.15 mg AAE/g dw, which was over twice that of its green cones (40.39 ± 0.73 mg AAE/g dw) [33]. Wild cherry bark showed the least FRAP value of 72.26 ± 1.08 mg AAE/g dw. These results were significantly higher compared to the FRAP values of bark extracts of *Fagus sylvatica* L. (49.69 ± 3.44 mg AAE/g dw) [30], and *Eucalyptus globulus* (7.81 mmol AAE/100 g dry bark) [42]. Other studies reported the FRAP antioxidant capacity in trolox equivalent such as for *Quercus robur* (640.30 ± 22.03 mg TE/g dried extract) [27], and *Goniothalamus velutinus* (80.11 ± 1.52 mg TE/g dried extract) [40]. In all of the antioxidant assays, aqueous ethanol proved to be a solvent with a very high extraction efficiency for the phenolic antioxidants, which has also been pointed out in a number of previous studies [27, 29]. Furthermore, the use of ultrasound to recover natural antioxidants proved to be an efficient method that enhanced the extraction yield with reduced solvent consumption and time; the mechanism of which is already established [6]. Thus, the bark species investigated here hold an immense potential for valorization due to their rich phenolic content and high antioxidant capacity, considering the growing interest for the possible utilization of natural bioactive compounds, especially in the food sector.

Antibacterial activity of bark extracts

The antibacterial properties of wild cherry, the European larch and sweet chestnut bark extracts were investigated using DDT, MIC assay and bacterial growth curves. Earlier studies have shown antimicrobial properties in extracts of larch bark [43–45]. The DDT is a qualitative method used for the detection of antimicrobial property [46]. In this study, the extracts exhibited a very weak inhibition against *S. aureus* and no inhibition against *E. coli*, as shown in Table 2.

The MIC assay results for bark extracts in the concentration range of 0.016–1.0 mg/mL showed that all extracts inhibited the growth of both standard strains after 15 h of incubation. Visible inhibition for *S. aureus* and *E. coli* was observed in the entire concentration range. As shown in Table 3, IC_{50} values were obtained for all extracts (≤ 0.5

Table 2 Antibacterial activity of bark extracts (1 mg/mL in 1% DMSO) by disk diffusion test

Standard bacterial strain	Positive control	Wild cherry	European larch	Sweet chestnut
S. aureus	++++	+	+	+
E. coli	++++	–	–	–

++++ Strong activity (zone of inhibition > 15 mm)

+++ Medium activity (zone of inhibition between 8 and 15 mm)

++ Weak activity (zone of inhibition < 8 mm)

+ Very weak activity (trace of activity)

– No activity

mg/mL) with *S. aureus*; however, with *E. coli*, IC_{50} value was obtained only for sweet chestnut (1.0 mg/mL). In general, the growth of *E. coli* was inhibited by all extracts, but as inhibitory concentration was 40% or lesser, hence IC_{50} with *E. coli* could not be obtained for the other two extracts, i.e., the European larch and wild cherry. Similar MIC values have been reported for larch wood extract for *S. aureus* (0.24 mg/mL) and *E. coli* (0.54 mg/mL) [15]. In contrast, cherry leaf extract gave MIC value of 50 mg/mL for the two strains [47], while chestnut leaf and shell extracts showed MIC values of 25 mg/mL and 50 mg/mL, respectively, for *S. aureus* [25].

Bacterial concentrations in the presence of bark extracts were recorded over 24 h to obtain the growth curves shown in Fig. 2. It was observed that the extracts had no antibacterial activity against *E. coli* (Fig. 2a, b, c) and supported their growth. In contrast, significant inhibitory effect of the extracts was observed on *S. aureus* (Fig. 2d, e, f). These results did not completely correlate with the MIC assay results, since IC_{50} values were obtained for both *S. aureus* and *E. coli*. With wild cherry, significant inhibition was shown at 0.063 mg/mL and higher extract concentrations for *S. aureus* (Fig. 2d). With the European larch and sweet chestnut, the inhibition effects were observed at extract concentrations of 0.500 mg/mL (Fig. 2e) and 0.250 mg/mL (Fig. 2f), respectively, or lower values. The fact that the extracts showed antibacterial activity against *S. aureus* and no activity against *E. coli* correlates with DDT results.

A noteworthy observation in this study is the higher efficacy of bark extracts against Gram-positive *S. aureus* than Gram-negative *E. coli*. This trend has also been observed in previous studies. For example, the larch bark extracts were reported to give MIC values of 1.2 mg/mL and 1.75 mg/mL against *S. aureus* [48, 49]. In contrast, it showed no antibacterial activity against Gram-negative strains [43, 48]. Similar results for MIC have also been found for wild cherry bark, as in this study. According to Abedini et al. (2020), the wild cherry bark extract was the most effective against Gram-positive and Gram-negative bacterial strains at MIC values of 0.125 mg/mL and 0.250 mg/mL, respectively [48]. Arora and Mahajan (2018) investigated the antimicrobial activity of a wild Himalayan cherry bark extract against several bacterial strains and observed MIC values of 1.0 mg/mL against *E. coli* and 5.0 mg/mL against *S. aureus* [50]. Likewise, the promising antibacterial activity of sweet chestnut bark has been demonstrated [51].

Table 3 IC_{50} values of bark extracts (1 mg/mL in 1% DMSO)

Standard bacterial strain	IC_{50} (mg/mL)		
	Wild cherry	European larch	Sweet chestnut
S. aureus	0.250	0.500	0.250
E. coli	–	–	1.000

– not determined

Polyphenolic composition of bark extracts

Altogether 123 compounds were tentatively identified and described from bark tissues of wild cherry, the European larch and sweet chestnut. Figure 3 depicts the UV chromatograms while Table 4 lists the major compounds identified in the extracts. Identification was based on literature data and tandem mass spectral databases. Some of the compounds including (+)-catechin (13), (−)-epicatechin (27), taxifolin-O-hexoside (32) and naringenin (81) were evidenced in all of the extracts, yet most of the compounds were specific to the species.

According to the height of the chromatographic peaks, the most abundant compounds in cherry bark were tentatively identified as iso/neosakuranin or luteolin-O-hexoside (76), apigenin-O-hexoside (50), formononetin-O-hexoside (87), daidzein-O-hexoside (73), kaempferol-O-hexosides (56, 60), taxifolin-O-hexoside (32), scopolin (20) as well as catechin isomers (13, 27), which are all flavonoids and flavonoid glycosides. The derivatives of dihydrowogonin and apigenin were first evidenced in wild cherry bark by Geibel and Feucht (1991) [52]. The presence of dihydrowogonin (91, 92), scopoletin (31), taxifolin (37), genistein (86), aromadendrin (51), and naringenin (81) in wild cherry bark has already been evidenced in a previous study [48]. However, the authors of the study found high diversity in flavonoids (mainly flavanones and flavonols) in aglycone form and only lower levels of glycosylated kaempferol, taxifolin and naringenin derivatives. On the other hand, in this study, mostly the glycoside conjugates of polyphenolic compounds were found rather than their respective free aglycones. The ratio of glycoside conjugates and their respective free aglycones in bark extracts depends on many factors including age, type of sample collection (ratio of inner and outer bark), storage and drying, as well as on extraction solvent and method [53]. The significance of polyphenolic compounds in contributing to the antibacterial properties of wild cherry bark extracts was reported by Oyetayo and Bada (2017) [47]. Abedini et al. (2020) also emphasized the role of dihydrowogonin as a potentially strong antimicrobial compound.

The most abundant compound identified in the European larch bark extract was piceatannol-O-hexoside (astringin) (39), also confirmed by earlier studies in larch bark extracts [45]. However, several studies on larch bark polyphenols [15, 54, 55] indicated the presence of other compounds as major constituents, which were also evidenced by this study in smaller amounts, including larixinol (54), quercetin-O-glycosides (36, 45, 48, 52, 58, 66), naringenin (81), aromadendrin (51), quercetin (75), kaempferol (89), astringenin (43), catechin isomers (13, 27) as well as procyanidin dimers

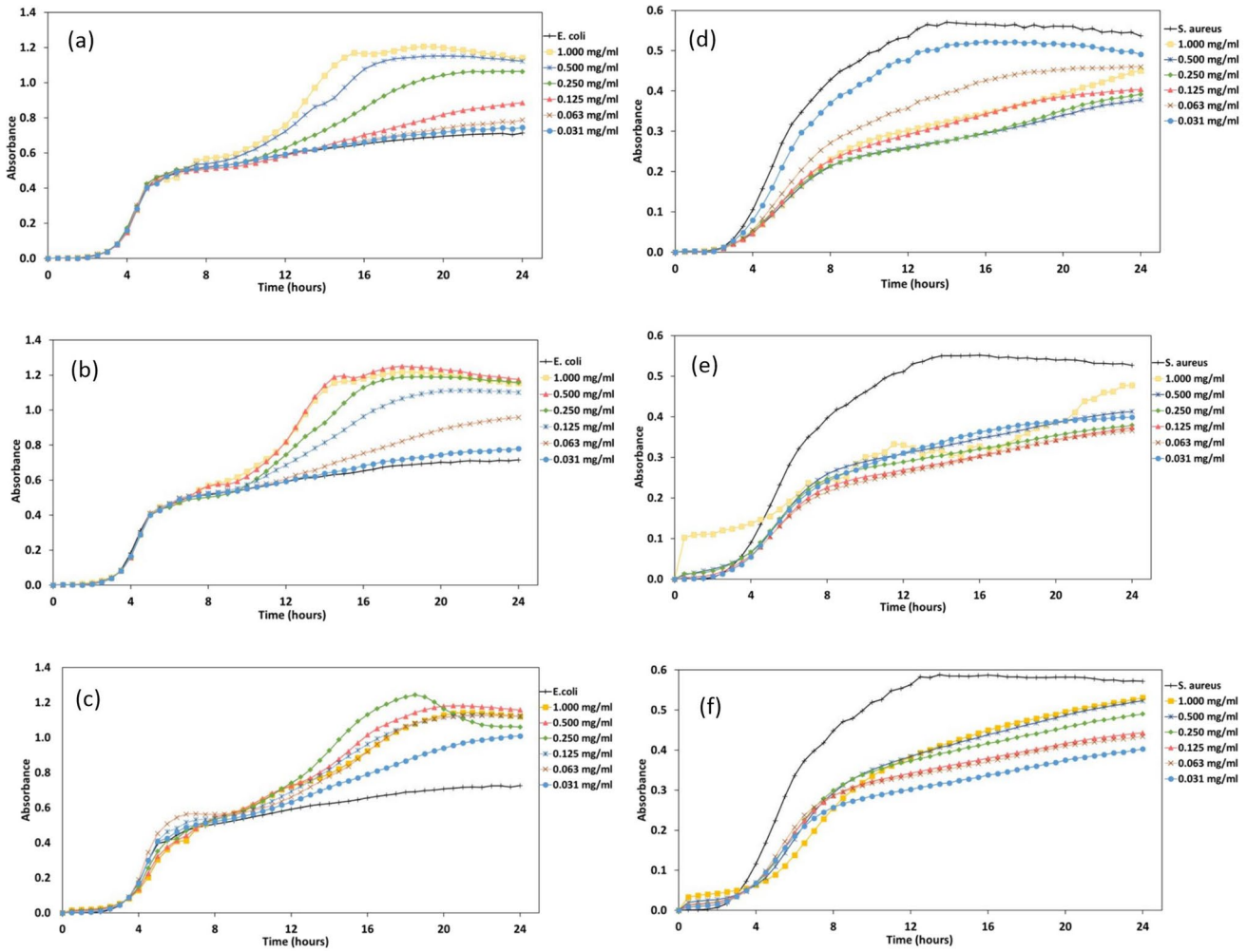


Fig. 2 Growth curves of *E. coli* (**a**, **b**, **c**) and *S. aureus* (**d**, **e**, **f**) with bark extracts of wild cherry (**a**, **d**); the European larch (**b**, **e**) and sweet chestnut (**c**, **f**)

(14, 24, 30). To the best of our knowledge, this is the first study to evidence pinocembrin (93)—a heartwood polyphenol of the European larch [56], also found in its bark. According to Wagner et al. (2019), it can be assumed that astringin is mainly responsible for the antimicrobial activity of methanol extracts of larch bark against *Staphylococcus aureus* [45]. Salem et al. (2016) pointed out that bark extracts have much higher antibacterial potential compared to wood extracts against all investigated bacteria [15].

The main polyphenolic constituents of sweet chestnut were identified as hydrolysable tannins (gallotannins and ellagitannins) with vescalagin (8), castalagin (10), monogalloyl (2,3,4,6) and digalloyl (16, 18) glucose isomers, gallic acid (5), unidentified gallotannin (29), trigalloyl-HHDP-glucose (40) and ellagic acid (49) showing the highest peaks. The great abundance of tannins in sweet chestnut bark is in accordance with the previous literature [38, 57–59]. Other major compounds include quinic acid (1), unidentified compounds (70, 90) and unidentified-O-pentoside, O-acetyl (88). Flavonoids and flavonoid glycosides were present in low amounts, including taxifolin (32), quercetin (36, 44, 48, 52), apigenin (50), isorhamnetin (53, 59, 67), daidzein conjugates (73) as well as catechins (13, 27). Only trace amounts of dihydrowogonin (91), naringenin (81), genistein (86), iso/neosakuranin or luteolin-O-hexoside (77) and genkwanin-O-hexoside (71) were evidenced, which together with daidzein, isorhamnetin and apigenin have been reported for the first time to be present in sweet chestnut bark. The presence of high amounts of hydrolysable tannins is advantageous for their potential anticancer, antiangiogenic, anti-inflammatory, anti-ulcerative, phytoestrogenic, and P-glycoprotein inhibiting effects [60–62]. Compared to flavonoid glycosides, these compounds are supposed to play a more dominant role in plant defence too, especially in plant–herbivore interactions [63]. Gallic and ellagic acids are important due to their proven anti-carcinogenic activity [64, 65].

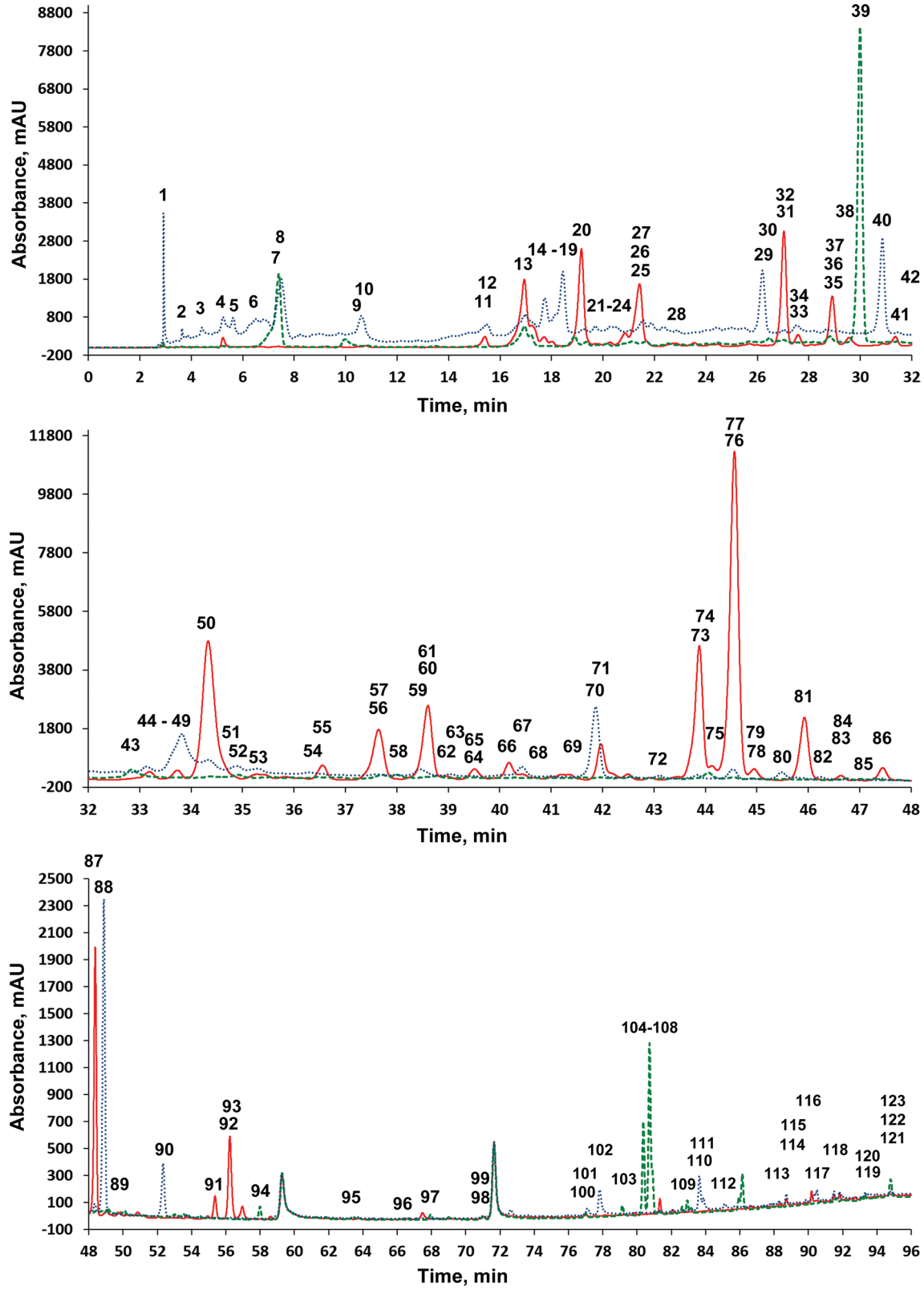


Fig. 3 The PDA (250–380 nm) chromatogram of the whole bark extracts of wild cherry (solid red line), the European larch (dashed green line) and sweet chestnut (dotted blue line)

Table 4 Chromatographic/mass spectrometric identification of the polyphenols in the bark tissues of wild cherry (W), the European larch (L) and sweet chestnut (S)

Peak	t_r (min)	Compound	W	L	S	$[M-H]^-$ m/z	MS/MS m/z
1	2.8	Quinic acid	–	x	x	191	173, 127, 111, 109,
2	3.6	Monogalloyl-glucose	–	–	x	331	313, 295, 271, 241, 211, 169, 125
3	4.4	Monogalloyl-glucose	–	–	x	331	313, 295, 271, 241, 211, 169, 125
4	5.2	Monogalloyl-glucose	–	–	x	331	313, 295, 271, 241, 211, 169, 125
5	5.6	Gallic acid	–	–	x	169	125
6	6.5	Monogalloyl-glucose	–	–	x	331	313, 295, 271, 241, 211, 169, 125
7	7.4	Unidentified	–	x	–	333	287, 161, 125, 113
8	7.5	Vescalagin	–	–	x	933	569, 493, 425, 301, 273, 249
9	10.4	Gallocatechin	–	–	x	305	261, 219, 179, 167, 137, 125
10	10.7	Castalagin	–	–	x	933	569, 493, 425, 301, 273, 249
11	15.4	Dihydroxycoumarin-O-hexoside (Aesculin)	x	–	–	339	177, 148, 133
12	15.5	Unidentified	–	–	x	469	425
13	16.9	(+)-Catechin	x	x	x	289	245, 203, 125, 123, 109
14	17.2	Procyanidin B dimer	x	x	–	577	425, 407, 289, 245, 125
15	17.7	Coumaric acid-O-hexoside	x	–	–	325	205, 187, 163, 145, 119
16	17.7	Digalloyl glucose	–	–	x	483	331, 313, 271, 241, 169, 125
17	18	Unidentified-O-pentoside	x	–	–	445	427, 313, 295, 233, 161
18	18.4	Digalloyl glucose	–	–	x	483	331, 313, 271, 241, 169, 125
19	18.9	Unidentified	–	x	–	423	193
20	19.2	Scopoletin-7-O-glucoside (Scopolin)	x	–	–	353	192, 191, 176, 148, 104
21	19.7	Digalloyl glucose	–	–	x	483	331, 313, 271, 241, 169, 125
22	20.3	Digalloyl glucose	–	–	x	483	331, 313, 271, 241, 169, 125
23	20.9	Unidentified	x	–	–	459	307, 265, 205, 163, 145, 119
24	21.2	Procyanidin B dimer	–	x	–	577	425, 407, 289, 245, 125
25	21.4	Aromadendrin-O-hexoside	x	–	–	449	421, 287, 259, 243, 215, 179, 151, 125
26	21.6	Digalloyl glucose	–	–	x	483	331, 313, 271, 241, 169, 125
27	21.7	(−)-Epicatechin	x	x	x	289	245, 203, 125, 123, 109
28	22.7	Unidentified gallotannin	–	–	x	467	313, 211, 169, 125
29	26.2	Unidentified gallotannin	–	–	x	469	331, 263, 169, 125
30	26.7	Procyanidin B dimer	–	x	–	577	425, 407, 289, 245, 125
31	27	Scopoletin	t	–	–	191	176, 147, 104
32	27	Taxifolin-O-hexoside	x	x	x	465	447, 437, 303, 285, 275, 259, 217, 179, 151, 125
33	27.5	Unidentified	–	–	x	425	299, 289
34	27.6	Taxifolin-O-hexoside	x	–	–	465	447, 437, 303, 285, 275, 259, 217, 179, 151, 125
35	28.7	Unidentified	–	x	–	509	463, 441, 373, 305, 283
36	28.7	Quercetin-O-hexoside	–	–	x	463	343, 301, 300, 273, 255, 179
37	28.9	Taxifolin	x	x	–	303	285, 275, 259, 241, 217, 179, 177, 151, 125
38	29.6	Eriodictyol	x	x	–	287	241, 151,135, 107
39	30	Piceatannol-O-hexoside (astringin)	–	x	–	405	243, 225, 201, 175, 159
40	30.9	Trigalloyl-HHDP-glucose	–	–	x	937	637, 619, 467, 423, 305, 260, 243, 169, 125
41	31.4	Apigenin-C-hexoside (vitexin)	x	–	–	431	341, 323, 311, 283, 225, 191
42	31.9	Trigalloyl glucose	–	–	t	635	483, 465, 313, 271, 211, 169, 125
43	32.9	Tetrahydroxy stilbene (astringenin)	–	x	–	243	225, 201, 175, 159, 147, 119
44	33.1	Quercetin-O-pentoside	–	–	x	433	301, 300, 273, 255, 179
45	33.2	Quercetin-O-hexoside	x	–	–	463	301, 300, 273, 255, 179, 151
46	33.6	Unidentified gallotannin	–	–	x	621	469, 451, 313, 271, 169, 125
47	33.7	Apigenin-C-hexoside (isovitexin)	x	–	–	431	341, 323, 311, 283, 225, 191
48	33.8	Quercetin-O-pentoside	–	–	x	433	301, 300, 273, 255, 179

Table 4 (continued)

Peak	t_r (min)	Compound	W	L	S	$[M-H]^-$ m/z	MS/MS m/z
49	33.9	Ellagic acid	–	–	x	301	284, 257, 229, 185
50	34.3	Apigenin-O-hexoside	x	–	x	431	311, 283, 269, 268, 239, 224, 211, 199
51	34.8	Aromadendrin	x	x	–	287	269, 259, 243, 201, 125
52	34.9	Quercetin-O-rhamnoside	–	–	x	447	301, 300, 271, 255, 243, 179
53	35.3	Isorhamnetin-O-pentoside	–	–	x	447	315, 314, 300, 299, 271, 243
54	36.5	Larixinol	–	x	–	541	513, 497, 415, 309, 308, 281, 267
55	36.6	Apigenin-O-hexoside	x	–	–	431	311, 283, 269, 268, 239, 224, 211, 199
56	37.7	Kaempferol-O-hexoside	x	–	–	447	327, 285, 284, 255, 227, 191
57	37.7	Unidentified	–	x	–	519	473, 357, 307, 247, 165, 125
58	38	Quercetin-O-rhamnoside (quercitrin)	–	x	–	447	301, 300, 271, 255, 243, 179
59	38.5	Isorhamnetin-O-pentoside	–	–	x	447	315, 314, 300, 299, 285, 271, 243
60	38.6	Kaempferol-O-hexoside	x	–	–	447	327, 285, 284, 255, 227, 191
61	38.6	Unidentified-O-hexoside	–	x	–	447	300, 285, 175
62	39	Isorhamnetin-O-hexoside	x	–	–	477	315, 314, 300, 299, 271, 257, 243
63	39.2	Tetragalloyl glucose	–	–	t	787	635, 613, 465, 447, 313, 215, 169
64	39.5	Unidentified	x	–	–	485	417, 255, 237, 211
65	39.5	Unidentified	–	x	–	447	379, 315, 285
66	40.2	Quercetin-O-hexoside	x	–	–	463	301, 300, 273, 255, 179, 151
67	40.4	Isorhamnetin-O-rhamnoside	–	–	x	461	446, 315, 314, 300, 299, 285, 271, 243
68	40.7	Unidentified	–	x	–	447	315, 285, 175
69	41.6	Kaempferol-O-rhamnoside	–	x	–	431	285, 284, 255, 227, 187
70	41.9	Unidentified	–	–	x	551	491, 343, 328, 313, 298, 285
71	42	Genkwanin-O-hexoside	x	–	t	445	325, 297, 283, 268, 240, 224
72	43.1	Unidentified-O-hexoside	–	–	x	475	460, 328, 328, 313, 298, 285, 270
73	43.8	Daidzein-O-hexoside	x	–	x	415	295, 253, 237, 224, 209
74	44	Daidzein-O-hexoside	x	–	–	415	295, 253, 237, 224, 209
75	44.1	Quercetin	x	x	–	301	273, 245, 179, 151, 107
76	44.5	Unidentified-O-hexoside	–	x	–	447	432, 285, 269, 241, 175
77	44.6	iso/neosakuranin or luteolin-O-hexoside	x	–	t	447	432, 285, 269, 241
78	44.9	iso/neosakuranin or luteolin-O-hexoside	x	–	–	447	432, 285, 269, 241
79	44.9	Unidentified	–	x	–	543	
80	45.5	Unidentified	–	–	x	329	314, 313, 299, 285, 271
81	45.9	Naringenin	x	t	t	271	177, 151, 119, 107
82	46.2	Unidentified	–	–	x	343	328, 313, 298, 285
83	46.6	Unidentified	x	–	–	298	283, 269, 255
84	46.7	Unidentified	–	x	–	291	273, 245, 229
85	47.1	Unidentified	–	x	–	567	405, 269, 255, 243, 237, 227
86	47.4	Genistein	x	–	t	269	241, 224, 201, 196, 159, 133
87	48.4	Formononetin-O-hexoside*	x	–	–	475	429, 267, 207
88	48.9	Unidentified-O-pentoside, O-acetyl	–	–	x	535	475, 343, 328, 313, 298, 285
89	49.1	Kaempferol	–	t	–	285	267, 229, 211, 159
90	52.3	Unidentified	–	–	x	343	328, 313, 298, 285
91	55.4	Dihydrowogonin isomer	x	–	t	285	270, 242, 213, 186, 166, 138, 110
92	56.2	Dihydrowogonin isomer	x	–	–	285	270, 242, 213, 186, 166, 138, 110
93	56.3	Pinocembrin	–	t	–	255	213, 211
94	58	Unidentified	–	x	–	505	490, 341, 326, 177, 163, 145, 119
95	63.4	Unidentified	–	t	–	431	413, 403, 377, 317, 255, 243, 213
96	66.2	Unidentified	–	t	–	445	417, 399, 371, 343, 315
97	68	Unidentified	–	x	–	443	415, 397, 369, 313, 269

Table 4 (continued)

Peak	t_r (min)	Compound	W	L	S	$[M-H]^-$ m/z	MS/MS m/z
98	71	Unidentified	–	x	–	311	293, 267, 249, 147
99	71	Unidentified	–	–	x	333	285, 265, 233
100	77	Unidentified	–	x	–	429	399, 387, 381, 299, 251
101	77.1	Unidentified-O-hexoside	–	–	x	795	633, 615, 603, 453, 179, 161, 135
102	77.8	Unidentified-O-hexoside	–	–	x	795	633, 615, 603, 453, 179, 161, 135
103	79.1	Unidentified	–	x	–	447	429, 387, 311, 99
104	80.4	Unidentified	–	x	–	431	401, 383, 335, 301, 253
105	80.7	Unidentified	–	x	–	687	641, 301
106	80.9	Unidentified	–	x	–	687	641, 301
107	81.3	Unidentified	x	–	–	447	429, 415, 345, 331, 317, 289, 271, 261, 193, 175, 160
108	81.4	Unidentified	–	x	–	447	415, 387, 345, 331, 271, 247, 193
109	82.9	Unidentified	–	x	–	713	550, 532, 296, 277
110	83.6	Unidentified	–	–	x	639	628, 617
111	83.9	Unidentified	–	–	x	617	587, 571, 438
112	85.1	Unidentified	–	–	x	603	
113	88.3	Unidentified	x	–	x	605	590, 531, 513, 445, 355, 175
114	88.7	Unidentified	x	–	–	539	521, 477, 285, 271, 267, 253, 241, 223
115	88.7	Unidentified	–	–	x	383	366, 338
116	89.8	Unidentified	x	–	x	633	618, 559, 541, 527, 473, 383, 359, 261, 175
117	90.4	Unidentified	x	–	x	633	618, 559, 541, 527, 473, 383, 359, 261, 175
118	91.5	Unidentified	–	–	x	369	323
119	93.3	Unidentified	–	x	–	469	443, 400, 163, 145, 133, 119
120	93.3	Unidentified	–	–	x	473	458, 429, 415, 360, 345
121	94.8	Unidentified	x	-	–	573	558, 513, 443, 415
122	94.8	Unidentified	–	x	–	471	428, 163, 145, 121, 117
123	94.8	Unidentified	–	–	x	501	486, 429, 401, 331, 317

x present, – not present, *t* in traces

*Detected as $[M-H+HCOOH]^-$ adduct

Thus, HPLC–PDA–ESI–MS/MS identification of polyphenols of all the three species confirms the presence of significant amounts of several bioactive compounds, which are potentially responsible for the antioxidant and antibacterial properties of the bark extracts. Although individual studies are available on the bioactivities of the bark of wild cherry [8, 48], the European larch [8, 9, 15] and sweet chestnut [27, 58], this study made a comparative investigation of the three species for their antioxidant and antibacterial properties along with the compounds responsible for the bioactivity. This work will encourage the exploration of secondary biomass as natural source of phenolic compounds with applications in food industry.

Conclusion

In summary, we have carried out a comparative study on the in vitro antioxidant and antibacterial properties of bark extracts of wild cherry, the European larch and sweet chestnut trees. Among them, the sweet chestnut bark showed the highest antioxidant potential, which also correlated with its highest total phenol content. All the extracts demonstrated significant antibacterial effect against Gram-positive *S. aureus* compared to Gram-negative *E. coli*. Interestingly, sweet chestnut also seemed to be the most effective against the tested bacterial strains. The presence of various polyphenolic compounds in the extracts contributing to the bioactivities was confirmed by HPLC–MS/MS analysis. Another notable aspect of the work was the use of ultrasound as a green route and aqueous ethanol as the solvent medium for the extraction of bioactive compounds from the bark. Since bark extracts are naturally rich in phenolic antioxidants, they hold promise for potential utilization as natural additives

particularly in the food sector. Further studies on animals or clinical trials are recommended to determine the in vivo effects of the bark extracts.

Acknowledgements The work was carried out as part of the "Sustainable raw material management thematic network—RING 2017", EFOP-3.6.2-16-2017-00010 project in the framework of the Széchenyi 2020 Program. The realization of this project is supported by the European Union, co-financed by the European Social Fund. In addition, financial support from ERDF "Multidisciplinary research to increase application potential of nanomaterials in agricultural practice", CZ. 02.1.01/0.0/0.0/16_025/0007314 project is gratefully acknowledged.

Author contributions CA: methodology, investigation, and writing—original draft preparation. TH: investigation, writing—review and editing. MV: investigation, writing—review and editing. NS: methodology (measuring of antibacterial activities). EV: formal analysis and validation. SV: supervision, formal analysis, and validation. ZP: conceptualization and project administration.

Funding Open access funding provided by University of Sopron.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Compliance with ethics requirements This article does not contain any studies involving human or animal subjects.

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