Chemical Engineering & Processing: Process Intensification xxx (xxxx) xxx

Contents lists available at ScienceDirect

Chemical Engineering and Processing - Process Intensification

journal homepage: www.elsevier.com/locate/cep

Low-frequency, green sonoextraction of antioxidants from tree barks of Hungarian woodlands for potential food applications

Charu Agarwal [a,*], Tamás Hofmann [b], Eszter Visi-Rajczi [b], Zoltán Pásztory [a]

[a] Innovation Center, University of Sopron, Bajcsy-Zsilinszky E. str. 4, Sopron, 9400, Hungary
[b] Institute of Chemistry, University of Sopron, Bajcsy-Zsilinszky E. str. 4, Sopron, 9400, Hungary

ARTICLE INFO

Keywords:
Tree bark
Ultrasound intensification
Polyphenols
Antioxidant potential

ABSTRACT

The present work evaluates and compares the antioxidant capacities of bioactive constituents in the barks of ten common wood species from Hungary *(Quercus rubra*, *Prunus serotina*, *Quercus robur*, *Betula pendula*, *Fraxinus excelsior*, *Robinia pseudoacacia, Carpinus betulus*, *Picea abies*, *Alnus glutinosa*, *Pinus sylvestris*). Low-frequency ultrasound was used for intensification of extraction from the bark to obtain extracts rich in polyphenolic antioxidants with potential applications in the food industry. The extractions were carried out in different aqueous organic solvents- ethanol 80 % and acetone 80 % at optimized conditions. The overall antioxidant capacity of the extracts was estimated by the combined evaluation of Folin-Ciocâlteu total phenol content (TPC), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and ferric reducing ability of plasma (FRAP) assays using a scoring system. Aqueous ethanol had better extraction efficiency than aqueous acetone as a solvent medium. Of all the investigated species, *Quercus robur* showed the maximum antioxidant capacity with TPC of 105.88 ± 17.75 mg GAE/g dw, DPPH (IC_{50}) of 1.90 ± 0.10 μg/mL, ABTS of 437.09 ± 36.22 mg TE/g dw and FRAP of 102.62 ± 8.69 mg AAE/g dw. The polyphenolic characterization of *Quercus robur, Robinia pseudoacacia* and *Fraxinus excelsior* was done by liquid chromatography/tandem mass spectrometry.

1. Introduction

The polyphenols constitute one of the most widely distributed groups of secondary metabolites in the plant kingdom. In recent decades, the polyphenols have attracted enormous attention and have become an emerging field of interest in biomedical healthcare, food and nutrition, as well as cosmetics [1,2]. They are well-known for their antioxidant property, i.e., the ability to donate an electron or a hydrogen atom from a hydroxyl group, to neutralize the highly reactive species such as superoxide ($O_2^{\bullet -}$) and hydroxyl ($OH^{\bullet -}$) radicals produced due to oxidative stress [3]. Other pharmacological effects of polyphenols include anti-inflammatory, antimicrobial, analgesic, antiallergenic, and antiproliferative activities [1]. Numerous studies have shown the key role of polyphenols, as second line defense antioxidants, in the regulation of metabolism for the prevention or treatment of cardiovascular diseases, neurodegenerative disorders (like Parkinson's and Alzheimer's), type 2 diabetes, osteoporosis, asthma, and other chronic conditions [2]. Polyphenols are used extensively in the food industry for fortification of foods and beverages as well as for enhancing the stability and shelf-life of foods [3,4]. In cosmetic formulations, the polyphenol-enriched extracts have been found to be effective to prevent premature skin aging, protect against UV damages, show antimicrobial and anti-inflammatory activities [5].

The polyphenols differ widely in their structures, arising from variations in the plant sources; thus, several techniques have been developed, which differ in their mechanism, to extract the targeted bioactive constituents from the plant matrix. The main factors governing the extraction process include time, temperature, pressure, pH, type of solvent, solid-to-solvent ratio, and particle size [6]. The conventional methods of extraction such as Soxhlet extraction, maceration, and hydrodistillation have significant drawbacks, notably in terms of the long extraction times and the large volumes of organic solvents consumed. These drawbacks are overcome by the novel "green" extraction intensification techniques such as those assisted by ultrasound and microwaves that offer advantages of faster kinetics, reduced solvent consumption, enhanced yield, improved selectivity of compounds, and a lower environmental footprint [1]. Ultrasound helps to achieve a higher extraction yield over a shorter time period compared to

* Corresponding author.
E-mail address: charu.agarwal3@gmail.com (C. Agarwal).

https://doi.org/10.1016/j.cep.2020.108221
Received 24 July 2020; Received in revised form 22 September 2020; Accepted 3 November 2020
Available online 7 November 2020

conventional extraction methods. The ultrasound degrades the plant cell walls to release the cell components by the cavitation phenomenon, which increases penetration of solvent into the plant matrix, leading to an increased mass transfer rate [7]. The extraction intensification of natural materials using ultrasonic irradiations has already been well--reviewed [8].

Tree bark is a major forest byproduct generated from the pulp and paper industries as well as during lumbering and processing of wood. The bark, comprising about 5–20 % of the wood, primarily serves as a physical and biological protection of the tree and facilitates the transport of nutrients. The annual bark production is estimated to be around 300–400 million m^3 globally [9]. Most of the bark generated goes into dump yards or is burned for energy (calorific value in the range of 16−22 MJ/kg) [9]. In comparison with wood, bark contains lower amounts of cellulose, more lignin, and relatively higher amounts of extractives including phenolic compounds such as tannins [10]. The phenolic compounds or polyphenols are bioactive compounds that play a crucial role in the defense mechanisms of bark and also contribute to the physiological and morphological functions in plants. The polyphenols from plants are increasingly preferred as natural antioxidants over their synthetic counterparts that may be unstable and highly toxic in nature [11].

Nevertheless, only a limited number of tree species have been investigated so far for the bark extractives and their properties. The alcoholic extracts of *Larix decidua* and *Solidago canadensis* barks have exhibited inhibitory effects on *S. aureus* owing to the high content of flavonoids that may hamper the nucleic acid synthesis and cytoplasmic membrane function [12,13]. Aqueous extracts of sugar maple and red maple barks have shown potential as safe dietary antioxidants and nutrients, having abundant proteins, total sugars and minerals [14]. In another work, *Fagus sylvatica* and *Picea abies* bark extracts were found to induce cytotoxicity in A375 human melanoma and stimulation in cell viability of A549 lung carcinoma cells in a dose-dependent manner [15]. Further, the antiproliferative properties of the extracts obtained at high doses could be correlated with their antioxidant effects. In the light of these studies, the bark extractives play a crucial role in health and nutrition by exerting several biological effects. The knowledge of the antioxidant properties of the bark extractives is fundamental to the development of dietary supplements and pharmaceutical drugs. The existing scientific data falls short of structured research on the antioxidant composition of bark extracts as a source of natural antioxidants. Furthermore, most of the literature studies on bark extraction have employed toxic organic solvents with energy-intensive conventional extraction techniques.

Thus, the objective of the present work was to extract bioactive compounds from tree barks using ultrasound at low-frequency, followed by a comprehensive assessment and comparison of their antioxidant properties obtained from multiple assays. In this study, ultrasound was applied as an efficient and environment-friendly technique for intensification of the extraction process on the tree bark to obtain extracts rich in polyphenolic antioxidants with potential utilization in the food industry. Aqueous solutions of ethanol and acetone were used as green solvents for extraction in view of their safety for food grade applications. The extractions were done bearing in mind the process and environment sustainability on the whole barks of ten commonly found tree species in Hungary. The antioxidant potential of bark extracts is a complex function of many factors and no single standard method can be adopted to assess their activity. Therefore, the *in vitro* antioxidant capacities were determined by several assays- the 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 ferric reducing ability of plasma (FRAP). To the best of our knowledge, the study on the antioxidant properties of whole barks extracted using the principle of ultrasound from multiple assays has not yet been reported. Their overall antioxidant potential was determined with a scoring system that combined the results of all the assays, with the idea of developing an approach for similar investigations on plant materials in the future. The identification of the major polyphenolic antioxidants was done for extracts showing the highest antioxidant activity, using high performance liquid chromatography/tandem mass spectrometry (HPLC–MS/MS) technique.

2. Materials & methods

2.1. Chemicals and reagents

Methanol, ethanol and acetone were obtained from Molar Chemicals Ltd., Hungary. Folin & Ciocâlteu's phenol reagent (2 N), DPPH, 2,4,6-tri (2-pyridyl)-1,3,5-triazine, sodium carbonate, gallic acid, ascorbic acid, ferric chloride, sodium acetate, acetic acid and hydrochloric acid were procured from Sigma-Aldrich, Hungary. Potassium persulfate, ABTS and trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) were purchased from Merck, Germany. LCMS grade acetonitrile was purchased from VWR International, Hungary. The chemicals used for the experiments were of analytical grade and used as received. Double distilled water was used for making all the standard solutions, reagents as well as for the extractions and HPLC-MS/MS analyses.

2.2. Sample collection and preparation

Whole bark samples were collected from the forests of Barcs (South-West Hungary) in autumn during October-November 2019 from ten common wood species viz., red oak (*Quercus rubra*), black cherry (*Prunus serotina*), pedunculate oak (*Quercus robur*), silver birch (*Betula pendula*), European ash (*Fraxinus excelsior*), black locust (*Robinia pseudoacacia*), European hornbeam (*Carpinus betulus*), Norway spruce (*Picea abies*), black alder (*Alnus glutinosa*) and Scots pine (*Pinus sylvestris*). The bark pieces were cut from 3 to 5 felled mature (50–70 years of age) and healthy trees of each species from a height above 5 m, within one month after felling. For each species, 1.5−2 kg of bark samples were collected. The samples were dried in an oven at 40 °C for 3 days and put into resealable plastic bags. The dried samples were subsequently ground and sieved. The meshed fraction was between 0.2−0.63 mm and was stored in the plastic bags at −20 °C until extraction. The moisture content of the bark samples was determined using an infrared moisture analyzer (Sartorius MA35) and was in the range of 6–10 %.

2.3. Extraction by ultrasound

Ultrasonic extractions of the bark were carried out on a low-frequency (20 kHz) probe sonicator (Tesla 150 WS) fitted with a titanium probe and having a maximum power output of 150 W. Two different organic solvents were used for extraction of each type of bark species: aqueous ethanol 80 % and aqueous acetone 80 %. In a typical extraction, 0.5 g of ground bark specimen was mixed with 50 mL of solvent in a beaker and extracted at gradually increasing maximum power for 15 min. The extracts were cooled down, filtered through filter paper into bottles and refrigerated at −20 °C until further analyses. The temperature during ultrasonication was about 74 °C using ethanolic solvent and 56 °C using acetonic solvent. The volume reduction of the solvents after ultrasonication was recorded and taken into account while determining the antioxidant potential of the extracts. The energy efficiency of the ultrasonic probe determined from the calorimetric measurements was around 34.9 %, as described in our previous work [16].

2.4. Estimation of antioxidant potential

All the assays were performed in triplicate using a UV–VIS spectrophotometer (Hitachi U-1500) for the measurement of absorbance at the respective wavelengths. Cuvettes with a 1 cm light pathway were used for spectrophotometric measurements. A test tube shaker (IKA, Germany) was used for vortexing the reaction mixtures prior to the

measurement of absorbance.

2.4.1. Determination of TPC

In the TPC assay, the Folin-Ciocâlteu (F-C) reagent reacts with the phenolic compounds in the extract, forming a blue complex due to electron transfer [17]. In a typical procedure, 500 µL of the extract was mixed thoroughly with 2.5 mL of the reagent (10-fold diluted) in a test tube. After 1 min, 2 mL of Na_2CO_3 solution (0.7 M) were added and the test tube was left in the water bath at 50 °C for 5 min. The absorbance was measured at 760 nm against the blank solution as the reference. Gallic acid standard solutions were used for the calibration curve. The TPC mean values were expressed in mg equivalents of gallic acid/g dry weight of bark (mg GAE/g dw).

2.4.2. Determination of DPPH antioxidant capacity

The DPPH assay, that measures the free radical scavenging potential of the extract, was done using the method of Sharma and Bhat with slight modifications [18]. A standard methanolic solution of DPPH of 2×10^{-4} M concentration was prepared; calibration was performed at different dilutions and the falling absorbance values were recorded at 515 nm. For the assay, 10 µl of extract was diluted with 2090 µl of unbuffered methanol followed by the addition of 900 µl of DPPH. The reaction mixture was incubated in the dark at room temperature for 30 min and the decrease in absorbance was measured at 515 nm. All the measurements were done in dim light. Results were expressed as IC_{50} (50 % inhibition concentration) values in µg extractives/mL, representing the amount of extractives reacting with 50 % of DPPH in the total assay volume (3 mL) under these conditions. The IC_{50} values of standard compounds (rutin, trolox, (+)-catechin) were also determined.

2.4.3. Determination of ABTS antioxidant capacity

The ABTS scavenging assay, which measures the inhibitory effect of the extract on the oxidation of the ABTS free radicals, was carried out according to an established procedure [19]. In a typical experiment, 40 µL of the extract was mixed with 1960 µL of $ABTS^{\bullet+}$ solution (aqueous solution of 7 mM ABTS and 12.5 mM potassium persulfate, with an absorbance of 0.70 ± 0.02 at 734 nm). The reaction mixture was incubated in the dark at ambient temperature for 10 min and the absorbance was measured at 734 nm. All the measurements were done in dim light. Serial dilutions of trolox standard solution (1 mM) were used for plotting the calibration curve. The ABTS mean values were expressed in mg equivalents of trolox/g dry weight of bark (mg TE/g dw).

2.4.4. Determination of FRAP antioxidant capacity

The FRAP was determined according to a standard method by Benzie and Strain that measures the antioxidant capacity by reducing the ferric ions to ferrous ions confirmed by their dark violet color [20]. A typical procedure involved mixing of 50 µL of the extract with 1500 µL of the reagent and allowing the reaction in the dark at ambient conditions for 5 min. The absorbance was measured at 593 nm against the blank solution using ascorbic acid standards for the calibration curve. The FRAP mean values were expressed in mg equivalents of ascorbic acid/g dry weight of bark (mg AAE/g dw).

2.5. Estimation of extractive content/extraction yield

The extraction yield measures the solvent efficiency to extract certain components from the plant material. Aliquots of the extracts (3 mL) were dried in plastic trays in an oven at 40 °C. The solids were weighed on a precision balance (Sartorius MSA225 P). The extractive content was determined and expressed in mg extractives/mL extract units. The extractive content was taken into account for the calculation of DPPH IC_{50} values.

2.6. Statistical analysis

The experimental data of different bark species was compared by analysis of variance (ANOVA) on Statistica 11 (StatSoft Inc., Tulsa, USA) software applying the Tukey HSD calculation method for a post-hoc test. The results were expressed as mean ± standard deviations of the three measurements. The correlations among data were calculated using the MS Excel software correlation and regression tools. For the ANOVA, the measurement values were first checked for normal distribution, and then the variables were checked for the homogeneity of variances using Bartlett's *Chi*-square test.

2.7. Chromatographic characterization of extracts

Selected bark extracts were identified for their polyphenolic composition using HPLC-MS/MS. Separation of the extracts was made using a Shimadzu LC-20 type high-performance liquid chromatograph coupled with a Shimadzu SPD-M20A type photodiode array (PDA) detector (Shimadzu Corporation, Kyoto, Japan) and an AB Sciex 3200 QTrap triple quadrupole/linear ion trap mass spectrometric (MS) detector (AB Sciex, Framingham, USA). A Phenomenex Synergy Fusion-RP 80A, 250 mm x 4.6 mm, 4 µm column was used for the separation with a Phenomenex SecurityGuard ULTRA LC type guard column (Phenomenex Inc., Torrance, USA) at 40 °C. The injection volume was 15 µL. The binary gradient of A (H_2O + 0.1 % HCOOH) and B (CH_3CN + 0.1 % HCOOH) solvents was run with 1.2 mL/min flow-rate using the following time gradient: 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 detector signal (250−380 nm) was recorded to monitor separation of peaks. A negative electrospray ionization mode was used for the MS detector by allowing 0.6 mL/min flow to enter the MS ion source using a split valve. Polyphenols were identified with the Information Dependent Analysis (IDA) scanning function of the mass spectrometer using a survey (Q1) scan between 150−1300 *m/z* and respective dependent (Q3) product ion scans between 80−1300 *m/z*. Ion source settings were as follows: spray voltage: −4500 V, source temperature: 500 °C, curtain gas (N_2) pressure: 40 psi, spray gas (N_2) pressure: 30 psi, drying gas (N_2) pressure: 30 psi. Chromatographic data were acquired and evaluated using the Analyst 1.6.3 software. Mass spectra evaluation and compound identification was done using the RIKEN tandem mass spectral database, via the scientific data found in the literature and by the use of fragmentation rules.

3. Results & discussion

3.1. Extraction method, solvents and process parameters

The choice of the extraction method, solvents and process conditions are the most critical aspects of extraction intensification using ultrasound. Ultrasound is widely preferred as a sustainable technology for extraction of bioactive compounds from plant materials for its versatile nature, simplicity of operation, reproducibility, energy-saving and potential for industrial scale-up [7]. In contrast to the classical solid-liquid extraction methods, the ultrasonic technique produces higher extraction yield and better selectivity of compounds due to enhanced rate of mass transfer [8]. Moreover, it has the ability to preserve the biological activity of the extracted constituents such as their antioxidant and antimicrobial properties [21]. Another factor affecting the yield is the efficiency of the solvent, which depends on its ability to dissolve the specific phenolic groups. Methanol, ethanol, acetone and ethyl acetate are the most widely used organic solvents for the extraction of polyphenols from plants. Several studies have reported better extraction efficiency of aqueous solvents than pure organic solvents due to their higher polarity resulting in synergistic effects [6]. Based on the optimization results of several studies, this study employed aqueous ethanol 80 % and aqueous acetone 80 % as green extraction solvents that have

Table 1
Values (mean ± standard deviation) of TPC[1], DPPH[2], ABTS[3] and FRAP[4] for the bark extracts of various species in aqueous ethanol 80 % and aqueous acetone 80 %. Different capital letters denote significant differences between different solvent extracts of a given species at the given p level (TPC $p<0.003$; DPPH IC_{50}, ABTS, FRAP $p < 0.05$). Different small letters indicate significant differences between the extracts of different species with a given solvent at the indicated p level.

	TPC (mg GAE/g dw)		DPPH (IC_{50}) (μg extractives/mL)		ABTS (mg TE/g dw)		FRAP (mg AAE/g dw)	
	$p<0.0001$ ethanol	$p<0.0001$ acetone	$p < 0.001$ ethanol	$p < 0.05$ acetone	$p<0.01$ ethanol	$p<0.0001$ acetone	$p < 0.001$ ethanol	$p<0.03$ acetone
Red oak	$17.34 \pm 2.97^{ab}_{B}$	$12.51 \pm 0.09^{bc}_{A}$	$6.37 \pm 0.13^{cd}_{A}$	$4.61 \pm 0.78^{cd}_{B}$	$63.04 \pm 7.05^{a}_{B}$	$48.38 \pm 1.99^{abc}_{A}$	$14.09 \pm 2.45^{ab}_{B}$	$9.43 \pm 0.76^{ab}_{A}$
Black cherry	$11.55 \pm 1.51^{a}_{B}$	$8.05 \pm 0.19^{a}_{A}$	$10.92 \pm 1.21^{ab}_{B}$	$15.47 \pm 1.51^{a}_{A}$	$46.17 \pm 3.46^{a}_{B}$	$24.72 \pm 1.60^{a}_{A}$	$9.37 \pm 0.30^{a}_{B}$	$5.86 \pm 0.15^{a}_{A}$
Pedunculate oak	$105.88 \pm 17.75^{e}_{B}$	$74.72 \pm 4.02^{h}_{A}$	$1.90 \pm 0.10^{e}_{A}$	$1.92 \pm 0.14^{d}_{A}$	$437.09 \pm 36.22^{c}_{A}$	$434.19 \pm 88.70^{d}_{A}$	$102.62 \pm 8.69^{d}_{B}$	$87.29 \pm 3.34^{f}_{A}$
Silver birch	$29.20 \pm 1.58^{bc}_{B}$	$23.97 \pm 0.25^{de}_{A}$	$7.50 \pm 0.47^{cd}_{B}$	$9.45 \pm 0.75^{b}_{A}$	$96.35 \pm 12.13^{a}_{A}$	$76.75 \pm 3.15^{abc}_{A}$	$21.14 \pm 2.98^{b}_{B}$	$13.71 \pm 1.05^{bc}_{A}$
European ash	$53.06 \pm 2.43^{d}_{B}$	$42.23 \pm 0.13^{g}_{A}$	$12.79 \pm 2.10^{a}_{A}$	$15.50 \pm 3.21^{a}_{A}$	$210.10 \pm 17.95^{b}_{B}$	$113.70 \pm 8.76^{bc}_{A}$	$41.64 \pm 0.78^{c}_{B}$	$35.50 \pm 1.03^{c}_{A}$
Black locust	$35.93 \pm 0.98^{cd}_{B}$	$29.17 \pm 0.61^{f}_{A}$	$7.29 \pm 0.25^{cd}_{A}$	$7.47 \pm 0.53^{bc}_{A}$	$165.00 \pm 30.07^{b}_{A}$	$120.96 \pm 23.69^{c}_{A}$	$32.68 \pm 0.76^{c}_{B}$	$21.95 \pm 1.30^{d}_{A}$
European hornbeam	$16.00 \pm 3.60^{ab}_{A}$	$11.10 \pm 0.28^{ab}_{A}$	$6.19 \pm 0.81^{d}_{A}$	$6.35 \pm 1.10^{bc}_{A}$	$36.36 \pm 3.02^{a}_{A}$	$35.52 \pm 1.26^{ab}_{A}$	$7.55 \pm 1.31^{a}_{A}$	$6.52 \pm 0.41^{a}_{A}$
Norway spruce	$27.96 \pm 1.39^{abc}_{A}$	$26.01 \pm 0.61^{ef}_{A}$	$9.35 \pm 1.93^{bc}_{A}$	$9.63 \pm 1.06^{b}_{A}$	$84.33 \pm 12.10^{a}_{A}$	$68.18 \pm 2.51^{abc}_{A}$	$16.16 \pm 2.40^{ab}_{A}$	$14.38 \pm 2.15^{c}_{A}$
Black alder	$21.25 \pm 2.59^{abc}_{B}$	$15.14 \pm 0.20^{c}_{A}$	$5.66 \pm 0.40^{d}_{A}$	$6.55 \pm 1.11^{bc}_{A}$	$68.42 \pm 5.76^{a}_{B}$	$45.19 \pm 4.14^{abc}_{A}$	$15.91 \pm 2.74^{ab}_{A}$	$11.93 \pm 0.83^{bc}_{A}$
Scots pine	$26.13 \pm 3.89^{abc}_{A}$	$20.90 \pm 0.26^{d}_{A}$	$6.51 \pm 0.56^{cd}_{A}$	$6.34 \pm 0.95^{bc}_{A}$	$74.08 \pm 4.03^{a}_{B}$	$59.21 \pm 5.01^{abc}_{A}$	$13.71 \pm 0.75^{ab}_{A}$	$13.92 \pm 0.55^{bc}_{A}$

[1] total phenol content (mg GAE/g dw).
[2] 2,2-diphenyl-1-picrylhydrazyl (IC_{50}, μg extractives/mL).
[3] 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (mg TE/g dw).
[4] ferric reducing ability of plasma (mg AAE/g dw).

low toxicity and are generally recognized as safe (GRAS) [22,23]. The nature of the plant material (moisture content, particle size, etc.) also plays a crucial role in the extraction, since reduced moisture content ensures proper contact with the solvent, and small solid-to-solvent ratio enhances extraction rates due to larger concentration gradients [7]. In this work, whole bark, with particle sizes between 0.2−0.63 mm was used with a solid-to-solvent ratio of 1:100 (g/mL). It was intended the use the whole bark comprised of the dead outer bark and the soft inner bark since the separation of the two is not practical or commercially viable for large-scale handling of the biomaterial.

In general, the highest efficiency of ultrasonic extraction, in terms of yield and composition of the extracts, can be accomplished by optimizing the extraction time and temperature with increasing ultrasonic power. The extraction yield generally increases with time, but up to a threshold limit, beyond which no substantial increase may be observed. The typical time range required for ultrasonic extractions is in the range of 120 s to 1 h, which is considerably lower than that required for the conventional approaches (1−10 h) [6,8]. Nonetheless, most studies have reported ultrasonication times between 15−30 min for polyphenols [6,7]. In this study, 15 min of ultrasonication was used for extractions based on the literature and our previous optimization study [6, 16]. The temperature of extraction significantly affects the rate of diffusion, thus impacting the yield. An increase in the extraction temperature leads to enhanced rates of heat and mass transfer, and higher solubility of the phenolic compounds. However, very high temperatures may cause the degradation of the thermolabile constituents in the plant matrix. In the present work, the temperature during ultrasonication was in the range of 70−80 °C for ethanolic extractions and 50−60 °C for acetonic extractions to maximize the intensification effect. Others have also reported high extraction efficiencies of antioxidants at elevated temperatures [24,25]. Another parameter is the frequency of ultrasound; low frequencies below 40 kHz are the most effective for polyphenol extraction due to the increased cavitation effect [21]. The normally recommended frequency of operation for extraction is 20 kHz since the liquid circulation currents and turbulence effects are dominant at this frequency [8]. The amplitude of 130−150 W was used for ultrasonication, in accordance with our earlier work [16]. At high ultrasonic power, degradation of polyphenols may occur due to the production of highly reactive hydroxyl radicals, especially in the presence of a high amount of water [21].

3.2. Evaluation of TPC

The F-C assay for the determination of TPC gives a general estimation of the total phenolics in the plant extract. It has a non-specific chemistry and can react with a range of phenolics including flavonoids, hydroxycinnamic acids, tannins, and anthocyanins. Nevertheless, it can also react with other antioxidant substrates such as reducing sugars, ascorbic acid, etc. The electron reduction potential of the phenolic radical is lower than that of the oxygen radical, which makes the former excellent oxygen radical scavengers [26]. Hence, it is very often done along with the common antioxidant assays to determine the overall antioxidant potential of the plant material. The structure of the bioactive compounds influences their solubility into a particular solvent. Ethanol dissolves most phenolic acids, flavonols, anthocyanins, tannins, alkaloids and terpenoids; whereas, acetone can dissolve flavonoids, phenolic diterpenes and tannins [6]. Table 1 shows the results from the TPC and antioxidant assays for various species of tree barks in aqueous ethanol and aqueous acetone. The polyphenol content varied widely among the different species as well as between the two solvents. Among the investigated species, pedunculate oak had the highest phenolic content, while black cherry had the lowest in ethanol (P. o.: 105.88 ± 17.75 mg GAE/g dw, b. c.:11.55 ± 1.51 mg GAE/g dw) as well as acetone (P. o.: 74.72 ± 4.02 mg GAE/g dw, b. c.: 8.05 ± 0.19 mg GAE/g dw). Other studies on pedunculate oak bark, using a stirring method with pure water for extraction, have reported far lower phenolic contents [27,28], which confirms the efficacy of using ultrasonication for the extraction of polyphenols from plant materials. The phenolic profile of a plant is primarily a function of the age of the tree, its location, climatic conditions, soil composition, and several other factors [29]. Strangely, the second highest TPC value was obtained for European ash (53.06 ± 2.43 mg GAE/g dw), which was considerably lower than that of pedunculate oak by an almost twofold magnitude. The exceptionally high phenolic content of pedunculate oak bark may be attributed to the presence of large amounts of high molecular weight tannins, phenolic acids, and proanthocyanidins, etc. [30]. According to the results, aqueous ethanol was a more efficient extraction medium than aqueous acetone for all types of bark with the exception of European hornbeam, Norway spruce and Scots pine, where results did not differ significantly from acetone. Several other studies have also found ethanol to be an excellent solvent for the recovery of phenolics, especially in combination with water, where a more polar medium is created that facilitates the extraction process, giving higher yield [6].

The F-C assay is performed with several other assays such as DPPH, ABTS and FRAP that measure the antioxidant potential or the free radical scavenging ability of the plant extracts, and have different sensitivities to different bioactive constituents due to the complex nature of the extract. Unfortunately, there is no standard procedure for carrying out the antioxidant assays and they are conducted using varying protocols differing widely in concentration of the radical or the reagent,

incubation time, and pH of the reaction mixture [18]. Furthermore, the reactions are also sensitive to light, oxygen, and solvent composition thus adding to the difficulty in comparing the results of these assays between laboratories. The results of the antioxidant capacities from DPPH, ABTS and FRAP assays are given in Table 1.

3.3. Evaluation of DPPH antioxidant activity

The DPPH scavenging activity was determined by calculating the IC_{50} values i.e., the antioxidant concentration necessary to reduce DPPH by 50 %. Among the various species, pedunculate oak exhibited the highest DPPH scavenging activity of 1.90 ± 0.10 μg/mL in ethanol and 1.92 ± 0.14 μg/mL in acetone, as also shown by TPC, resulting from the proanthocyanidin content. However, the lowest scavenging activities were shown by European ash and black cherry in ethanol (E. a.: 12.79 ± 2.10 μg/mL, b. c.: 10.92 ± 1.21 μg/mL) as well as in acetone (E. a.: 15.50 ± 3.21 μg/mL, b. c.: 15.47 ± 1.51 μg/mL). Further, the IC_{50} values of red oak, silver birch, black locust and Scots pine were comparable to one another. The quenching effect in the DPPH assay is primarily governed by the rate of initial reaction between the free radical and the antioxidants in the extract, which may occur due to electron transfer (very fast kinetics), or hydrogen atom transfer (diffusion-controlled) [31]. Interestingly, unlike TPC, there were no significant differences observed between the ethanolic and acetonic solvents for most of the species except for red oak, black cherry and silver birch. Comparing the IC_{50} values of bark extracts with those of standard antioxidants (trolox:4.29 μg/mL, (+)-catechin: 7.40 μg/mL, rutin: 13.94 μg/mL) using the same procedure, the DPPH activity of pedunculate oak was comparable to that of trolox, while that of European ash was similar to rutin [29]. Similar DPPH quenching activity (3.0 ± 0.1 μg/mL) has been reported for pedunculate oak bark extract in methanol using an ultrasonic bath at 30 °C for 60 min [32]. It is also important to consider the fact that molecular attributes of antioxidants such as the structure, number of phenolic −OH groups and the redox potential all limit the radical quenching capacity. The complexity of multiple rings or the bulky ring adducts may impede the accessibility of the −OH groups to the radical site, thus leading to steric hindrance [31]. This further emphasizes the need to understand the antioxidant potential in the light of several assays instead of a single assay.

3.4. Evaluation of ABTS antioxidant activity

The ABTS assay is widely used to screen anti-radical activity of bioextracts by measuring the extent of decolorization due to reduction of the ABTS free radicals in the presence of antioxidants [33]. As shown in Table 1, the ABTS radical quenching activity varies greatly among the studied species, ranging from 35.52 ± 1.26 to 437.09 ± 36.22 mg TE/g dw. The higher the ABTS value, the higher is the antioxidant capacity. The highest radical quenching activity was obtained for pedunculate oak, following a similar trend as with the TPC and DPPH assays. On the other hand, the poorest activity was seen in European hornbeam. No significant differences were observed in the antioxidant capacities of red oak, silver birch, Norway spruce, black alder and Scots pine in ethanolic as well as acetonic solvents. Further, the two solvents significantly differed from each other only for red oak, black cherry, European ash, black alder and Scots pine. A significant positive correlation was found between ABTS and the TPC assays ($r = 0.992$, $p < 0.001$), which indicates that the radical scavenging activity increases proportionally to the polyphenol content. Earlier studies have also found a strong correlation between the polyphenols and the antioxidant activity [11,13]. A previous work extracted inner barks of trees separate from outer barks using ultrasound in 80 % methanol and reported higher ABTS results for silver birch (300.4 ± 10.53 mg TE/g dw) and Scots pine (219.0 ± 13.98 mg TE/g dw), while lower values for black locust (63.7 ± 2.72 mg TE/g dw) [34]. The study found that on the whole, the inner bark exhibited higher antioxidant properties compared to the outer bark for most species, although there were some exceptions. Accordingly, the antioxidant capacity of extracts is strongly influenced by the presence of reducing compounds such as sugars, enzymes, and organic acids, other than polyphenols [34]. The intricacy of radical scavenging assays is augmented by the antioxidant action that differs widely in various compounds, making it almost impossible to study the activity of an enormous number of bioconstituents individually. Variations in results may be obtained for certain antioxidants with different incubation times, even within the same assay, since some compounds could attain stable end-points much faster than others [33]. The ABTS assay has an advantage over the DPPH assay of eliminating the color interference due to the wavelength absorption at 734 nm [14]. The radical scavenging assays employed here deal with radicals not found in nature; thus they do not provide information on the antioxidant reactivity in real life environments. Despite their limitations, the DPPH and ABTS assays are easy to implement and have been shown to deliver the most reproducible results between laboratories [11].

3.5. Evaluation of FRAP antioxidant activity

The FRAP assay offers a simple, quick, inexpensive and straightforward test to determine the antioxidant power of bioextracts with results that are reproducible over a wide range of concentration [20]. The reduction of colorless ferric-TPTZ complex to its blue-colored ferrous form at low pH is linearly related to its antioxidant concentration. A drawback of the assay is that it occurs at *in vitro* reaction conditions that are far from physiological environments, so the results may not reflect *in vivo* activities or hierarchies in real-life [20]. Also, the limited assay reaction time may not be enough for compounds with low activities to react, implying that they will be left out from the measured antioxidant capacity. As shown in Table 1, the FRAP antioxidant activities showed a trend similar to TPC and the radical scavenging assays. As expected, pedunculate oak (102.62 ± 8.69 mg AAE/g dw) exhibited the highest FRAP activity, while the lowest activity was found for black cherry (9.37 ± 0.30 mg AAE/g dw) and European hornbeam (7.55 ± 1.31 mg AAE/g dw) in ethanol. Ethanol was more efficient than acetone with significant differences in all the tested species of bark except European hornbeam, Norway spruce, black alder and Scots pine. As in the ABTS assay, the antioxidant activities of red oak, Norway spruce, black alder and Scots pine were comparable in an ethanolic medium, indicating a strong correlation between the two assays. In this work, very significant positive correlations were observed between FRAP and ABTS activities ($r = 0.996$, $p < 0.001$) and also between FRAP and TPC assays ($r = 0.990$, $p < 0.001$). This is in accordance with previous studies that have found a good correlation between of the FRAP activity with the phenolic content as well as the radical scavenging assays [11]. Similar results of FRAP antioxidant activity have been reported for whole tree bark extracts of *Fagus sylvatica* (38.28 ± 1.42 mg AAE/g dw) [35] and *Eucalyptus globulus* (8.2–20.9 mg AAE/g dw) [36], as well as for inner bark extracts of *Castanea sativa* (70.9 ± 3.47 mg AAE/g dw), *Quercus petraea* (44.5 ± 0.12 mg AAE/g dw), and *Populus alba* (34.6 ± 0.40 mg AAE/g dw) [34].

3.6. Assessment of overall antioxidant potential

None of the biochemical assays can individually measure the total antioxidant power of all compounds present in an extract, due to their selective preferences to various types of compounds with specific working principles and reaction mechanisms [11]. This makes the combined evaluation of the assays necessary to have a comprehensive assessment of the antioxidant efficiency of the bioextracts. The overall antioxidant potential was evaluated by a scoring system, as described in one of the previous works [37]. For TPC, ABTS, and FRAP assays, a score of “0” was assigned to the poorest value, while “1” to the best value in each of the assays, using linear approximation for the in-between values. In contrast, for the DPPH assay, the lowest IC_{50} value was assigned a

Table 2
Mean and scores for TPC[1], DPPH[2], ABTS[3] and FRAP[4] along with the sum of scores representing the overall antioxidant potential of barks in aqueous ethanolic extracts.

	Mean				Scores				Sum of scores
	TPC	DPPH	ABTS	FRAP	TPC	DPPH	ABTS	FRAP	
Red oak	17.34	6.37	63.04	14.09	0.061	0.589	0.067	0.069	0.786
Black cherry	11.55	10.92	46.17	9.37	0.000	0.172	0.024	0.019	0.215
Pedunculate oak	105.88	1.90	437.09	102.62	1.000	1.000	1.000	1.000	4.000
Silver birch	29.20	7.50	96.35	21.14	0.187	0.486	0.150	0.143	0.965
European ash	53.06	12.79	210.10	41.64	0.440	0.000	0.434	0.359	1.232
Black locust	35.93	7.29	165.00	32.68	0.258	0.505	0.321	0.264	1.348
European hornbeam	16.00	6.19	36.36	7.55	0.047	0.606	0.000	0.000	0.653
Norway spruce	27.96	9.35	84.33	16.16	0.174	0.316	0.120	0.091	0.700
Black alder	21.25	5.66	68.42	15.91	0.103	0.655	0.080	0.088	0.926
Scots pine	26.13	6.51	74.08	13.71	0.155	0.576	0.094	0.065	0.890

[1] total phenol content (mg GAE/g dw).
[2] 2,2-diphenyl-1-picrylhydrazyl (IC_{50}, μg extractives/mL).
[3] 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (mg TE/g dw).
[4] ferric reducing ability of plasma (mg AAE/g dw).

Table 3
Mean and scores for TPC[1], DPPH[2], ABTS[3] and FRAP[4] along with the sum of scores representing the overall antioxidant potential of barks in aqueous acetonic extracts.

	Mean				Scores				Sum of scores
	TPC	DPPH	ABTS	FRAP	TPC	DPPH	ABTS	FRAP	
Red oak	12.51	4.61	48.38	9.43	0.067	0.801	0.058	0.044	0.970
Black cherry	8.05	15.47	24.72	5.86	0.000	0.002	0.000	0.000	0.002
Pedunculate oak	74.72	1.92	434.19	87.29	1.000	1.000	1.000	1.000	4.000
Silver birch	23.97	9.45	76.75	13.71	0.239	0.445	0.127	0.096	0.908
European ash	42.23	15.50	113.70	35.50	0.513	0.000	0.217	0.364	1.094
Black locust	29.17	7.47	120.96	21.95	0.317	0.592	0.235	0.198	1.341
European hornbeam	11.10	6.35	35.52	6.52	0.046	0.673	0.026	0.008	0.754
Norway spruce	26.01	9.63	68.18	14.38	0.269	0.432	0.106	0.105	0.912
Black alder	15.14	6.55	45.19	11.93	0.106	0.659	0.050	0.075	0.890
Scots pine	20.90	6.34	59.21	13.92	0.193	0.675	0.084	0.099	1.050

[1] total phenol content (mg GAE/g dw).
[2] 2,2-diphenyl-1-picrylhydrazyl (IC_{50}, μg extractives/mL).
[3] 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (mg TE/g dw).
[4] ferric reducing ability of plasma (mg AAE/g dw).

score of "1" to represent the highest antioxidant capacity and the largest IC_{50} value was assigned "0", indicating the least antioxidant power. The individual assay scores for each species were added to obtain their overall antioxidant potential of the extracts. The scores were evaluated for both ethanolic as well as acetonic solvents, as shown in Tables 2 and 3, respectively. From the results, it is obvious that pedunculate oak bark scored the highest and thus has the maximum antioxidant potential, which is considerably higher than that of the other investigated species. This was followed by black locust and European ash in ethanol and acetone extraction media. On the other hand, black cherry and European hornbeam were found to have the least antioxidant potential in both the solvents.

The barks of silver birch, black alder and Scots pine showed medium antioxidant activity in the ethanolic medium, while red oak and Norway spruce showed low activity. A similar trend of scores for all the species can be seen in the individual assays except for the DPPH assay, which was also found in a previous work [29]. It was thus established that the polyphenols contributed significantly to the overall antioxidant potential of the bark extracts. This is also evident from pedunculate oak bark, which is particularly rich in tannins, phenolic acids, proanthocyanidins, and has shown the highest antioxidant activity in this study. The antioxidant potential of the extracts is directly related to the chemical profile of their bioconstituents in the extract, which are influenced by the environmental and genetic factors. Also, seasonal variation may play a critical role in altering their chemical composition, thus affecting the antioxidant potential of bark [37].

The type of evaluation used in this work presents a simple and appropriate method for the relative quantification of the antioxidant potential of bark extracts. Yet only a very limited studies have used the method for the determination of antioxidant capacity from plant materials such as barks, leaves and cones [29,34,37]. However, this is the first time that a comprehensive assessment of the total phenolic content as well as antioxidant properties of whole bark extracts using ultrasound has been done for the most abundant European tree species. According to the results of the study, the bark of pedunculate oak, black locust and European ash exhibited the highest antioxidant capacity. These species were further investigated for their polyphenolic profile using the HPLC-MS/MS technique. The knowledge on the molecular composition of the extracts is of prime importance because it gives information on the type of compounds that can account for the antioxidant and other potentially beneficial effects. This would be fundamental for the future development of drugs and food products/nutraceuticals and also for later extraction optimization.

3.7. Identification of polyphenolic constituents using HPLC-MS/MS

The identification of the molecular structure of the polyphenolic extractives in the bark extract solutions was done using high-performance liquid chromatography/tandem mass spectrometry from the ethanolic extract solutions for pedunculate oak, European ash and black locust, which were found to have the highest overall antioxidant activity. Fig. 1 depicts the HPLC chromatograms and Table 4 includes the major compounds found in the extracts. Altogether, 69 compounds have been described and tentatively identified by tandem mass spectrometric fragmentation (MS/MS) data by using earlier works of the authors [38,39] and other references [30]. The composition of the whole

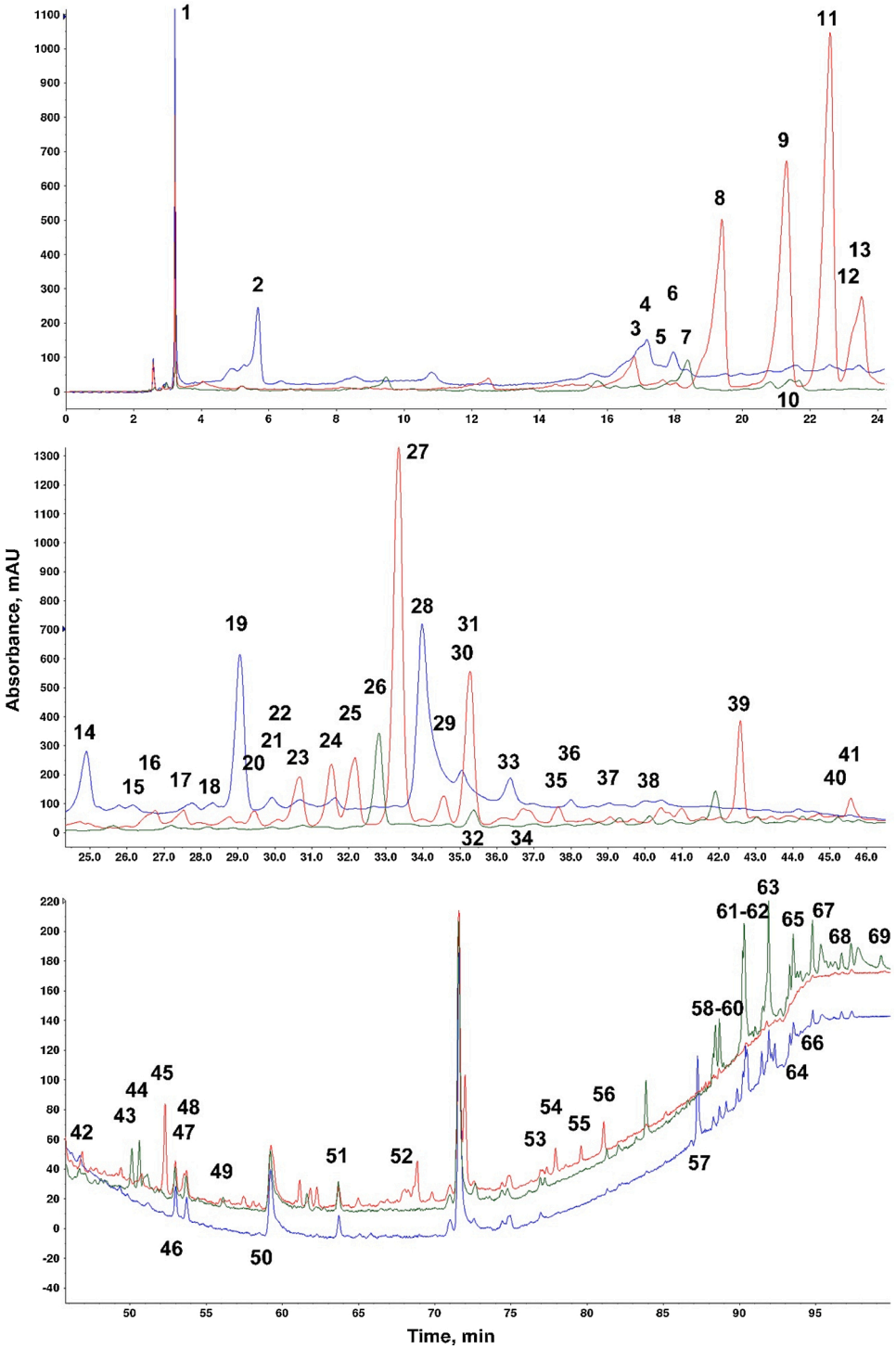

Fig. 1. The PDA (250-300 nm) chromatogram of the whole bark extracts of pedunculate oak (blue), European ash (red) and black locust (green) (For interpretation of the chromatograms to colour in this figure legend, the reader is referred to the web version of this article).

bark extracts of the three species is markedly different, yet procyanidin B dimer (3), (+)-catechin (4), taxifolin (19) and ellagic acid (28) were found in both pedunculate oak and black locust, while sinapaldehyde (13) was found in pedunculate oak and European ash barks, and an unidentified compound (51) was indicated in European ash and black locust. Most of the identified compounds were characteristic to a given species.

The pedunculate oak bark extracts contained flavan-3-ols, including (+)-catechin (4), procyanidin B dimer (3), (epi)-catechin-monogallate (21), however no (−)-epicatechin (10) was found, as already proven by an earlier study [40]. Besides the monogalloyl glucose (1), digalloyl glucose (5, 6) and pentagalloyl glucose (33) compounds, other tannin compounds were also indicated with unidentified structures (7, 20). Surprisingly, one of the largest peaks in the chromatogram of pedunculate oak belonged to ellagic acid (28), indicating that during sample processing and extraction, transformation of the tannins must have taken place, as according to the literature, the presence of ellagitannins over free ellagic acid in pedunculate oak bark is dominant [41]. Pedunculate oak bark also contained flavonoid glycosides, including taxifolin-O-hexosides (14, 15, 18) and quercetin-O-rhamnoside (35).

Table 4
Chromatographic/mass spectrometric identification of the polyphenols in the bark tissues pedunculate oak (Q), European ash (A) and black locust (L).

Peak	t_r (min)	Compound	Q	A	L	$[M-H]^-$ *m/z*	MS/MS *m/z*
1	3.2	Monogalloyl glucose	x			331	271, 211, 169, 151, 125
2	5.7	Gallic acid	x			169	125
3	17	Procyanidin B dimer	x		x	577	425, 407, 289, 245, 125
4	17.1	(+)-Catechin	x		x	289	245, 203, 125, 123, 109
5	17.7	Digalloyl glucose	x			483	331, 271, 211, 193
6	18	Digalloyl glucose	x			483	331, 271, 211, 193
7	18.1	Unidentified tannin	x			1205	$[M-2H]^{2-}$: 602; 457, 289
8	19.4	Unidentified hexoside		x		371	209
9	21.3	Sinapaldehyde-O-hexoside		x		369	354, 207, 192, 177, 163, 150, 135, 108
10	21.5	(−)-Epicatechin			x	289	245, 203, 125, 123, 109
11	22.6	Unidentified-hexoside*		x		429	383, 369, 221, 206, 191, 177, 163
12	23.2	Unidentified		x		221	206, 191, 177
13	23.5	Sinapaldehyde	x	x		207	192, 177, 164
14	24.9	Taxifolin-O-hexoside	x			465	339, 303, 285, 257, 151
15	26.2	Taxifolin-O-hexoside	x			465	339, 303, 285, 257, 151
16	26.8	Caffeoyl-hexoside conjugate		x		477	341, 315, 297, 179, 161, 135
17	27.5	Unidentified		x		357	342, 311, 151, 136
18	28.2	Taxifolin-O-hexoside	x			465	339, 303, 285, 257, 151
19	29	Taxifolin	x		x	303	285, 275, 217, 177, 151, 125
20	29.2	Unidentified tannin	x			497	313, 297, 169, 125
21	29.9	(Epi)-catechin monogallate	x			441	289, 245, 203, 169, 125
22	30.1	Caffeoyl-hexoside conjugate		x		477	341, 315, 297, 179, 161, 135
23	30.7	Caffeoyl-hexoside conjugate		x		477	341, 315, 297, 179, 161, 135
24	31.7	Unidentified	x			787	635, 617, 465
25	32.2	Caffeoyl-hexoside conjugate		x		623	461, 315, 297, 179, 161, 135
26	32.8	Unidentified			x	243	225, 201, 175, 159
27	33.3	Caffeoyl-hexoside conjugate		x		477	341, 315, 297, 179, 161, 135
28	34	Ellagic acid	x		x	301	284, 257, 229, 185
29	34.6	Unidentified hexoside		x		519	357, 342, 311, 193, 151, 136
30	35	Quercetin-O-rhamnoside	x			447	301, 300, 271, 255, 151
31	35.3	Caffeoyl-hexoside conjugate		x		623	461, 315, 297, 179, 161, 135
32	35.4	Unidentified			x	243	225, 201, 175, 159
33	36.3	Pentagalloyl glucose	x			939	768, 617, 465, 429, 169, 125
34	36.7	Caffeoyl-rhamnoside conjugate		x		461	315, 281, 179, 161, 135
35	37.7	Caffeoyl-rhamnoside conjugate		x		461	315, 281, 179, 161, 135
36	37.9	Unidentified	x			439	287, 274, 259
37	39.1	Caffeoyl-hexoside conjugate		x		491	329, 315, 297, 235, 191, 179, 161, 135
38	40.2	Unidentified			x	485	375, 357, 307, 291, 241, 229
39	42.6	Unidentified hexoside		x		523	362, 292, 260, 224, 139, 127, 101
40	45.2	Unidentified			x	469	375, 359, 241, 197
41	45.6	Unidentified		x		337	322, 307
42	46.9	Unidentified		x		619	559, 221
43	50.1	Unidentified			x	233	215, 205
44	50.6	Unidentified			x	243	225, 201, 175, 159
45	52.3	Unidentified		x		441	426, 221
46	53	Unidentified		x		457	353, 223, 103
47	53.5	Unidentified		x		441	426, 367, 337, 322, 221, 206, 163, 147
48	53.8	Unidentified			x	229	185, 167
49	56.2	Unidentified			x	323	279, 233, 217, 161, 133, 117
50	59	Unidentified	x			669	517
51	63.8	Unidentified		x	x	249	205
52	67.9	Unidentified		x		329	285, 257, 229, 191
53	77	Unidentified			x	339	321, 295, 277
54	77.9	Unidentified		x		309	266, 123, 97
55	79.6	Unidentified		x		353	183, 123, 97
56	81.3	Unidentified			x	447	415, 345, 271, 193, 175, 160
57	87	Unidentified	x			605	161
58	88.3	Caffeic acid derivative			x	403	359, 179, 161, 135
59	88.4	Caffeic acid derivative			x	431	389, 276, 179, 161, 135
60	88.7	Unidentified			x	531	500, 429, 356, 193, 175, 160, 134
61	90.2	Unidentified			x	356	338, 310
62	90.3	Caffeic acid derivative			x	432	179, 161, 135
63	91.9	Caffeic acid derivative			x	485	331, 261, 179, 161, 135
64	93.3	Unidentified			x	468	400, 383, 337
65	93.5	Caffeic acid derivative			x	489	179, 161, 135
66	94.8	Caffeic acid derivative			x	515	179, 161, 135
67	95.3	Caffeic acid derivative			x	541	179, 161, 135
68	96.7	Caffeic acid derivative			x	529	514, 502, 429, 345, 303, 261, 179, 161, 135
69	99.3	Unidentified			x	557	542, 133

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

According to Fig. 1, the presence of taxifolin in form of the free aglycone (19) is also dominant over the presence in conjugated (glycosilated) forms (14, 15, 18). In contrast, Lorenz et al. [41] indicated no free taxifolin in oak bark.

European ash bark contained several unidentified hexoside conjugates (8, 11, 29, 39), which was confirmed by the mass difference of a 162 *m/z* (hexosyl) unit in the MS/MS spectra. By the number of peaks and also by peak areas in the chromatogram, the most abundant groups of compounds found in European ash bark were derivatives of caffeic acid, out of which compounds 16, 22, 23, 25, 27, 31 and 37 were caffeoyl-hexoside conjugates, while compounds 34 and 35 were caffeoyl-rhamnoside derivatives. Here, again the presence of a hexosyl moiety was evidenced by the loss of a 162 *m/z* unit, while the presence

of a rhamnosyl unit was indicated by the mass difference of 146 m/z in the MS/MS spectra. The presence of the caffeoyl unit was justified by the simultaneous appearance of the 179, 161 and 135 m/z ions of the caffeoyl residues in the MS/MS spectra, belonging to the [caffeic acid−H]−, [caffeic acid−H–H_2O]− and [caffeic acid−H−CO_2]− structures, respectively. The presence and wide variety of caffeic and other phenolic acid derivatives in European ash was already shown by Kostova and Iossofova earlier [42].

Black locust bark contained (−)-epicatechin (10) and derivatives of caffeic acid (58, 59, 62, 63, 65, 66, 67, 68) besides the compounds also found in pedunculate oak bark (procyanidin B dimer (3), (+)-catechin (4), taxifolin (19), ellagic acid (28)). As opposed to the finding of Vek et al. [43], no robinetin and dihydrorobinetin traces were indicated in bark extracts, which may be attributed to different sample handling and extraction conditions.

Comparing the chromatograms in Fig. 1, it is apparent that overall the highest peaks were detected in European ash bark extracts. In fact, the extraction yield of the ethanolic extracts also justifies this trend: for the ethanolic extract solutions the extraction yield (described in section 2.5) was 1.83 g/L for pedunculate oak, 2.61 g/L for European ash and 1.03 g/L for black locust. Surprisingly, the antioxidant capacity of the samples did not reflect this trend, as pedunculate oak had outstanding antioxidant capacity using the assays and evaluation methods applied in this study. This may be attributed to the fact that different types of compounds make different contributions to the antioxidant capacity of the extracts, hence have different antioxidant "efficiency", which is further modulated by possible synergistic and antagonistic effects between compounds [44–46].

According to these findings, it is not the concentration of individual compounds but rather their ratio and efficiency that determines the antioxidant properties of an extract. The antioxidant power of the compounds found in pedunculate oak bark was superior to that of European ash and black locust, which may be important considering the extraction optimization and use of the extracts in the future. The free taxifolin and ellagic acid content may be especially important in this regard, as glycosilation of flavonoids may have a significant impact on the antioxidant potential. However, this effect depends very much on the type of the aglycone and the sugar unit, as well as on the type (C-, or O- glycoside) and the location of aglycosilation [47]. Indeed, studies have proven the higher in vitro antioxidant activity of ellagic acid compared to the ellagitannins, although ellagic acid is predominately found in plants esters-linked to sugars in the form of ellagitannins, that is freed during hydrolysis or degradation [48,49]. The unexpectedly high free aglycone content in pedunculate oak bark extracts combined with the outstanding overall antioxidant power of this sample highlights the importance of pretreatment and extraction optimization of the samples in order to improve the antioxidant power of the extracted solutions, which would also contribute to use of the extracts.

4. Conclusion

This work assesses the polyphenolic content and antioxidant capacity of whole bark taken from commonly found wood species in Hungary. Low-frequency ultrasound was applied for intensification of the extraction process to obtain polyphenol-rich extracts from the bark. Aqueous ethanol 80 % was more efficient than aqueous acetone 80 % for the extraction of antioxidants. A similar trend was observed among TPC, ABTS and FRAP assays, however, the DPPH activity showed a different behavior, possibly due to its different selectivity. The study establishes a methodology for future investigations of antioxidant properties of bark or parts of other tree species. Overall, pedunculate oak, black locust and European ash barks were found to have the highest antioxidant potential. It would thus be fascinating to consider the possible utilization of bioactive chemical constituents from these types of bark for possible uses in the food industry. Future work can focus on exploring properties such as antimicrobial and anti-proliferative, which are based on the behavior of the phenolic antioxidants. The HPLC-MS/MS analysis showed that the efficiency of the various types of compounds was the major factor contributing to the antioxidant potential of the bark extracts. These promising results highlight the enormous potential of bark extracts as sources of bioactive antioxidants for food applications, thus contributing to the valorization of waste bark biomass. Further lab studies and clinical tests are required in order to determine the feasibility/toxicity of the extracts for dietary intake, which could be a subject of future work.

CRediT authorship contribution statement

Charu Agarwal: Methodology, Investigation, Writing - original draft. **Tamás Hofmann:** Supervision, Writing - review & editing. **Eszter Visi-Rajczi:** Formal analysis, Validation. **Zoltán Pásztory:** Conceptualization, Project administration.

Declaration of Competing 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.

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.

References

[1] M.K. Khan, L. Paniwnyk, S. Hassan, Polyphenols as natural antioxidants: sources, extraction and applications in food, cosmetics and drugs, in: Y. Li, F. Chemat (Eds.), Plant Based "Green Chem. 2.0". Green Chem. Sustain. Technol., Springer, Singapore, Singapore, 2019, pp. 197–235, https://doi.org/10.1007/978-981-13-3810-6_8.

[2] H. Cory, S. Passarelli, J. Szeto, M. Tamez, J. Mattei, The role of polyphenols in human health and food systems: a mini-review, Front. Nutr. 5 (2018) 1–9, https://doi.org/10.3389/fnut.2018.00087.

[3] M. Carocho, P. Morales, I.C.F.R. Ferreira, Antioxidants: Reviewing the chemistry, food applications, legislation and role as preservatives, Trends Food Sci. Technol. 71 (2018) 107–120, https://doi.org/10.1016/j.tifs.2017.11.008.

[4] A.S.C. Galván, M.O. Sánchez, C.E.O. Álzate, C.A.C. Álzate, Potential of antioxidants for functional beverages to improve health through good business, Value-Added Ingredients and Enrichments of Beverages (2019) 325–352, https://doi.org/10.1016/b978-0-12-816687-1.00010-2.

[5] F. Rodrigues, M.D.L.L. Cádiz-Gurrea, M.A. Nunes, D. Pinto, A.F. Vinha, I.B. Linares, M.B.P.P. Oliveira, A.S. Carretero, Cosmetics, Polyphenols Prop. Recover. Appl. (2018) 393–427, https://doi.org/10.1016/B978-0-12-813572-3.00012-9.

[6] A. Oreopoulou, D. Tsimogiannis, V. Oreopoulou, extraction of polyphenols from aromatic and medicinal plants: an overview of the methods and the effect of extraction parameters, Polyphenols in Plants (2019) 243–259, https://doi.org/10.1016/b978-0-12-813768-0.00025-6.

[7] I. Lavilla, C. Bendicho, Fundamentals of ultrasound-assisted extraction, Water Extr. Bioact. Compd. From Plants to Drug Dev. (2017) 291–316, https://doi.org/10.1016/B978-0-12-809380-1.00011-5.

[8] S.R. Shirsath, S.H. Sonawane, P.R. Gogate, Intensification of extraction of natural products using ultrasonic irradiations-A review of current status, Chem. Eng. Process. Process Intensif. 53 (2012) 10–23, https://doi.org/10.1016/j.cep.2012.01.003.

[9] Z. Pásztory, I.R. Mohácsiné, G. Gorbacheva, Z. Börcsök, The utilization of tree bark, BioResources 11 (2016) 7859–7888, https://doi.org/10.15376/biores.11.3.Pasztory.

[10] R. Nosek, M. Holubcik, J. Jandacka, The impact of bark content of wood biomass on biofuel properties, BioResources 11 (2016) 44–53, https://doi.org/10.15376/biores.11.1.44-53.

[11] Dudonné, et al., 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. 57 (2009) 1768–1774, https://doi.org/10.1021/jf803011r.

[12] K. Wagner, C. Roth, S. Willför, M. Musso, A. Petutschnigg, G.J. Oostingh, T. Sclmabel, Identification of antimicrobial compounds in different hydrophilic larch bark extracts, BioResources 14 (2019) 5807–5815, https://doi.org/10.15376/biores.14.3.5807-5815.

[13] Y. Deng, Y. Zhao, O. Padilla-Zakour, G. Yang, Polyphenols, antioxidant and antimicrobial activities of leaf and bark extracts of Solidago canadensis L, Ind. Crops Prod. 74 (2015) 803–809, https://doi.org/10.1016/J.INDCROP.2015.06.014.

[14] S. Bhatta, C. Ratti, P.E. Poubelle, T. Stevanovic, Nutrients, antioxidant capacity and safety of hot water extract from sugar maple (Acer saccharum M.) and red maple (Acer rubrum L.) bark, Plant Foods Hum. Nutr. 73 (2018) 25–33, https://doi.org/10.1007/s11130-018-0656-3.

[15] S.L. Coşarcă, E.A. Moacă, C. Tanase, D.L. Muntean, I.Z. Pavel, C.A. Dehelean, Spruce and beech bark aqueous extracts: source of polyphenols, tannins and antioxidants correlated to in vitro antitumor potential on two different cell lines, Wood Sci. Technol. 53 (2019) 313–333, https://doi.org/10.1007/s00226-018-1071-5.

[16] C. Agarwal, K. Máthé, T. Hofmann, L. Csóka, Ultrasound-assisted extraction of cannabinoids from Cannabis sativa L. optimized by response surface methodology, J. Food Sci. 83 (2018) 700–710, https://doi.org/10.1111/1750-3841.14075.

[17] V.L. Singleton, J.A. Rossi, Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents, Am. J. Enol. Vitic. 16 (1965) 144–158. http://www.ajevonline.org/content/16/3/144.abstract.

[18] O.P. Sharma, T.K. Bhat, DPPH antioxidant assay revisited, Food Chem. 113 (2009) 1202–1205, https://doi.org/10.1016/j.foodchem.2008.08.008.

[19] P. Stratil, B. Klejdus, V. Kubáň, Determination of phenolic compounds and their antioxidant activity in fruits and cereals, Talanta 71 (2007) 1741–1751, https://doi.org/10.1016/j.talanta.2006.08.012.

[20] I.F.F. Benzie, J.J. Strain, The ferric reducing ability of plasma (FRAP) as a measure of "Antioxidant power": the FRAP assay, Anal. Biochem. 239 (1996) 70–76, https://doi.org/10.1006/ABIO.1996.0292.

[21] C.S. Dzah, Y. Duan, H. Zhang, C. Wen, J. Zhang, G. Chen, H. Ma, The effects of ultrasound assisted extraction on yield, antioxidant, anticancer and antimicrobial activity of polyphenol extracts: a review, Food Biosci. 35 (2020) 100547, https://doi.org/10.1016/j.fbio.2020.100547.

[22] G.D.A.R. Oliveira, A.E. De Oliveira, E.C. Da Conceição, M.I.G. Leles, Multiresponse optimization of an extraction procedure of carnosol and rosmarinic and carnosic acids from rosemary, Food Chem. 211 (2016) 465–473, https://doi.org/10.1016/j.foodchem.2016.05.042.

[23] B.B. Li, B. Smith, M.M. Hossain, Extraction of phenolics from citrus peels: I. Solvent extraction method, Sep. Purif. Technol. 48 (2006) 182–188, https://doi.org/10.1016/j.seppur.2005.07.005.

[24] S. Hemwimol, P. Pavasant, A. Shotipruk, Ultrasound-assisted extraction of anthraquinones from roots of Morinda citrifolia, Ultrason. Sonochem. 13 (2006) 543–548, https://doi.org/10.1016/j.ultsonch.2005.09.009.

[25] Y. Zhou, J. Zheng, R.Y. Gan, T. Zhou, D.P. Xu, H. Bin Li, Optimization of ultrasound-assisted extraction of antioxidants from the mung bean coat, Molecules 22 (2017) 1–13, https://doi.org/10.3390/molecules22040638.

[26] E.A. Ainsworth, K.M. Gillespie, Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent, Nat. Protoc. 2 (2007) 875–877, https://doi.org/10.1038/nprot.2007.102.

[27] L. Skrypnik, N. Grigorev, D. Michailov, M. Antipina, M. Danilova, A. Pungin, Comparative study on radical scavenging activity and phenolic compounds content in water bark extracts of alder (Alnus glutinosa (L.) Gaertn.), oak (Quercus robur L.) and pine (Pinus sylvestris L.), Eur. J. Wood Wood Prod. 77 (2019) 879–890, https://doi.org/10.1007/s00107-019-01446-3.

[28] P. Dróżdż, K. Pyrzynska, Assessment of polyphenol content and antioxidant activity of oak bark extracts, Eur. J. Wood Wood Prod. 76 (2018) 793–795, https://doi.org/10.1007/s00107-017-1280-x.

[29] T. Hofmann, E. Visi-Rajczi, L. Albert, Antioxidant properties assessment of the cones of conifers through the combined evaluation of multiple antioxidant assays, Ind. Crops Prod. 145 (2020) 111935, https://doi.org/10.1016/j.indcrop.2019.111935.

[30] P. Lorenz, M. Heinrich, M. Garcia-Käufer, F. Grunewald, S. Messerschmidt, A. Herrick, K. Gruber, C. Beckmann, M. Knoedler, R. Huber, C. Steinborn, F. C. Stintzing, C. Gründemann, Constituents from oak bark (Quercus robur L.) inhibit degranulation and allergic mediator release from basophils and mast cells in vitro, J. Ethnopharmacol. 194 (2016) 642–650, https://doi.org/10.1016/j.jep.2016.10.027.

[31] J. Xie, K.M. Schaich, Re-evaluation of the 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) assay for antioxidant activity, J. Agric. Food Chem. 62 (2014) 4251–4260, https://doi.org/10.1021/jf500180u.

[32] H.O. Elansary, A. Szopa, P. Kubica, H. Ekiert, M.A. Mattar, M.A. Al-Yafrasi, D.O. El-Ansary, T.K. Zin El-Abedin, K. Yessoufou, Polyphenol profile and pharmaceutical potential of quercus spp. Bark extracts, Plants. 8 (2019) 1–14, https://doi.org/10.3390/plants8110486.

[33] L. Zheng, M. Zhao, C. Xiao, Q. Zhao, G. Su, Practical problems when using ABTS assay to assess the radical-scavenging activity of peptides: importance of controlling reaction pH and time, Food Chem. 192 (2016) 288–294, https://doi.org/10.1016/j.foodchem.2015.07.015.

[34] E. Tálos-Nebehaj, L. Albert, E. Visi-Rajczi, T. Hofmann, Combined multi-assay evaluation of the antioxidant properties of tree bark, Acta Silv. Lignaria Hungarica. 15 (2019) 85–97, https://doi.org/10.2478/aslh-2019-0007.

[35] T. Hofmann, E. Nebehaj, É. Stefanovits-Bányai, L. Albert, Antioxidant capacity and total phenol content of beech (Fagus sylvatica L.) bark extracts, Ind. Crops Prod. 77 (2015) 375–381, https://doi.org/10.1016/j.indcrop.2015.09.008.

[36] G. Vázquez, J. González-Alvarez, J. Santos, M.S. Freire, G. Antorrena, Evaluation of potential applications for chestnut (Castanea sativa) shell and eucalyptus (Eucalyptus globulus) bark extracts, Ind. Crops Prod. 29 (2009) 364–370, https://doi.org/10.1016/j.indcrop.2008.07.004.

[37] E. Tálos-Nebehaj, T. Hofmann, L. Albert, Seasonal changes of natural antioxidant content in the leaves of Hungarian forest trees, Ind. Crops Prod. 98 (2017) 53–59, https://doi.org/10.1016/j.indcrop.2017.01.011.

[38] T. Hofmann, E. Nebehaj, L. Albert, The high-performance liquid chromatography/multistage electrospray mass spectrometric investigation and extraction optimization of beech (Fagus sylvatica L.) bark polyphenols, J. Chromatogr. A 1393 (2015) 96–105, https://doi.org/10.1016/j.chroma.2015.03.030.

[39] T. Hofmann, E. Nebehaj, L. Albert, Antioxidant properties and detailed polyphenol profiling of European hornbeam (Carpinus betulus L.) leaves by multiple antioxidant capacity assays and high-performance liquid chromatography/multistage electrospray mass spectrometry, Ind. Crops Prod. 87 (2016) 340–349, https://doi.org/10.1016/j.indcrop.2016.04.037.

[40] Á.N. Makk, T. Hofmann, T. Rétfalvi, Isolation of (+)-catechin from the bark of different oak species, Faipar. 61 (2013) 16–25.

[41] P. Lorenz, M. Heinrich, M. Garcia-käufer, F. Grunewald, S. Messerschmidt, A. Herrick, K. Gruber, C. Beckmann, M. Knoedler, R. Huber, C. Steinborn, F. C. Stintzing, C. Gründemann, Constituents from oak bark (Quercus robur L.) inhibit degranulation and allergic mediator release from basophils and mast cells in vitro, J. Ethnopharmacol. 194 (2016) 642–650, https://doi.org/10.1016/j.jep.2016.10.027.

[42] I. Kostova, T. Iossifova, Chemical components of Fraxinus species, Fitoterapia. 78 (2007) 85–106, https://doi.org/10.1016/j.fitote.2006.08.002.

[43] V. Vek, I. Poljanšek, P. Oven, Variability in content of hydrophilic extractives and individual phenolic compounds in black locust stem, Eur. J. Wood Wood Prod. 78 (2020) 501–511, https://doi.org/10.1007/s00107-020-01523-y.

[44] T. Hofmann, E. Tálos-Nebehaj, L. Albert, L. Németh, Antioxidant efficiency of Beech (Fagus sylvatica L.) bark polyphenols assessed by chemometric methods, Ind. Crops Prod. 108 (2017) 26–35, https://doi.org/10.1016/j.indcrop.2017.06.016.

[45] F. Cebeci, N. Şahin-Yesilcubuk, The matrix effect of blueberry, oat meal and milk on polyphenols, antioxidant activity and potential bioavailability, Int. J. Food Sci. Nutr. 65 (2014) 69–78, https://doi.org/10.3109/09637486.2013.825699.

[46] M. D'Archivio, C. Filesi, R. Varì, B. Scazzocchio, R. Masella, Bioavailability of the polyphenols: status and controversies, Int. J. Mol. Sci. 11 (2010) 1321–1342, https://doi.org/10.3390/ijms11041321.

[47] J. Xiao, Dietary flavonoid aglycones and their glycosides: Which show better biological significance? Crit. Rev. Food Sci. Nutr. 57 (2017) 1874–1905, https://doi.org/10.1080/10408398.2015.1032400.

[48] Y. qing Sun, X. Tao, X. ming Men, Z. wei Xu, T. Wang, In vitro and in vivo antioxidant activities of three major polyphenolic compounds in pomegranate peel: ellagic acid, punicalin, and punicalagin, J. Integr. Agric. 16 (2017) 1808–1818, https://doi.org/10.1016/S2095-3119(16)61560-5.

[49] D.D. Evtyuguin, S. Magina, D.V. Evtuguin, Recent advances in the production and applications of ellagic acid and its derivatives. A review, Molecules 25 (2020) 2745, https://doi.org/10.3390/molecules25122745.