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Electrospray Ionization—Mass Spectrometry Characterization of Pine Bark Extracts

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Abstract: This study explored the potential application of polyphenols from Pinus halepensis bark in leather tanning. The primary objective was to characterize these polyphenols. The extraction and atomization processes proved efficient, reducing moisture content to 7.4%, increasing tannin content from 26.2% to 45.1%, and reducing insoluble substances by 77.5%. High-performance liquid chromatography (HPLC) coupled with mass spectrometry identified and quantified various polyphenolic compounds, including (+)-catechin, (+)-taxifolin, protocatechuic acid, and procyanidin B2. Notably, tannic catechin dimers were detected. Lignin was effectively removed through filtration. Concentrations of protocatechuic acid, (+)-catechin, (+)-taxifolin, (−)-epicatechin, and procyanidin B1 were significantly higher in the extract than in the powder, with the extract showing 1214.3 mg/kg of protocatechuic acid, 2098.0 mg/kg of (+)-catechin, 4017.0 mg/kg of (+)-taxifolin, 2163.0 mg/kg of (−)-epicatechin, and 917.0 mg/kg of procyanidin B1.

Keywords: *Pinus halepensis*; pine bark extract; polyphenols; tanning; leather; electrospray ionization (ESI); mass spectrometry (MS)

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1. Introduction

Polyphenols, a class of chemical compounds found in abundance in nature, have been gaining significant attention in various industries due to their antioxidant properties and potential health benefits. One notable source of polyphenols is the bark of pine trees, often discarded as waste in the timber industry. Recent studies have suggested that these polyphenols can be extracted and characterized for various applications, including the leather tanning industry [1–3].

Leather tanning, a process that transforms raw hides into durable material, traditionally relies on chemicals harmful to the environment. Currently, the leather industry is focused on reducing its environmental impact and seeking alternatives to chrome tanning by developing new, more sustainable technologies to reduce chromium waste. Alternatives include new chrome-free tanning processes such as organic tanning, which involves tanning without heavy metals and aluminum salts (metal-free). These processes use syntans, vegetable tannins, polymers, and aldehydes, among others [4–7]. In recent years, triazine tanning agents have garnered considerable interest as viable and environmentally friendly alternatives notable for being free from metals, formaldehyde, and phenols [8–10].

Polyphenols used in leather tanning are typically extracted from plant sources rich in tannins, a type of polyphenol. Common plant materials used for tannin extraction for leather tanning include oak bark, chestnut wood, quebracho, mimosa, and tara pods.

Pine forests are essential components of the Mediterranean biome and have significant economic value in the Mediterranean region. Pine trees are harvested for timber, used in construction, furniture making, and paper production. Additionally, pine nuts, the edible seeds of certain pine species, are a valuable culinary ingredient and income source for local

communities. Pine resin is also harvested for various industrial applications, such as adhesives, varnishes, and fragrances. However, Mediterranean pine forests tend to accumulate large amounts of combustible biomass, such as needle fall and pine cones, which create a flammable layer on the soil facilitating the spread of wildfires. The Mediterranean climate, characterized by mild, dry winters and hot, dry summers, creates a conducive environment for forest fires, with high temperatures, low humidity, and strong winds increasing fire likelihood and spread. Therefore, effective forest management is crucial [11–13].

Recovering waste from pine forestry operations, such as pine bark, pine cones, and pruning residues, as a sustainable and renewable source of tannins, could promote their use as a tanning material and improve forest management in the Mediterranean region. This paper aims to explore the characterization of polyphenols from *Pinus halepensis* bark extract and discuss their potential use in leather tanning.

Previous studies have demonstrated the presence of tannins in pine bark and pruning residues, classified as condensed tannins [14].

The traditional industrial method of tannin extraction in the leather sector involves boiling plant substances with water, followed by concentration through evaporation. Industrial producers of plant extracts perform extraction in countercurrent autoclaves, under specific conditions of temperature, pressure, extraction time, and water/plant substance ratio. After extraction, clarification is done by decantation, and the solution is concentrated by evaporation until it reaches 40–50% dry matter. The tannin solution can be stored with a stabilizing agent or subjected to spray drying to achieve a powder with less than 10% moisture content [15,16].

The efficiency of tannin extraction can be enhanced by using organic solvents such as ethanol, methanol, acetone, ethyl acetate, or mixtures thereof. Alkaline solutions can also be useful. Seabra et al. concluded that alkaline and acidic additives were not selective for condensed tannins and promoted the extraction of extraneous substances, including carbohydrates and lignin. In contrast, ethanol was selective for these phytochemicals [1].

Various extraction techniques have been employed to isolate polyphenols from pine bark, including conventional methods such as Soxhlet extraction, maceration, and hydro-distillation, as well as modern techniques like supercritical fluid extraction (SFE), ultrasound-assisted extraction (UAE), and microwave-assisted extraction (MAE). Each method has its advantages and limitations in terms of efficiency, yield, and environmental impact [17,18].

Soxhlet extraction is a conventional method used for the extraction of polyphenols from solid samples. In this technique, finely ground pine bark is placed in a thimble, within a Soxhlet extractor, filled with a suitable solvent, typically ethanol or a mixture of ethanol and water. The solvent is heated, causing it to vaporize and condense in a condenser, then drip onto the sample, leaching out the polyphenols. The process is repeated cyclically until the desired extraction is achieved. Soxhlet extraction is known for its efficiency in extracting a wide range of compounds but can be time-consuming and may require large amounts of solvent [19–21].

Maceration is a simple and widely used extraction technique suitable for polyphenols from pine bark. In this method, finely ground pine bark is soaked in a solvent, such as ethanol or water, for a specific period with occasional agitation. The solvent gradually penetrates the bark, dissolving the polyphenols and other bioactive compounds. After sufficient extraction time, the mixture is filtered to separate the solid residue from the liquid extract containing the polyphenols. Maceration is relatively straightforward and requires minimal equipment but may be less efficient compared to other extraction methods [22–24].

Hydro-distillation, also known as water distillation, is employed to extract essential oils and volatile compounds from plant materials, including pine bark. In this method, the bark is submerged in water and heated to generate steam. The steam passes through the plant material, carrying volatile compounds, including some polyphenols. The steam is then condensed, resulting in a mixture of water and essential oil. The oil layer, which contains polyphenols and other lipophilic compounds, can be separated from the aque-

ous layer. Hydro-distillation is suitable for extracting polyphenols with low molecular weights but may not be efficient for extracting higher molecular weight compounds such as proanthocyanidins [25–27].

Supercritical fluid extraction is an advanced technique that utilizes supercritical fluids, typically carbon dioxide (CO_2), as a solvent to extract polyphenols from pine bark. In SFE, CO_2 is pressurized above its critical point, where it exhibits properties of both liquids and gases. The supercritical CO_2 is passed through the pine bark, selectively dissolving polyphenols and other target compounds. The extract is then collected as the pressure is reduced, allowing the CO_2 to revert to its gas phase, leaving behind the extracted polyphenols. SFE offers several advantages, including high selectivity, minimal solvent residue, and the ability to control extraction parameters. However, it requires specialized equipment and expertise [28–31].

Ultrasound-assisted extraction utilizes ultrasonic waves to enhance the extraction of polyphenols from pine bark. In this method, the bark is immersed in a solvent, and ultrasonic waves are applied to the mixture. The high-frequency waves create cavitation bubbles in the solvent, which implode near the plant material, facilitating the release of polyphenols into the solvent. UAE can improve extraction efficiency and reduce extraction time compared to conventional methods. However, optimal parameters such as frequency, power, and extraction time need to be carefully optimized [32–35].

Microwave-assisted extraction employs microwave energy to accelerate the extraction of polyphenols from pine bark. In this method, the bark is mixed with a solvent in a microwave-transparent container and subjected to microwave irradiation. The microwaves penetrate the sample, causing rapid heating and promoting the release of polyphenols from the plant matrix into the solvent. MAE offers advantages such as shorter extraction times, higher yields, and reduced solvent consumption compared to conventional methods. However, careful control of microwave power and extraction conditions is necessary to prevent degradation of heat-sensitive compounds [36–38].

These extraction techniques vary in terms of efficiency, selectivity, and complexity, and the choice of method depends on factors such as the desired polyphenols, solvent compatibility, equipment availability, and desired scale of extraction. Optimization of extraction parameters is crucial to maximize the yield and quality of polyphenols extracted from pine bark while minimizing costs and environmental impact.

As previously mentioned, investigations into the utilization of pine bark powder in leather processing have been documented [2,3]. However, the inherent reactivity of untreated pine bark powder tends to lead to superficial fixation. Therefore, a sulfitation procedure will be implemented, involving the solubilization of pine bark powder to yield a less astringent pine extract. Herein, an autoclave extraction method has been performed using sodium metabisulfite as the solubilizing agent. The primary objective of this study is to conduct a comparative analysis of the chemical composition between *Pinus halepensis* bark extract and *Pinus halepensis* bark powder, focusing on polyphenolic compounds, tannins, and non-tannins. This evaluation aims to ascertain whether the extraction process yields a polyphenol profile more conducive to tanning.

2. Material and Methods

Spain holds the tenth position globally and the fourth in Europe concerning forest science production, reflecting its efficacy in socio-economic roles pertaining to forest resources. Recent trends reveal a notable decline in employment within the forest industry, amounting to a reduction of 39%. This decline coincides with a focus on exploiting timber sources from fast-growing species such as Pinus Pinaster and Eucalyptus spp. on privately owned lands. This shift is underscored by a substantial disparity emerging between rural agricultural regions and urban centers.

The abandonment of agricultural and forested areas exacerbates the accumulation and exposure of significant biomass volumes, elevating the susceptibility to fire hazards. Spanish forests harbor a timber volume surpassing one billion cubic meters, predominantly

composed of *Pinus sylvestris*, *Pinus Halepensis* and *Pinus pinaster* species. Consequently, the study focuses on bark samples in a pulverized state originating from the exploitation residues of Pinus halepensis pine forests in Spain. The diminished commercial value of its timber relative to other species stems from the copious resin content and the diminutive, contorted morphology it exhibits.

In this context, the use of pine bark extract aims to investigate a novel approach to obtaining tannins from a sustainable source, namely residues from Mediterranean pine forest logging. To start this approach, the aim of this work is characterizing polyphenols in pine bark powder and pine bark extract to identify and quantify the different types of polyphenolic compounds present.

The initial phase entails the preparation of pine bark samples, both in powdered form and as an extract, for subsequent analysis. This process involves comminuting the pine bark material into a finely powdered state with particle sizes of 50 μm (referred to as pine bark powder), alongside the extraction of polyphenolic compounds utilizing a suitable solvent (referred to as pine bark extract).

The heterocyclic reactivity of tannins occurs under both acidic and alkaline conditions. Catalyzed rearrangements, such as hydrolysis and self-condensation, are common reactions for tannins. Interflavonoid bond cleavage can be catalyzed either through acidic pathways or by sulfonation reaction induction [39]. Hydrolysis and self-condensation processes may take place under strongly acidic conditions. Degradation in acidic medium allows for the formation of catechins and anthocyanins. If acidic hydrolysis of heterocyclic tannins is followed by condensation, a type of reddish tannic substance called flavaphenes is formed. In basic hydrolysis, the rearrangements differ. These rearrangements are based on the cleavage of the C_4–C_8 interflavonoid bond. Reactivity depends on the nature of the tannins. The formed products may undergo self-condensation, leading to alkaline condensation. A second possible reaction involves heterocyclic opening, which enhances reactivity, resulting in partial self-condensation, as observed with catechin monomers. In this study, to obtain the pine bark extract, sulfonation was performed. Sulfonation is a process for tannin solubilization involving sodium bisulfite or sodium bisulfate, both of which facilitate heterocycle opening and insertion of the sulfonic group ($-SO_3^-Na^+$) at position 2 after ring opening. This polar group increases solubility and reduces tannin viscosity.

To obtain the pine bark extract, the ground pine bark powder is subjected to a temperature of 100 °C for 1 h in an autoclave, along with water in a 1:5 ratio and the addition of a 2% sodium metabisulphite. Subsequently, the obtained liquid is filtered and atomized, resulting in a powder form of pine bark extract.

The following step is to determine the polyphenol profiling in both samples: pine bark powder and pine bark extract by using high-performance liquid chromatography (HPLC) and HPLC coupled with mass spectrometry HPLC DAD-ESI-TOF/MS (electrospray ionization, high resolution). Its basic principle revolves around the elution of molecules within the chromatographic column, which are subjected to pressure. Direct detection of these molecules by the mass spectrometer detector (MSD) is unfeasible due to the spectrometric unit operating under vacuum conditions. Therefore, it necessitates the liquid to pass through an interface, the electrospray ionization (ESI) system (Figure 1), which eliminates the mobile phase used in the chromatograph and transfers the analyte to the chromatographic unit. The time-of-flight (TOF) analyzer measures the time ions take to travel a fixed distance, enabling the calculation of the mass-to-charge ratio. The equation linking the m/z ratio with time is as follows:

$$m/z = e \times \mathrm{E} \times (\frac{t}{d})^2$$

where

e = electron charge ($1.60217733 \times 10^{19}$ Coulomb)

E = potential electric field of the extraction pulse (V)

t = ion time-of-flight (s)
d = length of the free-field drift region (m)

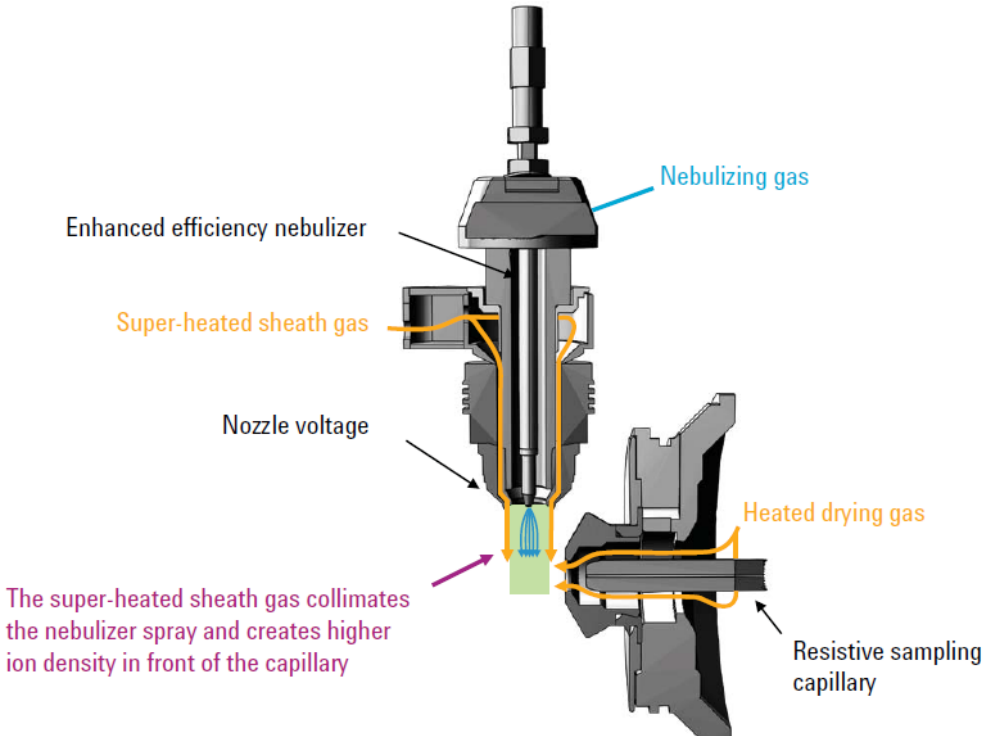


Figure 1. ESI-MS Agilent Jet Stream technology.

The integration of the ESI source with the TOF analyzer in an ESI-TOF/MS tandem setup serves as a valuable tool for conducting comprehensive characterization studies of plant extracts.

The mass detector measures the m/z ratio of ions by subjecting them to an electric or magnetic field, which can manipulate ion movement, allowing for their classification based on mass. This detector is capable of measuring and amplifying the current ion to quantify the quantity of classified ions.

In scenarios involving two mass detectors, molecules exhibiting specific m/z ratios can be selected for further analysis, wherein ions undergo fragmentation via collision-induced dissociation or other fragmentation processes. Subsequently fragmented ions can be detected by a secondary mass spectrometric unit.

To evaluate the response characteristics of various samples and optimize operational parameter values, direct infusion [ESI (−)] analysis was conducted on prepared solutions of all samples. Direct infusion involves the introduction of the analyte without passage through any chromatographic column, traversing through the interface, analyzer, and mass detector.

The assay was performed using an LC/MSD-TOF (liquid chromatography/mass selective detector − time-of-flight) system from Agilent Technologies by direct introduction. This setup employs electrospray ionization (ESI-MS) for the mass spectrometry technique, specifically configured to read negative ions. The spectrometer conditions were established using a capillary voltage of 4000 V in positive mode and 3500 V in negative mode, with a drying gas flow rate of 10.0 L/min, a nebulizer pressure of 30 Psi, a fragmenter voltage of 175 V for positive mode, and a scanning range from 105 to 2000 m/z. The source was equipped with a dual nebulizer, enabling the simultaneous introduction of an internal reference through an independent nebulizer for accurate mass measurement.

For the analysis of the samples, solutions with a concentration of 800 ppm of each were prepared in ultrapure water. After preparation, the solutions were homogenized by

vortexing, followed by 10 min of sonication, and filtration through filter paper, followed by a 0.45-micron syringe filter before analysis by LC/MS. Subsequently, 10 µL of the prepared sample solution were injected into the spectrometer via the Agilent 1100 HPLC system using a mixture of acetonitrile and water (CH_3CN/H_2O) 1:1 acidified with 0.1% formic acid (HCOOH) as the eluent at a flow rate of 220 µL/min.

Once identified, the total polyphenol content had been quantified according to standard ISO 14088:2020 Quantitative analysis of tanning agents by filter method used in the leather sector [39]. Additionally, the individual polyphenols had been quantified using calibration curves generated from standard solutions of the respective compounds. Quantification is typically expressed as milligrams or micrograms of specific polyphenols per gram of sample. The equipment used is an HPLC Alliance from Waters. An XBridge Phenyl separation column (3.5-micron particle size, 15 cm length, 130 Å pore diameter from Waters) is used with a PDA detector between 200 and 400 nm. Specifically, at 271.1 nm. XBridge Phenyl columns have applicability in separations where alternative selectivity is needed, especially as the analytes of interest contain an aromatic ring. These columns offer alternative selectivity over straight chain alkyl columns, providing great flexibility in difficult-to-resolve separations. The mobile phase was composed of eluent A (ultrapure water acidified with 0.1% formic acid) and eluent B (acetonitrile acidified with 0.1% formic acid). The reverse phase working method is constituted by a gradient of eluents according to the following work program: 95% A-5% B from 0 to 6 min, 74% A-26% B, from 6 to 30 min, 0% from A-100% B from 30 to 34 min. The mobile phase flow is 1 mL/min. The working temperature of the column was set at 35 °C. All the reagents used are of the necessary quality for HPLC assays. All the patterns and reagents used meet the quality standards required for HPLC tests. All patterns analyzed are purchased from Extrasynthése (France). Millipore quality ultrapure water is used for the preparation of the solutions. All solutions are filtered through 0.45 µm nylon filters prior to HPLC-DAD analysis.

Finally, the data obtained from the analytical techniques have been analyzed to compare the polyphenolic composition of pine bark powder and pine bark extract.

3. Results and Discussion

Polyphenols are secondary metabolites encompassing a diverse array of chemical structures such as flavonoids, phenolic acids, and lignans. Pine bark polyphenols are primarily composed of oligomeric and polymeric proanthocyanidins, also known as condensed tannins. These compounds are characterized by their flavan-3-ol units, primarily catechin and epicatechin, linked through C4-C8 or C4-C6 bonds. Additionally, pine bark contains monomeric flavonoids such as taxifolin and catechin, along with phenolic acids like ferulic acid and caffeic acid.

In Figure 2, powdered pine bark and pine bark extract are depicted for the identification and quantification of diverse polyphenolic compounds. Initially, both samples underwent analysis according to ISO 14088:2020 standards to determine the content of tannins and non-tannins within the two vegetal matrices [39]. This method entails the assessment of tannin content, quantities of insoluble and soluble substances, as well as the total solids and water content using gravimetric techniques.

The results obtained for both samples can be seen in Table 1.

Table 1. Results of the ISO 14088:2020 assay [39].

Parameter	Units	Pine Bark Powder	Pine Bark Extract
Non-tannins	%	4.9	33.5
Soluble substances	%	31.0	78.6
Tannins	%	26.2	45.1
Insoluble substances	%	62.2	14.0
Water	%	6.8	7.4
pH solution		6.0	4.5

Figure 2. Samples. **Left**: pine bark powder, **right**: pine bark extract.

The results presented in Table 2 corroborate the effective performance of the extraction and atomization process, yielding an average moisture percentage of only 7.4%. The tannin content increases from 26.2% to 45.1%, representing a 72.1% increment. Additionally, there is a 77.5% reduction in insoluble substances. These findings suggest that pine bark extract will not merely react superficially with the leather surface but rather facilitate its penetration into the leather and enhance binding with the amino acid groups of collagen within the leather.

After quantifying the tannins in both samples in accordance with leather sector standards, polyphenol profiling was conducted using HPLC coupled with mass spectrometry, specifically HPLC DAD-ESI-TOF/MS.

In order to facilitate compound identification, specific polyphenolic compounds were selectively chosen from our database, encompassing lignan compounds previously identified in pine bark samples [40], as well as lignin monomers such as sinapyl, coniferyl, and p-coumaryl alcohols. Notably, substantial concentrations of lignin (23%) have been detected in the bark of Pinus pinaster [41]. Lignins are characterized by their composition of phenolic compounds polymerized with sugars; they exhibit insolubility in acidic conditions but solubility in strong alkalis like NaOH. Soluble lignin derivatives exhibit a broad range of molecular masses, although the majority of values for isolated lignins typically fall within the range of 1000 to 1200. The rationale behind selecting the aforementioned monomers stems from the fact that lignins are copolymers derived from three fundamental monomeric units of phenylpropane (monolignols): p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol.

Figure 3 depicts the ESI-TOF (−) mass spectrum corresponding to the pine bark powder sample, while Figure 3 illustrates the ESI-TOF (−) mass spectrum corresponding to the pine bark extract.

Using ESI (−), ions ionized with negative charge are detected, where compounds lose a proton upon ionization. The mass spectra depicted in Figure 3 exhibit m/z values ranging from 105 to 1000 Da, despite the extended range up to 2000 Da. This indicates that the pine bark powder sample did not contain precursor ions or mass fragments with values exceeding 1000 Da. The sample predominantly exhibits m/z values of 303, 191, 160, 248, 162, 304, 311, and 577. Those m/z values exceeding 500 meet the minimum measure of tannins, characteristic polyphenols with a tanning effect.

The mass spectra depicted in Figure 4 exhibit m/z values ranging from 105 to 1000 Da, despite the extended range up to 2000 Da, indicating the absence of precursor ions or mass fragments exceeding 1000 Da in the pine bark extract sample. Employing ESI (−), the samples predominantly present m/z values of 303, 369, 161, 261, 657, and 198. Those m/z values surpassing 500 meet the minimum criterion for tannins, characteristic polyphenols with a tanning effect.

Table 2. Polyphenol profiling in both samples: pine bark powder and pine bark extract.

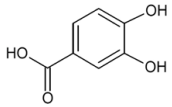
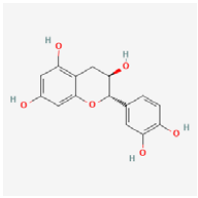
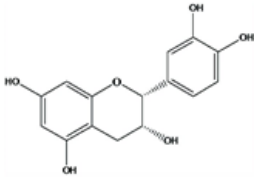
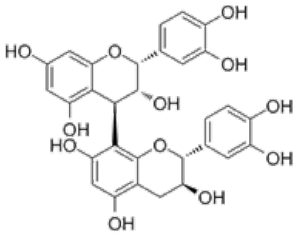
	Formula	Sample	Retention Time (minutes)	[M-H]$^-$ Theoretical	[M-H]$^-$ Experimental	Error (ppm)	Compound Defined by [M-H]$^-$
1	$C_7H_6O_4$ 	Powder	5.38	153.0193	153.0196	1.88	Protocatechuic acid
		Extract	5.46	153.0193	153.0193	0.33	Protocatechuic acid
2	$C_{15}H_{14}O_6$ 	Powder	12.22	289.0718	289.0719	0.36	(+)-catechin
		Extract	12.33	289.0718	289.0714	1.09	(+)-catechin
3	$C_{15}H_{14}O_6$ 	Powder	16.08	289.0718	289.0718	0.08	(−)-epicatechin
		Extract	16.77	289.0718	289.0715	0.85	(−)-epicatechin
4	$C_{30}H_{26}O_{12}$ 	Powder	11.58	577.1351	577.1357	0.99	Procyanidin B1
		Extract	11.71	577.1351	577.1353	0.93	Procyanidin B1

Table 2. *Cont.*

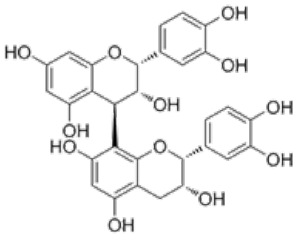
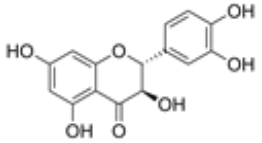
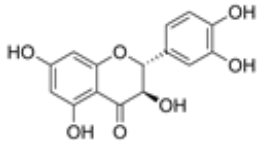
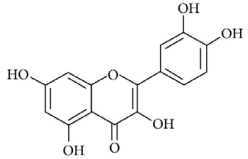
	Formula	Sample	Retention Time (minutes)	[M-H]$^-$ Theoretical	[M-H]$^-$ Experimental	Error (ppm)	Compound Defined by [M-H]$^-$
5	$C_{30}H_{26}O_{12}$ 	Powder	12.59	577.1351	577.1350	0.18	Procyanidin B2
		Extract	12.64	577.1351	577.1362	1.79	Procyanidin B2
6	$C_{15}H_{12}O_7$ 	Powder	22.71	303.0510	303.0505	1.84	(+)-taxifolin
		Extract	22.83	303.0510	303.0509	0.44	(+)-taxifolin
7	$C_{15}H_{12}O_7$ 	Powder	23.75	303.0510	303.0505	1.61	(−)-taxifolin
		Extract	23.87	303.0510	303.0508	0.66	(−)-taxifolin
8	$C_{15}H_{10}O_7$ 	Powder	- - -	301.0354	n.d.	n.d.	- - -
		Extract	32.21	301.0354	301.0352	0.69	Quercetin

Table 2. *Cont.*

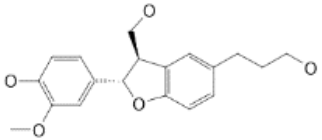
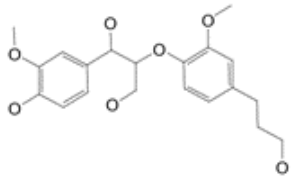
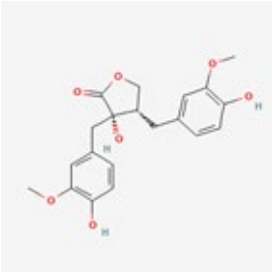
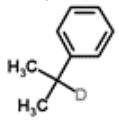
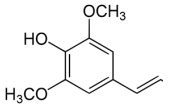
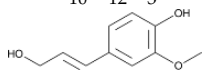
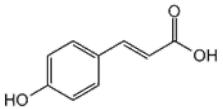
	Formula	Sample	Retention Time (minutes)	[M-H]$^-$ Theoretical	[M-H]$^-$ Experimental	Error (ppm)	Compound Defined by [M-H]$^-$
9	$C_{19}H_{22}O_5$ 	Powder	30.44 (1)	329.1394	329.1392	0.69	4-[3-hydroxymethyl-5-(3-hydroxypropyl)-2,3-dihydroxyben-zofuran-2-yl]-2-methoxyphenol
		Extract	30.55 (1)	329.1394	329.1391	1.01	4-[3-hydroxymethyl-5-(3-hydroxypropyl)-2,3-dihydroxyben-zofuran-2-yl]-2-methoxyphenol
10	$C_{20}H_{26}O_7$ 	Powder	n.d.	377.1606	n.d.	n.d.	1-[-4-hydroxy-3-methoxyphenyl)-2-[4-(3-hydroxypro-pyl)-2-ethoxypheno-xy]-propane-1,3-diol
		Extract	n.d	377.1606	n.d.	n.d.	1-[-4-hydroxy-3-methoxyphenyl)-2-[4-(3-hydroxypro-pyl)-2-ethoxypheno-xy]-propane-1,3-diol
11	$C_{20}H_{22}O_7$ 	Powder	31.76 (1)	373.1293	373.1296	1.32	Pinopalustrin
		Extract	31.87 (1)	373.1293	373.1298	1.82	Pinopalustrin
12	C_9H_{12} 	Powder	n.d.	119.0866	n.d.	n.d.	Phenylpropane
		Extract	n.d.	119.0866	n.d.	n.d.	Phenylpropane

Table 2. *Cont.*

	Formula	Sample	Retention Time (minutes)	[M-H]$^-$ Theoretical	[M-H]$^-$ Experimental	Error (ppm)	Compound Defined by [M-H]$^-$
13	$C_{11}H_{14}O_4$ 	Powder	n.d.	209.0819	n.d.	n.d.	Sinapic alcohol
		Extract	n.d.	209.0819	n.d.	n.d.	Sinapic alcohol
14	$C_{10}H_{12}O_3$ 	Powder	n.d.	179.0714	n.d.	n.d.	Coniferyl alcohol
		Extract	n.d.	179.0714	n.d.	n.d.	Coniferyl alcohol
15	$C_9H_{10}O_2$ 	Powder	n.d.	149.0608	n.d.	n.d.	p-Coumaric alcohol
		Extract	n.d.	149.0608	n.d.	n.d.	p-Coumaric alcohol

n.d. Not detected. (1) Possible presence is detected as a mixture of compounds.

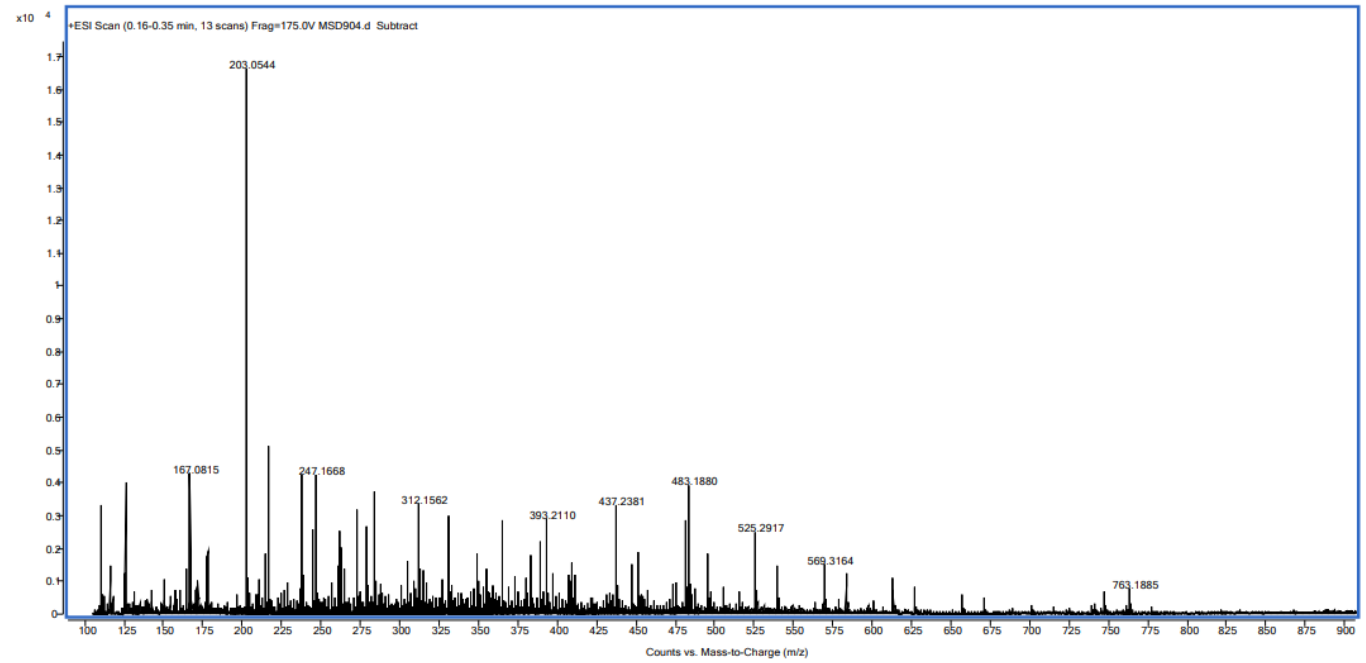


Figure 3. ESI-TOF (−) mass spectrum of pine bark powder.

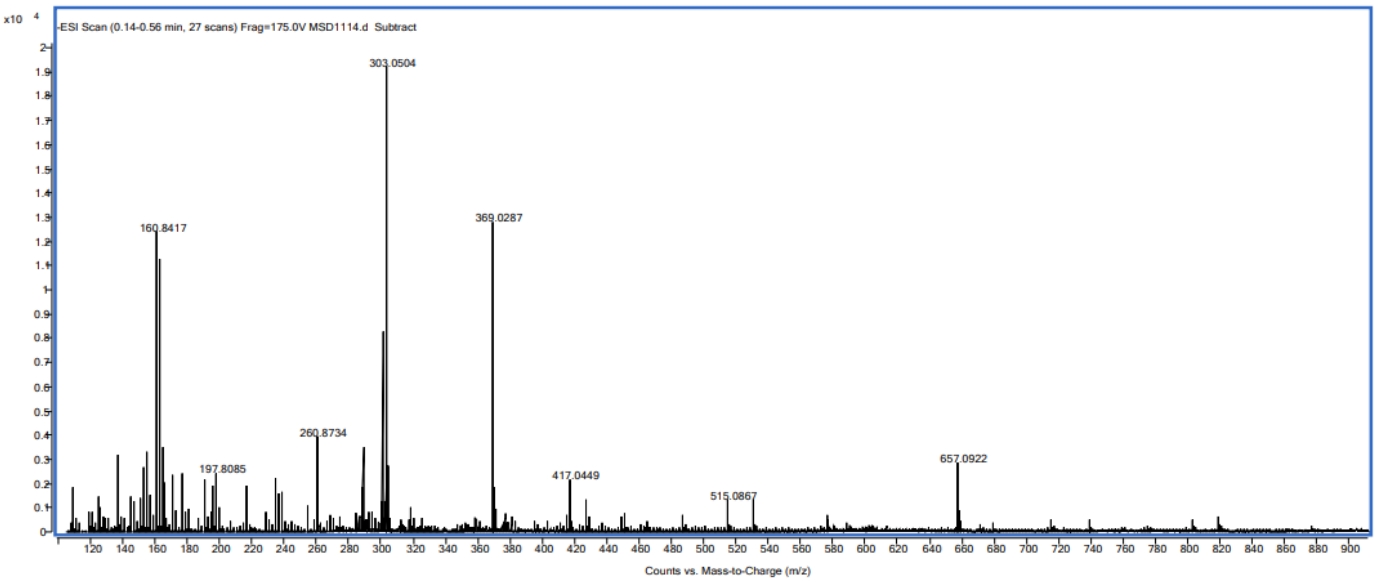


Figure 4. ESI-TOF (−) mass spectrum of pine bark extract.

Table 2 provides a comprehensive breakdown, including the empirical formula of the compound, the theoretical and experimental molecular mass following the loss of a proton $[M\text{-}H]^-$, the associated error expressed in parts per million (*ppm*), and the potential compound corresponding to the empirical formula.

The computation of the error in *ppm* is derived from the formula:

$$E(ppm) = \frac{(m/z\ theoretical - m/z\ experimental) \times 10^6}{m/z\ theoretical}$$

The presence of (+)-catechin, (+)-taxifolin, protocatechuic acid, and procyanidin B2 in both samples can be confirmed. Compounds such as pinopalustrin, (−)-taxifolin, quercetin, (−)-epicatechin, and 4-[3-hydroxymethyl-5-(3-hydroxypropyl)-2,3-dihydro-xybenzofuran-

2-yl]-2-methoxyphenol have been indicated as possible constituents in the samples. Their presence in the samples should be validated through testing against the respective compound standards. Among the non-tannic substances, derivatives of quercetin, flavan-3-ols, and possible lignans corresponding to the empirical formulas introduced in the chromatographic program and supported by relevant literature can be distinguished.

Regarding tannic substances, catechin dimers with tanning capability are detected. Lignin, a high molecular weight polyphenolic compound (10,000 u), is highly insoluble. Its presence in the tanning bath hinders the penetration of other polyphenolic compounds with tanning capability into the leather. In a quest for soluble lignin fractions resulting from tannin extraction, the results have been negative: (1) no m/z values exceeding 1000 are detected, and (2) m/z values of the basic monomers forming lignin (sinapic acid, coniferyl acid, and p-coumaric acid) are not detected. The possible lignin present in the two samples under study has been removed through physical separation via filtration.

Finally, the individual polyphenols had been quantified using calibration curves generated from standard solutions of the respective compounds.

Specifically, protocatechuic acid, (+)-catechin, (+)-taxifolin, and (−)-epicatechin have been quantified utilizing the high-performance liquid chromatography (HPLC-DAD) method. Chromatograms obtained for the two respective samples can be observed in Figures 5 and 6. In both figures, there are three overlapping lines because the experiment was performed in triplicate. In Table 3, the quantities of individual polyphenols mentioned present in the two samples can be observed.

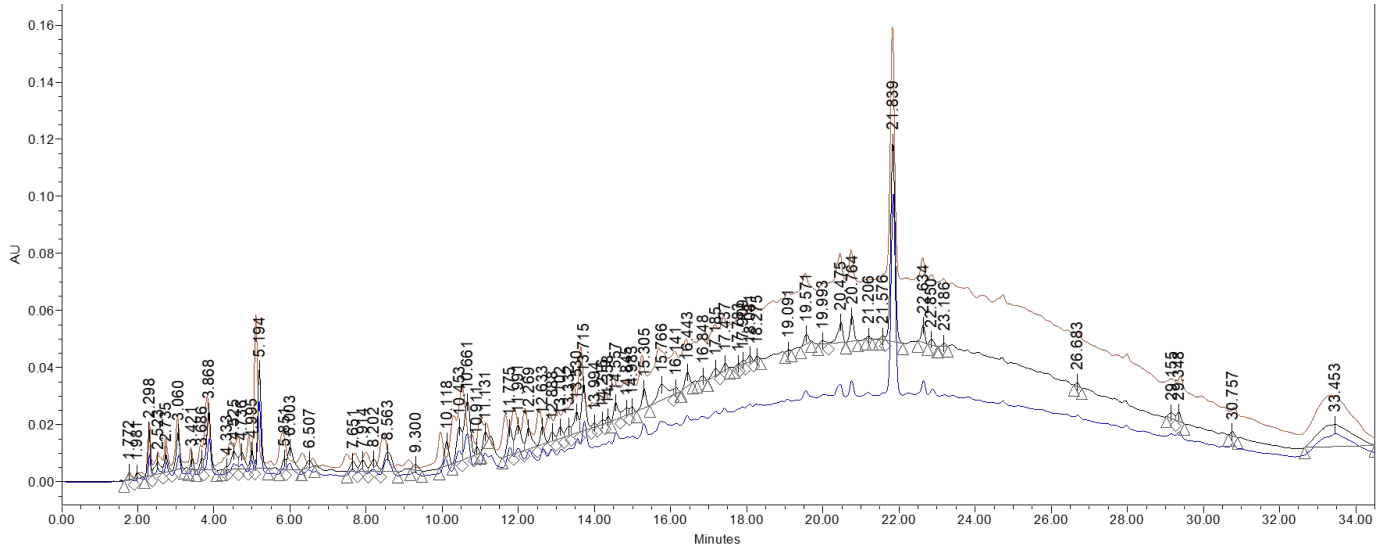


Figure 5. HPLC_DAD spectrum of pine bark powder.

Protocatechuic acid, (+)-catechin, (+)-taxifolin, and (−)-epicatechin are present in both powder and extract forms, with significantly higher concentrations in the extract form compared to the powder form.

Procyanidin A1, A2, B1, and B2 are mostly present in the extract form, with only a few being detected in the powder form.

The R^2 values for the calibration curves are very high (close to 1), indicating strong linear relationships between the known concentrations and the measured values, which suggests the reliability of the analytical method used.

These results provide valuable insights into the composition and concentration of polyphenols in the analyzed samples, showing that Pinus halepensis bark extract may have an application for the leather industry.

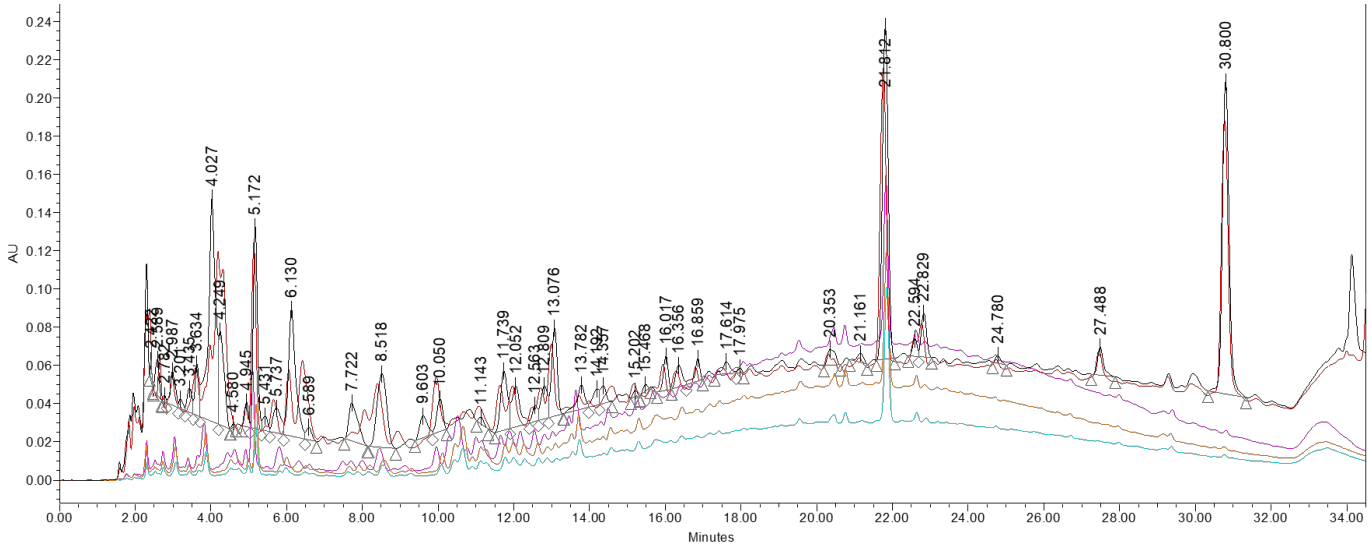


Figure 6. HPLC_DAD spectrum of pine bark extract.

Table 3. Quantities of individual polyphenols.

Polyphenol	Sample	Retention Time (min)	R^2 Calibration Curve	Concentration mg/kg
Protocatechuic acid	Powder	5.10	0.9995	68.4
Protocatechuic acid	Extract	5.17	0.9995	1214.3
(+)-catechin	Powder	11.78	0.9996	98.4
(+)-catechin	Extract	11.74	0.9996	2098.0
(+)-taxifolin	Powder	21.84	0.9994	242.2
(+)-taxifolin	Extract	21.81	0.9994	4017.0
(−)-epicatechin	Powder	16.13	0.9979	360.3
(−)-epicatechin	Extract	16.02	0.9979	2163.0
Procyanidin A1	Powder	21.57	0.9999	19.47
Procyanidin A1	Extract	n.d.	0.9999	n.d.
Procyanidin A2	Powder	n.d.	0.9999	n.d.
Procyanidin A2	Extract	n.d.	0.9999	n.d.
Procyanidin B1	Powder	11.13	1.0000	59.3
Procyanidin B1	Extract	11.13	1.0000	917.0
Procyanidin B2	Powder	15.76	1.0000	85.9
Procyanidin B2	Extract	15.66	1.0000	101.0

4. Conclusions

The research focused specifically on characterizing polyphenols in *Pinus halepensis* bark powder and *Pinus halepensis* bark extract. The effective performance of the extraction and atomization process, yielding an average moisture percentage of only 7.4% has been demonstrated. The tannin content increases from 26.2% to 45.1%, representing a 72.1% increment. Additionally, there is a 77.5% reduction in insoluble substances.

The use of HPLC coupled with mass spectrometry for the identification and quantification of diverse polyphenolic compounds present in the samples has been described. The presence of (+)-catechin, (+)-taxifolin, protocatechuic acid, and procyanidin B2 in both samples have been confirmed. Regarding tannic substances, catechin dimers with tanning capability have been detected. The possible lignin present in the two samples under study has been removed through physical separation via filtration. Protocatechuic acid, (+)-catechin, (+)-taxifolin, and (−)-epicatechin are present in both powder and extract forms, with significantly higher concentrations in the extract form compared to the powder form.

Specifically, Pinus halepensis bark extract contained 1214.3 mg/kg of protocatechuic acid, 2098.0 mg/kg of (+)-catechin, 4017.0 mg/kg of (+)-taxifolin, 2163.0 mg/kg of (−)-epicatechin and 917.0 mg/kg of Procyanidin B1.

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Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

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

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