P48: EXTENSIVE CHARACTERIZATION OF PHENOLIC COMPOSITION FROM PINE BARK CONCENTRATED EXTRACT AS A FOOD SUPPLEMENT BY HPLC-ESI/DAD-QTOF-MS

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Abstract – Pine bark is used as a dietary supplement and phytochemical remedy for several diseases. A comprehensive characterization of the bioactive compounds using advanced and powerful techniques as HPLC-ESI-QTOF-MS and their antioxidant capacity are crucial in food supplement studies. A total of 37 compounds were identified belonging to various structural classes being the most representative ones flavan-3-ols (oligomeric forms). Moreover, the extract showed antioxidant activity decreasing the generation of ROS and high values were obtained by TPC and flavan-3-ol content assays.

Keywords: pine bark, proanthocyanidins, HPLC, antioxidant, food

1. INTRODUCTION

Medicinal and spice plants, which are well known for their pharmacological activity, contain many substances that exhibit radical-scavenging properties as phenolic compounds. These compounds, which are secondary plant metabolites, are an essential part of the human diet. They are of considerable interest due to their suggested advantageous health effects and possibility for use as natural food additives, since they influence the quality and stability of foods by acting as flavourants, colourants, and antioxidants [1].

Proanthocyanidins are found in many woody plants. The two most common sources of them are grape seeds (*Vitis vinifera*) and white pine (*Pinus maritima*, *Pinus pinaster*) but are also abundant in green tea (*Camellia sinensis*), hawthorn (*Crataegus oxyacantha*. These compounds are oligomers and polymers of flavan-3-ol monomer units most frequently linked either C4 → C6 or C4 → C8 (B-type proanthocyanidins). A-type proanthocyanidins possess a second interflavanoid bond, resulting in oxidative coupling between the C2 → O7 positions. The most common classes are procyanidins consisting of catechin, epicatechin and/or their gallic acid esters [2, 3].

Pine (*Pinus sylvestris L.*) tree bark is also valued medicinally for its rich content of proanthocyanidins. Pine bark extracts have been used as a folk medicine, and are used as a dietary supplement and phytochemical remedy for several diseases (pycnogenol) [4]. It has also been shown to be a very powerful antioxidant and free radical-scavenger, even more powerful than either vitamin C or vitamin E. Pine bark extract is used in cardiovascular and heart formulas, and has also been shown to be beneficial to those with chronic venous insufficiency. Procyanidins occurring in pine bark consist mainly of the flavan-3-ol units of (+)-catechin [5, 6].

In this work, procyanidin-rich extract from pine bark was analysed and compared by HPLC coupled to a quadrupole time-of-flight (QTOF) mass spectrometer and equipped with an ESI interface. Additionally, we wanted to determinate the antioxidant potential present in the extract by three complementary antioxidant activity methods: TEAC, FRAP, and ORAC. We also wanted to evaluate the total phenolic and flavan-3-ol contents by Folin-Ciocalteu and Vanillin assays.

2. EXPERIMENTAL

2.1. Sample preparation

Concentrated pine bark extract (Nutrafur, Spain) was used in this study. The polyphenols from whole extract were analytically characterised using a 10 mg/mL solution of the extract in DMSO.

2.2. Instrumentation

Analytical characterisation of pine bark extract was performed using an Agilent 1200 series rapid-resolution LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a binary pump, an autosampler, and a diode array detector (DAD). The HPLC system was coupled to a quadrupole time-of-flight mass spectrometer (QTOF) mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ESI interface (model G1607A from Agilent Technologies, Palo Alto, CA). Fluorescence (ORAC) and absorbance (Folin-Ciocalteu assay, Vanillin assay, FRAP, and TEAC) measurements were carried out on a Synergy Mx Monochromator-Based Multi-Mode Micro plate reader (Bio-Tek Instruments Inc.) using 96-well polystyrene microplates.

2.3. Chromatographic, UV, and spectrophotometric conditions

The compounds from pine bark extract were separated at room temperature using a Zorbax Eclipse Plus C18 column (1.8 µm, 150 × 4.6 mm). The mobile phases consisted of 0.5% acetic acid (A) and methanol (B). The following multi-step linear gradient was applied: 0 min, 0% B; 5 min, 25% B; 15 min, 35% B; 20 min, 39% B; 38 min, 60% B; 40 min, 70% B; 42 min, 80% B; 44 min, 100% B; 46 min, 0% B; and 48 min, 0% B. The initial conditions were held for 10 min. The injection volume was 10 µL and the flow rate was 0.3 mL/min. For the spectrophotometric conditions for antioxidant assays, the excitation and emission wavelengths were 485 and 520 nm, respectively, for the ORAC assay. The absorbance wavelengths for Folin-Ciocalteu, Vanillin assay, FRAP, and TEAC assays were 760, 500, 593, and 734 nm, respectively.

2.4. ESI-QTOF-MS detection

The HPLC system was coupled to a QTOF mass spectrometer equipped with an ESI interface operating in negative ion mode using a capillary voltage of +3.5 kV. The other optimum values of the source parameters were: drying gas temperature, 220 °C; drying gas flow, 9 L/min; and nebulising gas pressure, 2.5 bar. The detection was performed for a mass range of 50–1200 m/z.

The accurate mass data of the molecular ions were processed through the DataAnalysis 4.0 software (Bruker Daltonics), which provided a list of possible elemental formulas using Generate Molecular Formula Editor.

2.5. Total phenolic and flavan-3-ol contents and antioxidant capacity assays

The Total phenolic content was measured by the Folin-Ciocalteu method reported by [7], with some modifications. The phenol content was calculated based on the calibration curves of gallic acid and expressed as mg GAE/g of dry matter. Measurements were made in triplicate.

Flavan-3-ol content was calculated based on the calibration curves of (+)-catechin using a method described in [8] and expressed as mg CE/g of dry matter. Measurements were made in triplicate.

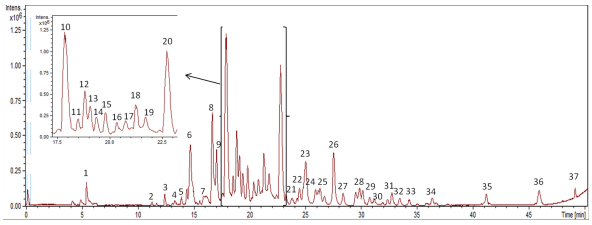
The TEAC, FRAP and ORAC antioxidant assays were performed by using a previously described method [9]. TEAC values were calculated using Trolox as the standard. FRAP values were calculated using $FeSO_4 \cdot 7H_2O$ as the standard. And the final ORAC values were calculated using a regression equation between the Trolox concentration and the net area of the fluorescence decay curve (area under curve, AUC), as previously described in *Laporta et al.* [10]. Measurements were made in triplicate.

3. RESULTS AND DISCUSSION

3.1. Characterization of polar compounds by HPLC-ESI-QTOF-MS

A comprehensive characterisation of phenolic compounds using advanced and powerful techniques is crucial. For this reason, suitable methods need to be established for their characterisation in vegetable matrices. The use of QTOF technologies allows for the exact mass measurements of both MS and MS/MS ions to be achieved, which is essential for elemental composition assignment and, thus, for the characterisation of small molecules [9, 11].

Figure 1. Base peak chromatogram of pine bark extract.



A total of 37 compounds distributed in three major categories (flavan-3-ols and derivatives, flavonols, and other compounds) were analysed in the present study. Figure 1 shows the base peak chromatogram (BPC) of the pine bark extract. The major peaks, which were identified based on elution order, are listed in Table 1. All of the compounds were characterised by interpretation of their mass spectra obtained by the QTOF-MS, and also by taking into account previously reported data.

Table 1. Compounds characterized in pine bark extract by HPLC-ESI-QTOF-MS and MS/MS in negative mode.

Peak	Proposed compounds
1	Sucrose
2	Procyanidin C
3	Gardenoside
4	Procyanidin A (isomer 1)
5	Procyanidin A (isomer 2)
6	Procyanidin B (isomer 1)
7	Procyanidin B (isomer 2)
8	Chalcan-flavan-3-ol dimer (isomer 1)
9	Procyanidin trimer A-type (isomer 1)
10	(-)-Epicatechin
11	Chalcan-flavan-3-ol dimer (isomer 2)
12	Chalcan-flavan-3-ol dimer (isomer 3)
13	Chalcan-flavan-3-ol dimer (isomer 4)
14	Procyanidin trimer A-type (isomer 2)
15	Chalcan-flavan-3-ol dimer (isomer 5)
16	Chalcan-flavan-3-ol dimer (isomer 6)
17	(Epi)fisetinidol-(epi)catechin (isomer 1)
18	Procyanidin A (isomer 3)
19	(Epi)fisetinidol-(epi)catechin (isomer 2)
20	(+)-catechin
21	(Epi)fisetinidol-(epi)catechin (isomer 3)
22	(Epi)fisetinidol-(epi)catechin (isomer 4)
23	Procyanidin A (isomer 4)
24	(Epi)fisetinidol-(epi)catechin (isomer 5)
25	Procyanidin A (isomer 5)
26	Procyanidin A (isomer 6)
27	(Epi)fisetinidol-(epi)catechin (isomer 6)
28	(Epi)fisetinidol-(epi)catechin (isomer 7)
29	Procyanidin A (isomer 7)
30	(Epi)fisetinidol-(epi)catechin (isomer 8)
31	Procyanidin A (isomer 8)
32	(Epi)fisetinidol-(epi)catechin (isomer 9)
33	Quercetin rhamnosylrutinoside
34	Rutine
35	Isorhamnetin rutinoside
36	Quercetin
37	Kaempferol

3.2. Total phenolic and flavan-3-ol contents and in vitro antioxidant activities of pine bark extract

The obtained values for each assay are shown in Table 2. By comparing all our assays, the extract showed high values of antioxidant activities, and total phenolic and flavan-3-ol contents. This could be a result of our sample being rich in flavan-3-ol, mainly the oligomeric forms. Other sources, which have been reported to contain oligomeric flavan-3-ols (i.e. cocoa), showed similar antioxidant capacity values [9].

Table 2. Values for different antioxidant measurements performed. Values are expressed as mean ± SD.

Assays	Values
Folin-Ciocalteu[a]	847, 62 ± 39,74
Vanillin assay[b]	883,33 ± 76,38
TEAC[c]	5,72 ± 0,78
FRAP[d]	4,83 ± 0,15
ORAC[c]	8,4 ± 0,4

[a]Expressed in mg gallic acid equivalents g^{-1} extract (dw)
[b]Expressed in mg (+)-catechin equivalents g^{-1} extract (dw)
[c]Expressed in mmol Trolox equivalents g^{-1} extract (dw)
[d]Expressed in mmol $FeSO_4$ equivalents g^{-1} extract (dw)

4. CONCLUSIONS

In the present study, HPLC-ESI-QTOF-MS has been confirmed as a powerful analytical technique for separating and detecting phenolic and other polar compounds in concentrated pine bark extract.

With this method, 37 compounds were tentatively identified based on their chromatographic retention, MS data, and MS/MS fragmentation pattern. The most representative groups of compounds tentatively identified were flavan-3-ols (oligomeric forms). Of these compounds, (epi)fisetinidol-(epi)catechin isomers and other chalcan-flavan-3ol isomers have been tentatively identified for the first time in pine bark.

This extract possess significant antioxidant capacity to reduce peroxyl radicals determinate by ORAC assay. Moreover, pine bark extract showed a strong capacity to donate electrons by FRAP and TEAC assays. Additionally, it had high phenolic and flavan-3-ol contents.

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