Short Term Scientific Mission Report

Valorization of NWFP's for tannin and antioxidants production:

Study of pine and oak barks and pine nut shells

Cost Action FP 1203

Ali Umut Şen

15.05.2014

Preface

The main scientific findings of the short term scientific mission (STSM)carried out by Ali Umut Şen at Institute of Advanced Chemisty of Catalonia (Institut de QuimicaAvançada de Catalunya) Barcelona, Spain under the supervision of Dr. JosepLluís Torres between 26.03.2014 and 25.04.2014 are reported.

1. Introduction

Tannins are described as water-soluble phenolic compounds with molecular weights between 500 and 3000 Da (Bate-Smith and Swain, 1962). Tannins are found in most plants but their concentration is particularly high in tree barks. Among tree barks softwood barks contain considerable amounts of tannins.

Tannins are generally classified as hydrolyzable tannins and condensed tannins. Hydrolyzable tannins are composed of a polyol core (commonly glucose) which is esterified with galloyl groups. Galloyl glucoses and ellagi tannins are examples of hydrolyzable tannins. Condensed tannins are oligomers or polymers of two or more flavan-3-ols (Barbehenn and Constabel, 2011).

Tannins are traditionally used for tanning leathers. Beginning from the last 60 years new utilizations for tannins are studied. These studies include development and production of adhesives for wood-based panelssuch as particleboard, plywood and glulam (i), extraction of nutraceuticals (ii), and production of tannin-based foams (iii) for insulation of doors and wall-panels (Pizzi, 1982; Packer et al. 1999; Tondi et al., 2008).

Antioxidants are molecules that inhibit oxidation reaction which produce free radicals such as hydroxyl and peroxyl radicals and damage cells with these radicals causing several diseases including cancer. Tree barks can be used for extraction of antioxidants as they are often rich in reducing agents such as polyphenols and tannins. The commercial bark extract of *P. pinaster*, pycnogenol is not only a powerful natural antioxidant but also it is amongtop-selling cardiovascular dietary supplements in USA and Europe.

The main objective of the study is to contribute to an efficient valorization of the bark biomass of two oak species (*Quercussuber*and *Q. cerris*) and two pine species (*Pinus pinaster* and *P. pinea*) as well as of pine nut shells (*P. pinea*) by studying the chemical composition of their phenolic extractives from three different granular fractions and by evaluating their antioxidant capacities using electron paramagnetic resonance (EPR) spin-trap method.

2. Materials and Methods

2.1. Materials

Barks samples were obtained from two hardwood species (*Q. suber* and *Q. cerris*), two softwood species (*P. pinaster* and *P. pinea*) and pine nut shells from *P. pinea* from Portugal (*Q. suber*, *P. pinaster*, *P. pinea*) and Turkey (Q. cerris).

2.2. Methods

2.2.1. Extraction of bark polyphenols

40-60 mesh bark samples were defatted with hexane to remove lipophilic extractives. The extracted bark samples were treated with water: acetone: acetic acid (70: 29.5: 0.05 v/v) solution to obtain polyphenol

extracts. The obtained polyphenol extracts were freeze-dried and kept at -20 ºC until the time of MALDI and EPR analyses.

2.2.2. MALDI TOF/ TOF MS Analyses

Matrix-assisted laser desorption adsorption Time of Flight Mass Spectroscopy (MaldiTofMs) is a soft ionization technique that allows to study large molecules such as polyphenols and tannins. In our study we used a tandem mass spectrometer (AutoFLEX III, BrukerDaltonics, Bremen, Germany) and the following conditions (Mateos et al. 2012): The accelerating voltage was 20 kV and the reflectron voltage 21 kV. Spectra were the sum of 500 shots with a frequency of 200 Hz. Both positive and negative reflectron modes were tried. The MS/MS spectra were obtained in the collusion-induced dissociation (CID) mode using Argon as the collusion gas.

2.2.3. EPR Analyses

Electron paramagnetic resonance (electron spin resonance, EPR, ESR) is a powerful technique to measure and identify samples with unpaired electrons. Since the technique is selective to molecules with unpaired electrons it is very useful to detect free radicals.

In this technique, short-lived free radicals are converted to stable free radicals (spin adducts) using spin traps. The most common spin trap is 5,5-dimethyl-1-pyrolline-N-oxide (DMPO). The DMPO reacts with free radicals and forms spin adducts which is measured by EPR Spectroscopy. In our study we used the following conditions: EPR Spectrometer (Bruker EMX-Plus 10/12), microwave frequency 9.876 GHz, microwave power 30.27 mW, modulation frequency 100 kHz, modulation amplitude 1.86 G.

3. Main Scientific Results

3.1. Polyphenol composition of the biomass samples

3.1.1 *P. pinaster* and *P. pinea*

MaldiTOF MS and MaldiTOF/TOF MS analyses revealed that *P. pinaster* and *P. pinea* barks have similar polyphenol compositions. 18 and 20 different polyphenol series were observed in *P. pinaster* and *P. pinea* bark extracts respectively. The main polyphenol is (epi)catechin in both extracts. In *P. pinaster*

bark up to 9 (epi)catechin monomers were observed while in *P. pinea* bark up to 7 (epi)catechin monomers were observed. Jerez et al. (2007) showed that *P. pinaster* bark is composed mainly by epicatechin monomers therefore the 9 monomers observed in *P. pinaster* bark are likely to be epicatechin type. The presence of 763, 1051, 1339 and 1627*m/z* peaks in *P. pinea* bark suggests that bark contains glucosyl-catechin chain.

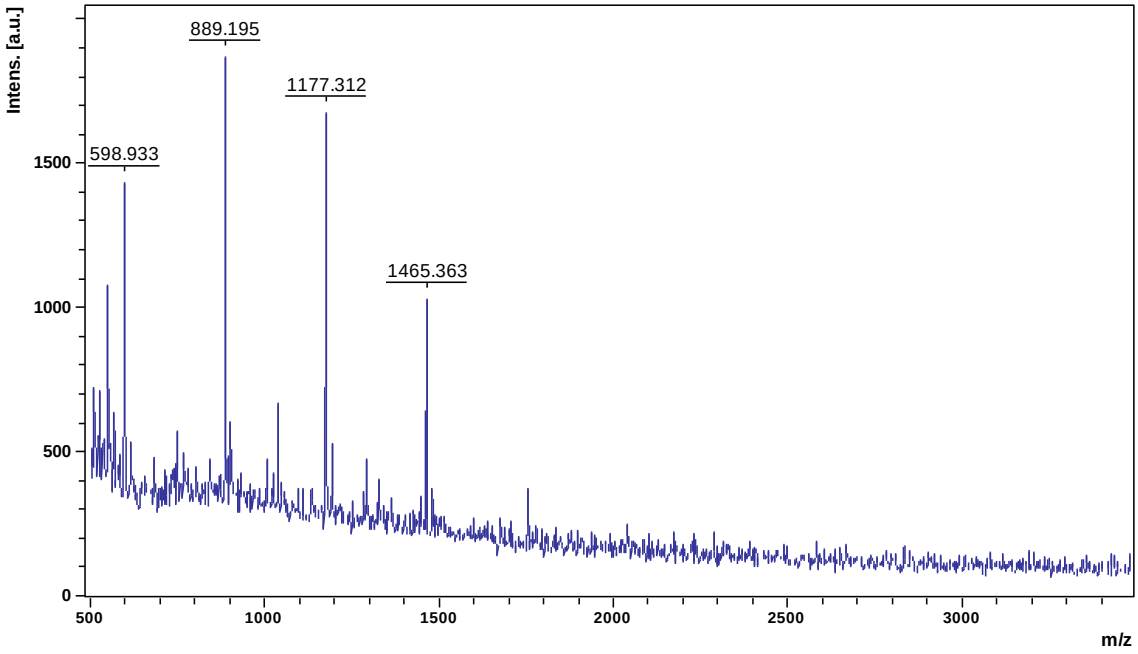

Fig 1. MaldiTOF spectrum of *P. pinaster* bark extract

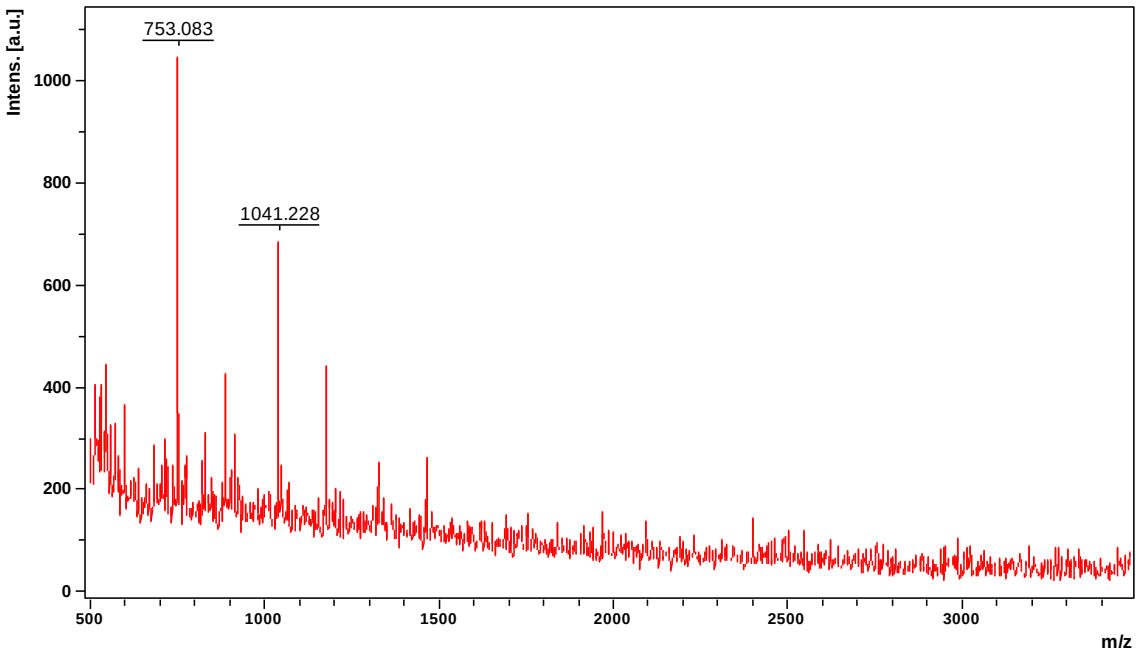

Fig 2. MaldiTOF spectrum of *P. pinea*bark extract

3.1.2 *Q. suber* and *Q. cerris*

MaldiTOF spectra of *Q. suber* and *Q. cerris* cork extracts were studied for the first time. *Q. suber* cork extract contains 17 distinct detected compounds while *Q. cerris* cork contains 28 distinct detected compounds. Seven signals are common to both species (599, 736, 754, 839, 867, 957, and 1229 *m/z*). As *Q. cerris* cork contains phloemic impurities these peaks (without considering possible protein peaks of 736 and 754 *m/z*) indicate common cork polyphenols. The 957 *m/zm/z* peak is the most intens peak in *Q. suber* bark extract.This peak corresponds probably to castalagin/vescalagin, an ellagitannin. *Q. cerris* bark extract contains more compounds and possibly (gallo)catechin monomers (905 *m/z* peak). *Q. suber*extract is reported to contain gallic acid, protocatechuic acid, flavanones (naringenin, eriodictyol) (Santos et al. 2010)

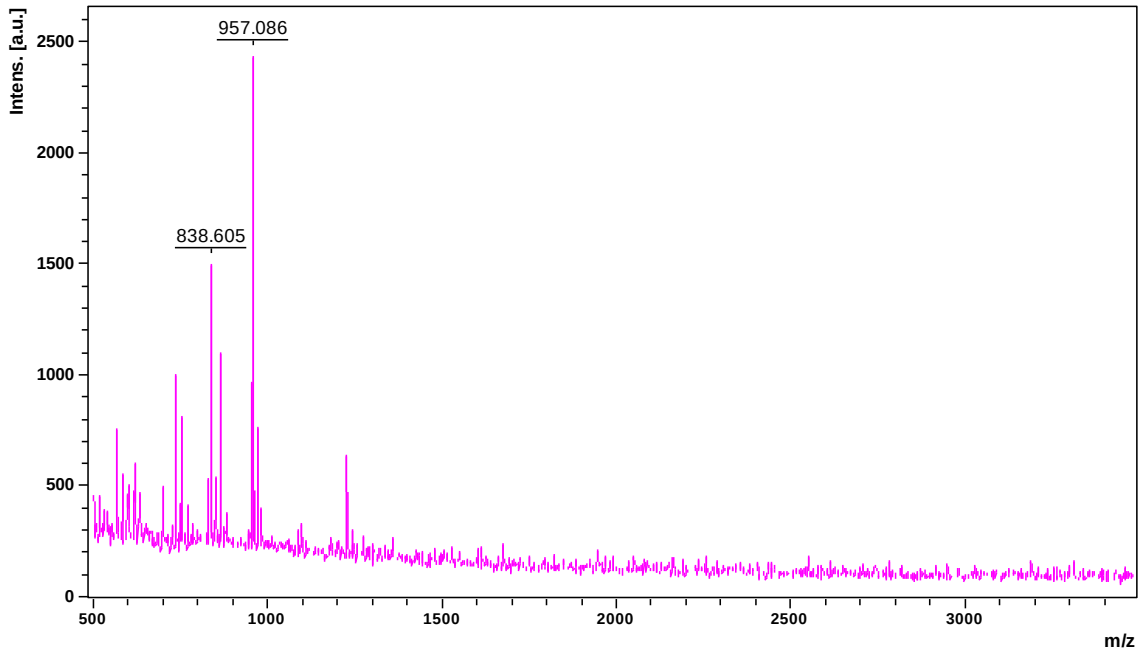

Fig 3. MaldiTOF spectrum of *Q. suber*bark extract

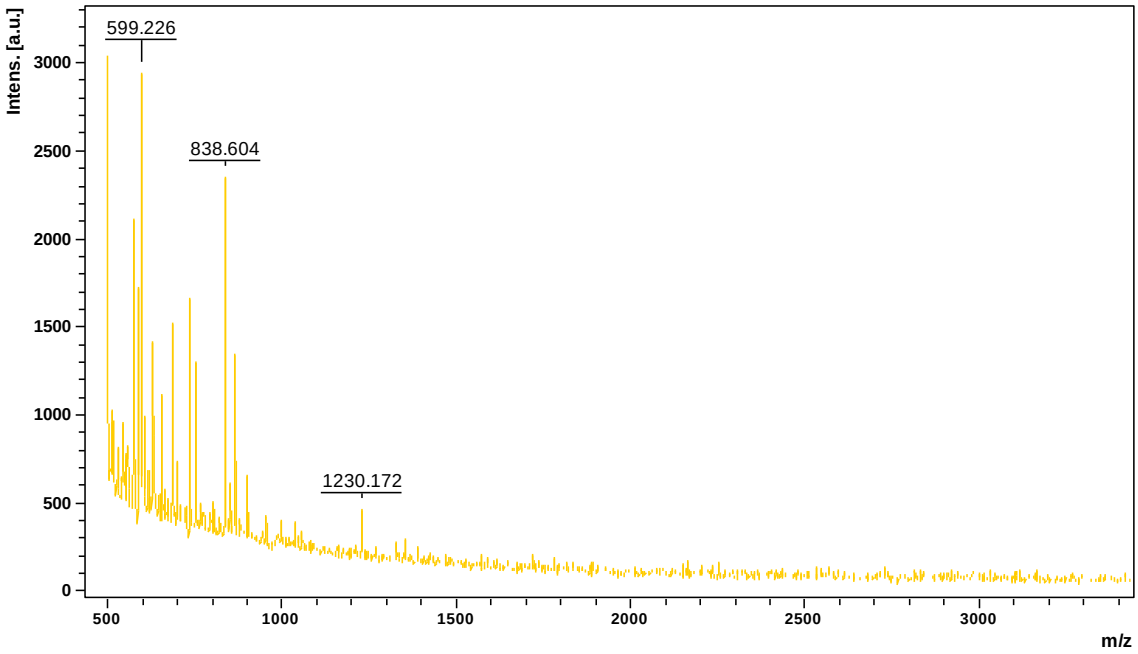

Fig 4. MaldiTOF spectrum of *Q. cerris*bark extract

3.1.3. Pine nut shells

MaldiTOF spectra of Pine nut shells were studied for the first time. Pine nut shells contain 19 distinct detected compounds. 8 of them are possibly of protein nature as they have even numbers (632, 636, 648, 652, 664, 738, 754 and 868 *m/z*).

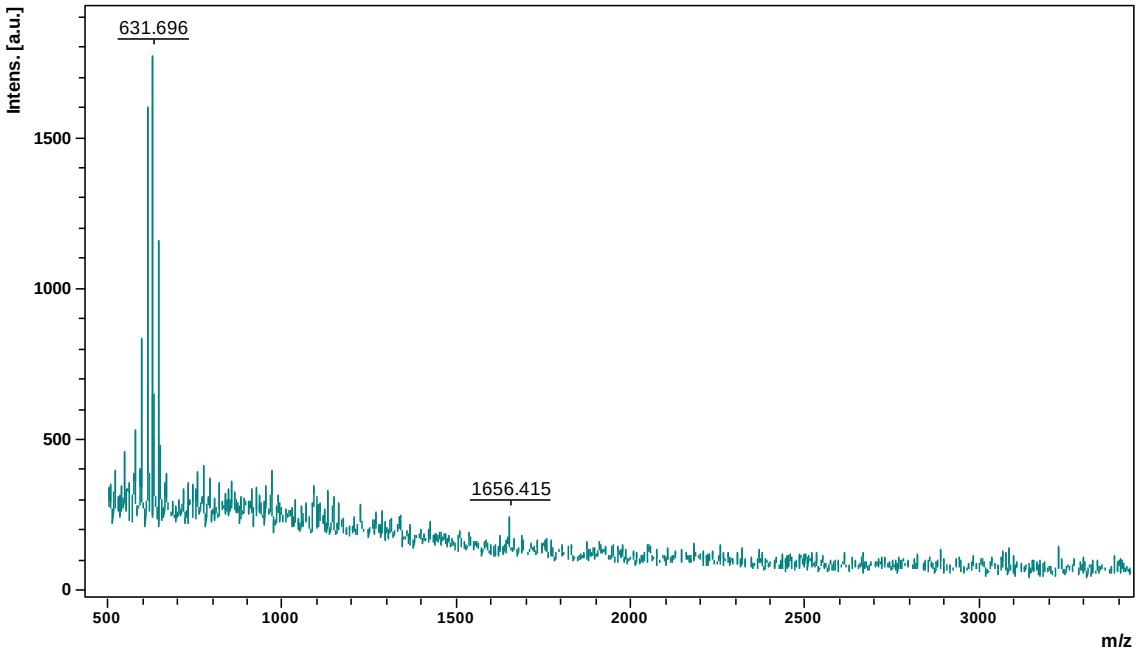

Fig 5. MaldiTOF spectrum of pine nut shell extract

3.2. Antioxidant activity

3.2.1 *P. pinaster* and *P. pinea*

P. pinaster and *P. pinea* bark extracts showed high antioxidant capacity 72% and 69% of antioxidant Trolox. These results are quite similar and promisingly high. The similar antioxidant capacities of these bark extracts must be related to their similar polyphenols composition.

*3.2.2. Q. suber*and *Q. cerris*

*Q. suber*and *Q. cerris* corks showed similar antioxidant capacities (33 and 40 % of Trolox). The antioxidant capacity of *Q. suber* was slightly higher than that of *Q. cerris*. Their antioxidant capacity was lower than that of pine samples. Dudonné et al. (2009) demonstrated using DPPH and ABTS radical scavenging essays that Pine bark (*Pinusmaritima*) and Oak wood (*Quercusrobur*) are strong antioxidants. Santos et al (2010) studied the antioxidant capacity of cork of *Q. suber* by DPPH method and obtained high antioxidant capacity (IC_{50} value of 2.79 µg/ml compared to IC_{50} value of 2.12 µg/ml of ascorbic acid in methanol). An interesting difference between pine and oak samples is that pine samples showed higher antioxidant capacity in our study. Also it was observed that when the granule size is increased the antioxidant capacity reduces in *Q. suber* and *Q. cerris* cork samples (64, 40, and 25% of 60-80 mesh, 40-60 mesh and 20-40 mesh extracts of *Q. suber*, respectively). This granule effect was not observed in *P. pinea* bark samples.

3.2.3. Pine nut shells

Surprisingly pine nut shells showed the highest antioxidant capacity (74% of Trolox activity) of all biomass samples. This result implies that pine nut shells can be extracted for phenolic contents before industrial burning operations to make use of this potential.

4. Conclusions

Maldi-TOF and EPR analyses of bark extracts showed a clearpotential forphenolics and antioxidants production. Maldi-TOF analysis revealed that biomass samplesvary in polyphenolcomposition. *P. pinaster* and *P. pinea* barks have similar polyphenol compositions (procyanidin type polyphenols) but they have different catechin series and different polymerization degrees. *Q. suber* and *Q. cerris* barks have some common polyphenol peaks but their general compositions are different.The main polyphenol

of these barks is probablycastalaginor vescalagin. Pine nut shells contain low molecular weight polyphenols which have considerable antioxidant effect. *P. pinea* and *P. pinaster* barks also contain polyphenols of high antioxidant activity. Antioxidant activity of pine bark extracts is higher than that of oak bark extracts. The higher procyanidin content of pine barks might be determining factor in antioxidant activity.

Taken together, pine and oak barks as well as pine nut shells have great potential to be extracted for polyphenol and antioxidants production.

5. Foreseen publications

The following publications are considered after carefully evaluating the overall results of the study which are tentatively titled as:

1. Comparative polyphenolic composition and antioxidant activities of *P. pinaster* and *P.pinea* barks studied by Maldi TOF/TOF MS and EPR
2. Phenolic composition and antioxidant activity of *Q. suber* and *Q. cerris* corks
3. Maldi TOF/TOF and EPR studies of Pine nut shells

6. Future collaboration

Collaboration with the Host Institute under the EU Framework Programme for Research and Innovation (Horizon 2020) to research polyphenolic composition of forest biomass samples, particularly bark biomass, is under consideration.

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