MALDI-TOF Analysis of Aleppo Pine (*Pinus halepensis*) Bark Tannin

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Matrix-assisted laser desorption/ionisation time of flight (MALDI-TOF) mass spectrometry is a suitable method for examining polyflavonoid tannin oligomers because it has the capability to determine aspects of their oligomeric structure and characteristics that are too difficult to determine by other techniques. For non-purified industrially extracted Aleppo pine polyflavonoid tannin, it was possible to determine by MALDI-TOF that: (i) oligomers formed by catechin/epicatechin are present in tannin, as are mixed oligomer units with fisetinidin and robinetinidin units; the presence of flavonoid gallate and other structures was confirmed; (ii) oligomers up to 12 to 13 repeating monoflavonoid units, in which the repeating unit is at 264 Da, have been confirmed; and (iii) oligomers of the two types covalently linked to each other also occur. The presence of a small proportion of hydrolysable tannins by chemical analysis can also be explained by gallate residues attached to some of the flavonoid oligomers.

Keywords: MALDI; Mass spectrometry; Polyflavonoids; Tannins; Structure; Structural composition; Oligomer distribution

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INTRODUCTION

Polyflavonoid tannins are natural polyphenolic materials that can be used for a variety of industrial applications (Pizzi 1994), and the tannin extracts are primarily composed of flavan-3-ols repeating units and smaller fractions of polysaccharides and simple sugars. The polyflavonoid tannins have been promoted both as wood adhesives (Pizzi 1994), as the basis of fire resistant rigid foams (Meikleham and Pizzi 1994; Tondi et al 2008; 2009a,b; Tondi and Pizzi 2009), as well as heavy metal complexing agents for pollution control in water (Tondi *et al.* 2009a,b).

Aleppo pine trees are extensively present all around the Mediterranean region, especially in north African coastal countries such as Tunisia. They also constitute a potential reserve of bark from which commercially usable tannins can be extracted. Recently, awareness of the potential of pine bark tannin as a resource has come to the forefront with the production of a few industrial batches of maritime pine bark tannin extract (Navarrete *et al.* 2010). The actual structures of the primary monomers constituting the Aleppo pine tannin oligomers are not known, and their degree of

polymerisation distribution has yet to be defined in the bark of any species, including Aleppo pine. As different polyflavonoid tannins present different structures, different average molecular mass distribution, and different degrees of polymerisation (Navarrete *et al.* 2010; Oo *et al.* 2008; Pasch *et al.* 2001), it is necessary to define their characteristics to understand which of the existing tannin resin technologies can be used for industrial exploitation. Matrix-assisted laser desorption/ionisation (MALDI) mass spectrometry has greatly expanded the use of mass spectrometry with large molecules and has revealed itself to be a powerful method for the characterisation and elucidation of the distribution of different oligomers of polyflavonoid tannins (Navarrete *et al.* 2010; Oo *et al.* 2008; Pasch *et al.* 2001).

EXPERIMENTAL

Material

The bark from the trunk of the Aleppo pine (TPAE; *Pinus halepensis*, otherwise called "Debaghessnawbar" in Arabic) was gathered from Souk el "Blat", Tunisia, in 2010. The bark was air-dried and reduced to a fine powder (particle size about 50 µm) with a Retsch SK1 Crossbeater Mill (Haan, Germany).

Methods

Extraction of total polyphenols

The crushed sample (0.5 g) was placed in 10 mL of 80% methanol for 2 h at room temperature (three replicates) and extracted under gentle agitation (30 rpm). Following filtration of the solution on a sintered glass funnel, approximately 65% of the methanol volume was evaporated with a rotary evaporator (Heidolph Vaporota 4000; Fisher Scientific; Illkirch, France). Two drops of hydrochloric acid (HCl, 6N) was added to the remaining extract and the volume adjusted to 10 mL with distilled water. Using a separatory funnel, diethyl ether (3 x 5 mL) was added to the extract, resulting in two phases. The volume of the aqueous phase was adjusted to 10 mL with distilled water. Water was eliminated from the ether phase with the addition of 2 g of anhydrous magnesium sulphate. The ether then was evaporated, and the residue dissolved in 5 mL of methanol (Scalbert 1989).

Determination of polyphenols and tannins

Polyphenols were determined by the method of Folin-Ciocalteu (Singleton and Rossi 1965). The ether extract was diluted 10 times with absolute methanol, while the aqueous extract was diluted 100 times with water. A polyphenol control (gallic acid) was added to an aqueous medium to provide a reference range from 10 to 90 ppm. To determine the concentration of polyphenols, 0.5 mL of the diluted solution was mixed with 2.5 mL of Folin-Ciocalteu reagent (diluted 10 times with water) and 2 mL of a solution of sodium carbonate (75 g/L), which was added 30 s to 8 min after the addition of the Folin-Ciocalteu reagent. The test tubes were placed for 5 min in a water bath at 50 °C and then immediately transferred to cold water. The absorbance was read at 760 nm on a Pharmaspec 1700 UV-Vis spectrophotometer (Shimadzu; Marne la Vallée, France). The results were expressed in gallic acid equivalents based on the amount of dried sample (GAE/g DS).

The humidity was determined by placing 1 g of the sample in a drying balance or in an oven at 105 °C for 24 h. The humidity was calculated from Eq. 1,

$$H\% = [m_o - m_1 / m_0] * 100 \quad (1)$$

where m_0 is the mass of the sample before drying and m_1 is the mass of the sample after drying.

Determination of proanthocyanidins

The vanillin test (Broadhurst and Jones 1978; Price *et al.* 1978) was performed with 1 mL of the aqueous extract, which was mixed with 2 mL of freshly prepared vanillin (1 g/100 mL in 70% sulfuric acid). The mixture was placed in a water bath at 35 °C for 15 min, and the absorbance was read at 500 nm (Scalbert 1989; Schofield *et al.* 2001). The results were expressed in catechin equivalents based on the amount of wood extracted (mg eq (+)catechin/g DS). Each result is the average of three measurements.

For the butanol-HCl test (Porter *et al.* 1986), 0.5 mL of the aqueous extract was mixed with 5 mL of a solution of iron (77 mg $FeSO4 \bullet 7H_2O$ dissolved in 500 mL of a mixture of butanol and hydrochloric acid (2:3 v/v)). The mixture was placed in a water bath at 95 °C for 15 min, and the absorbance was determined at 530 nm (Scalbert 1989). The results are expressed in cyanidine equivalents based on the amount of the extracted dried sample (mg eq. cyanidine/g DS).

$$\mathbf{[PA] = (A\ V\ M\ V'\ D) / (m\ v\ \varepsilon)} \quad (2)$$

where [PA] is the proanthocyanidins contents (CYA mg/g DS); V is the volume of extract (mL); M is the molecular weight of cyaniding (287 g/mol); V' is the volume of reaction medium (mL); m is the mass of the sample (g); v is the sample volume; and ε is the molar extinction coefficient (34,700 L/mol.cm).

Determination of ellagitannins

In a covered Teflon tube, 0.2 mL of the aqueous extract was mixed with 1.8 mL of 50% methanol (in the case of low sample concentration, 1 mL of the aqueous extract was mixed with 1 mL of 90% methanol), and 0.16 mL of 6% acetic acid was added to the mixture. The sample was degassed with nitrogen for 5 to 10 min, followed by the addition of 0.16 mL of 6% sodium nitrite and another degassing that lasted a few seconds. The determination of ellagitannins was based on their oxidation by nitrous acid in the absence of oxygen, which causes a blue colour (Bate-Smith 1972; 1973; 1977; Wilson and Hagerman 1990). The tubes were closed and placed in a water bath at 25 °C for 10 min. The results were expressed in mg eq. of 4,6-hexahydroxydiphenoyl-glucose based on the amount of the extracted dried sample (Scalbert *et al.* 1989)

$$[ET] = (A\ V\ M\ V'\ D) / (m\ v\ \varepsilon) \quad (3)$$

Quantities in Eq. 3 can be given as [ET]: ellagitannins content (4.6 mg HHDP-G/g DS)/ M: the molecular weight of 4,6 hexahydroxydiphenoyl-glucose (482 g/mol); ε: The molar extinction coefficient (2169 mL/mol.cm).

Determination of gallotannins

A 5-mL solution of 2.5% (w/v) potassium iodate was heated to 30 °C for 7 min, followed by the addition of 1 mL of the extract and heating for 2 min. The absorbance was measured at 550 nm (Bossu *et al.* 2006), and the results are expressed in equivalent mg tannic acid/g DS. With the presence of the gallotannins, a red colour is formed; otherwise, a yellow colour results.

Extraction of tannin in powder form

The bark sawdust was extracted with water at room temperature at a ratio of 1:5 (w/w). The mixture was heated to exactly 70 °C; 0.5% sodium bicarbonate and 2.5% sodium bisulfite were added, and the mixture was mechanically stirred for 6 h. After filtration on filter paper and washing the dry residue with water, the filtrate was concentrated by evaporation for 3 h at 40 °C. A dry residue was obtained after drying the concentrated filtrate at 40 °C.

MALDI-TOF operating conditions

The spectra were recorded on a AXIMA Performance MALDI TOF instrument (Shimadzu Scientific Instruments; Manchester, UK). The irradiation source was a pulsed nitrogen laser with 3-ns intervals at a wavelength of 337 nm. The measurements were carried out using the following conditions: polarity-positive, flight path-linear, mass-high (20-kV accelerating voltage), and 100 to 150 pulses per spectrum. The delayed extraction technique was used to apply delay times of 200 to 800 ns.

MALDI-TOF sample preparation

The samples were dissolved in acetone (4 mg/mL, 50/50 volume), and the solutions were mixed with the matrix solution (10 mg/mL in acetone). The matrix, which facilitates the deposition of the sample in the instrument, was 2,5-dihydroxy benzoic acid. For the enhancement of ion formation, sodium chloride (NaCl) was added to the matrix (10 mg/mL in distilled water). The sample and the matrix solutions were mixed as 3 parts of the matrix solution, 3 parts of the sample solution, 1 part NaCl solution, and 0.5 to 1 µL of the resulting mix was placed on the MALDI target. After evaporation of the solvent, the MALDI target was introduced into the spectrometer. The dry droplet sample preparation method was used. Each peak value in the resulting positive mode spectrum must be subtracted of 23 Da, this being the molecular weight of the Na^+ included as NaCl in the matrix and attached to the oligomers, to obtain the molecular weight of the chemical species of the peak.

Condensed flavonoid tannins greatly predominated, with a minority of tannins appearing to be hydrolysable-type tannins or their derivatives. Such a low proportion of hydrolysable tannins indicates that the gallic/ellagic acid groups detected may not belong to hydrolysable tannins but may instead be gallate moieties attached to flavonoid units of epicatechin gallate. The bark of Aleppo pine is rich in polyphenols. The values shown in Table 1 are typical but can vary depending on the amount of bark and the tree age.

The interpretation of the MALDI spectra in Fig. 1 yielded the series of oligomer structures shown in Table 2. In general in condensed tannins the flavonoid units involved in the formation of the oligomers were of three types, A, B, and C, corresponding to the masses 274.3 Da, 290.3 Da, and 306.3 Da, respectively. Two coexisting structures contributed simultaneously to the mass of B.

RESULTS AND DISCUSSION

Dosage of Tannins

The concentrations of tannins in the bark are summarised in Table 1.

Table 1. Results of the Dosage of Total Polyphenols and Tannins

Concentration of total polyphenols in the aqueous phase (mg GAE/gDS)	48.873 ± 0.0001
Concentration of total polyphenols in the ether phase (mg GAE/gDS)	5.902 ± 0.0002
Concentration of proanthocyanidins, Vanillin test (mg CE/g DS)	13.535 ± 0.016
Concentration of proanthocyanidins, Butanol-HCl test (mg CYA/g DS)	16.754 ± 0.016
Concentration of gallotanins (mgAT/g DS)	1.958 ± 0.802

The combinations of these masses (A = 274, B= 290, C= 306) can be used to calculate the masses of the oligomer peaks in the spectra according to the expression M+ Na+=23.0(Na)+2.0 (end groups, 2X H)+ 272.3 A+288.3 B + 304,3 C when NaCl enhancer has been used. Table 2 reports the main oligomer compositions in the absence of Na^+ enhancer and oligomer compositions in the presence of Na^+ enhancer are reported in Table 3.

The four A, B, and C structures are linked C4 to C6 and C4 to C8 depending on which are linked together, to form the oligomers. In the case of units of type A (fisetinidin) and of type B having a third –OH on the B ring (robinetinidin), these are linked C4 to C6. When the other type of B unit (catechin) or a C unit (gallocatechin) is involved this can be linked either C4 to C6 but predominantly C4 to C8 to other flavonoid units. Even so-called "angular" tannins (in reality a branching point in the structure of higher oligomers) can occur having the following structure (Pasch *et al.* 2001):

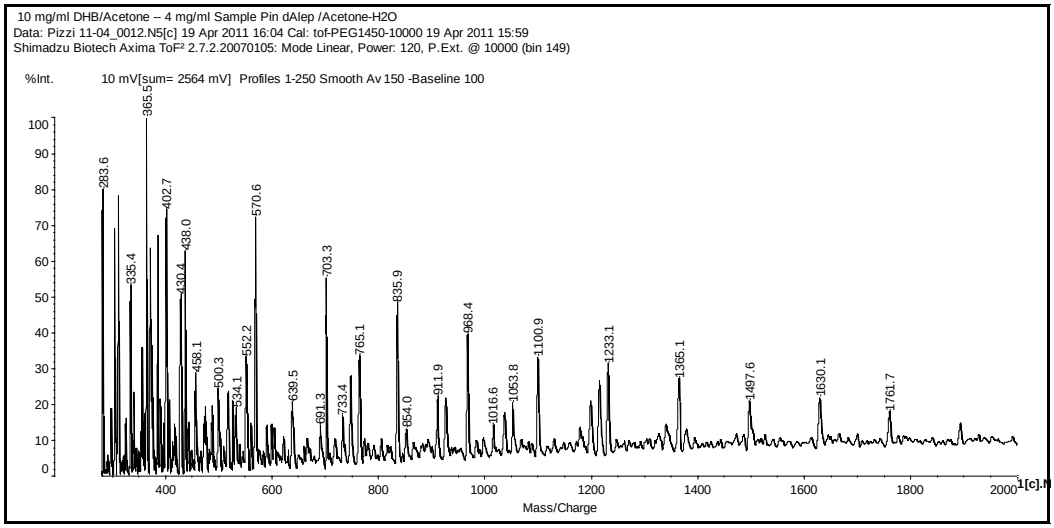

Fig. 1. MALDI TOF of Aleppo pine tannin extract in the range 280 Da to 2000 Da

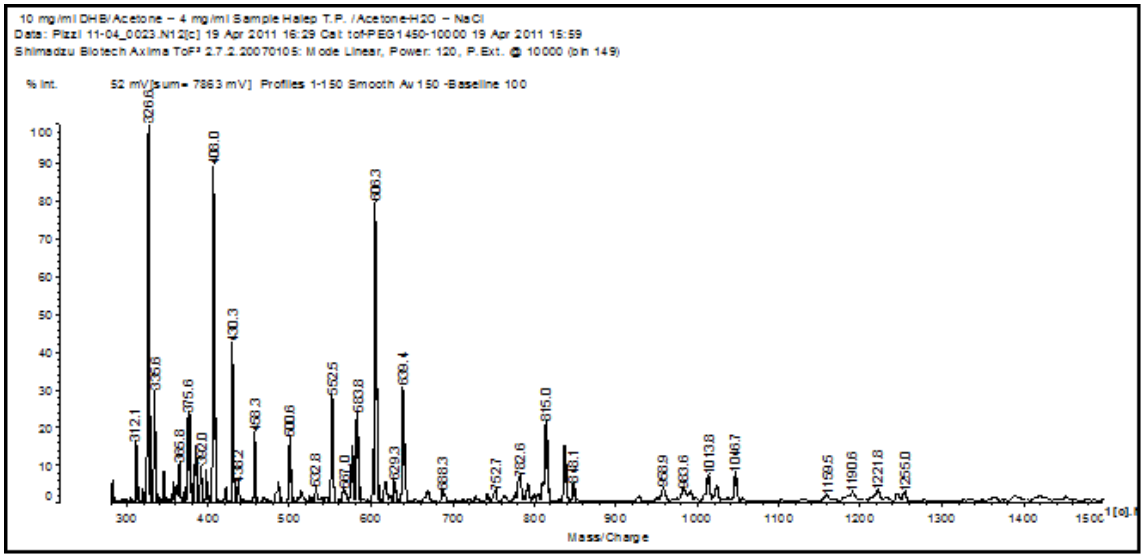

Fig. 2. MALDI TOF of Aleppo pine tannin extract in the range 280 Da – 1500 Da with NaCl enhancement

Table 2. Results of MALDI TOF Spectra of the Tannin Extracted from the Bark of the Trunk of Aleppo Pine without Na^+ Enhancement

Experimental M(Da)	Calculated M(Da)	Flavonoid unit types	Oligomer type
306	306	C_1	Monomer
438	442	B_1G_1	Monogallate flavonoid monomer
534.1	533	D_1	Dimer, protonated
552.2	549	A_2	Dimer, protonated
570.6	566	A_1B_1	Dimer, protonated
703.3	702	A_2G_1	Monogallate flavonoid dimer
835.9	836	C_1D_1	Trimer
968.4	970	$D_1B_1G_1$	Monogallate flavonoid trimer
1100.9	1099	$D_1A_1B_1$	Tetramer, protonated
1233.1	1232	$D_1A_2G_1$	Monogallate flavonoid tetramer
1365.1	1365	C_1D_2	Pentamer
1497.6	1498	$D_2B_1G_1$	Monogallate flavonoid pentamer
1630.1	1629	$D_2A_1B_1$	Hexamer
1761.7	1762	$D_2A_2G_1$	Monogallate flavonoid hexamer
1895	1895	C_1D_3	Heptamer

In the above tables, the oligomers are indicated for example as A_2B_1, meaning that the oligomer is a trimer formed by 2 A units and a B unit linked together.

One can note that in normal MALDI, NaCl is used as enhancer, thus the peaks must be subtracted by 23 Da (for Na+) to obtain the molecular weight. When NaCl has not been used, the value of the peak is the molecular weight of the oligomer itself.

In some flavonoids, there is either a monosaccharide or a gallate residue attached to one unit of the flavonoid. For the gallate, this has been indicated by G.

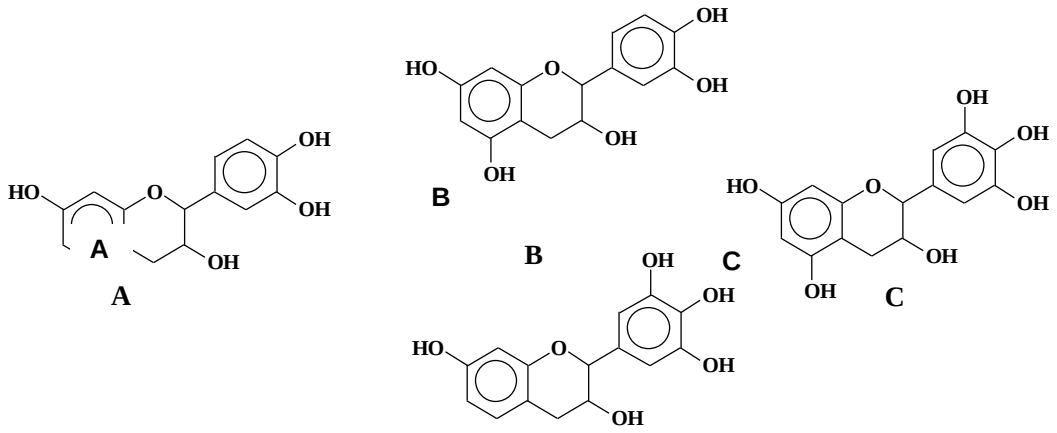

Fig. 3. Flavonoid units A, B, and C involved in the formation of the oligomers

Table 3. Results of MALDI TOF Spectra of the Tannin Extracted from the Bark of the Trunk of Aleppo Pine with Na^+ Enhancement

Experimental $M+Na^+$(Da)	Calculated $M+Na^+$(Da)	Flavonoid unit types	Oligomer type
312.1	313	B_1	Honomer
326.6	326	C_1	Honomer
552.5	553	A_2 – 1x-OH	Dimer – 1x-OH
569	569	A_2	Dimer
583.8	585	A_1B_1	Dimer
606.2	603	B_2	Dimer, multiprotonated
639.1	635	C_2	Dimer, multiprotonated
752.7	753	B_2G_1	Monogallate flavonoid dimer
840	841	A_3	Trimer
1159.5	1161	A_1B_3 or $A_2B_1C_1$	Trimer
1190.6	1193	B_3C_1 or $A_1B_1C_2$	Tetramer
1225.0	1225	$B_1C_2G_2$	Digallate flavonoid trimer

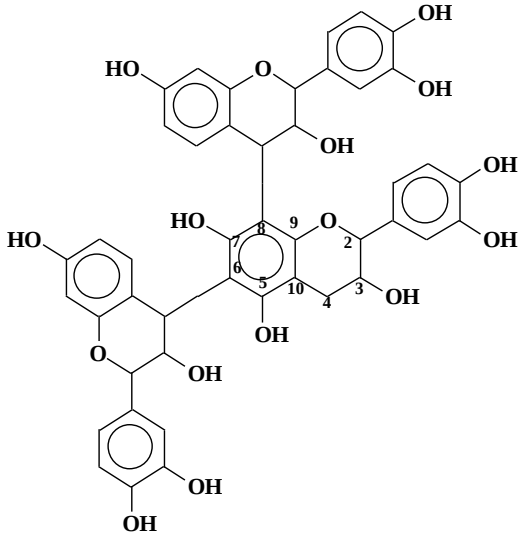

Fig. 4. Example of angular tannin with a catechin unit linked C4-C6 and C4-C8 to the other two flavonoid units

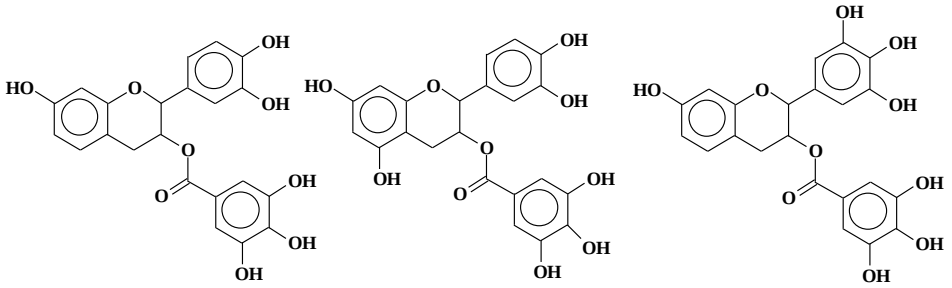

Fig. 5. A, B, and C units to which a gallate residue is linked at the flavonoid unit C3

Thus, Fig. 5 shows units of the type present in this tannin. No gallic acid residue attached to a C unit has been found in this tannin, contrary to what is found in other tannins (Navarrete *et al.* 2010; Oo *et al.* 2008).

Contrary to other pine tannins, while procyanidin type oligomers are still predominant, structures of type A are very much present, indicating some fundamental differences in such a tannin. Thus, while dimers of different kinds appear to be in the majority, oligomers up to a heptamer are present (Table 2). The MALDI –TOF spectrum in Fig. 1 shows repeating mass increments of 132-132.5 Da. This indicates possibly the presence of other monomers than those shown above and/or different combinations of various structures. Similar types of structure have already been identified before for other pine tannins (Navarrete *et al.* 2010). In this case the corresponding structure has molecular mass 530 causing the observed repeating increments of 132 Da. Thus, 530/4 = 132.5 Da of the increments. We will call the 530 Da structure, structure D (Fig. 6).

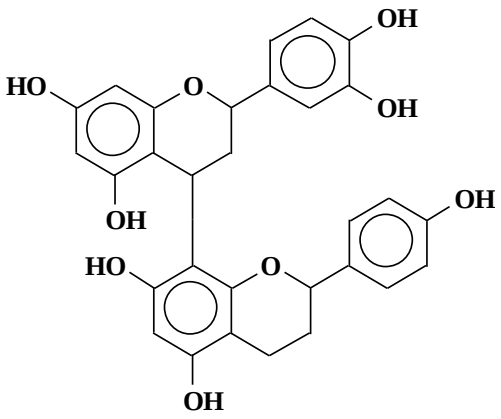

Fig. 6. Flavonoid dimer (structure D) of mass **530** Da composed of two different units

From the peak intensities in Fig. 1 and Table 2 its participation is well defined in the formation of some Aleppo pine tannin oligomers. Four overlapping series of the most evident higher MALDI molecular mass peaks rely on the repetition of this 530 Da structure (Fig. 1, Table 2). The appearance is then of a 132 Da recurring increment caused by the regular overlapping of the series presenting the real 530 Da increment. The four series are (i) the peaks 306 Da, 835 Da, 1365 Da, and 1895 Da corresponding respectively to C_1, C_1D_1, C_1D_2, C_1D_3; (ii) 438 Da, 968 Da, and 1497 Da corresponding respectively to B_1G_1, $D_1B_1G_1$, and $D_2B_1G_1$ hence a monogallated series; (iii) 570 Da,

1101 Da, and 1630 Da corresponding respectively to A_1B_1, $D_1A_1B_1$, and $D_2A_1B_1$; (iv) 703 Da, 1233 Da, and 1762 Da corresponding respectively to A_2G_1, $D_1A_2G_1$, and $D_2A_2G_1$, a second monogallated series of oligomers. On the basis of the first of these, the first series corresponds to a 306 Da monomer to (306 Da, Fig. 1) to which a number of units of type D are added, giving as a consequence a flavonoid units trimer (835 Da), a flavonoid units pentamer (1101 Da), and a flavonoid units heptamer (1895 Da). The second series to the oligomers of which is always attached a gallate residue corresponds to a monogallated monomer (438 Da), to a monogallated trimer (968 Da) and to a monogallated pentamer (1497 Da). The third series of oligomers corresponds to a dimer (570 Da), tetramer (1101 Da) and hexamer (1630 Da). The fourth series, it is the second of monogallated oligomers corresponds to a monogallated dimer (703 Da), monogallated tetramer (1233 Da), and a monogallated hexamer (1762 Da). This distribution indicates the somewhat more complex nature of the structures present in the tannin of Aleppo pine.

Regarding the analysis MALDI-TOF of this tannin when enhanced by addition of NaCl, the pattern of the oligomers species observed is less evident (Fig. 3). Thus, the oligomers observed are in general from monomers to tetramers in which the structures A, B, and C are connected. The repeating 132 Da increment is not observed at all in this case, hence structure D is not observed. The question to be asked then is: is structure D a degradation of a different type of flavonoid dimer, which under the conditions of use of the instrument may have caused detachment of some hydroxyl group? This is possible, but seems rather unlikely on the basis of previous analysis of tannins using this method. That modifications might occur in the spectrometer is correct as, while gallate species are clearly present also in Table 3 and Fig. 2, even one digallate species occurs in Table 3, Fig. 2, while only monogallate species occurs in Fig. 1, Table 2. Especially in species where gallic acid residues are present, it is often the case that some of such gallic residues are separated either by action of the equipment or by the tannin extraction method. Clear indication of this was obtained in the analysis of chestnut hydrolysable tannin where such phenomenon has been shown to be rather extensive (Pizzi *et al.* 2009).

CONCLUSIONS

1. The results of this study show that the bark of the Aleppo pine is rich in polyphenolic tannins.
2. Classic wet chemistry analysis allows the conclusion that a great majority of these tannins are of the condensed polyflavonoid type, with a minor percent of hydrolysable gallic-type tannins.
3. The MALDI-TOF analysis provided the identification of the structural units involved in the polyflavonoid oligomers.
4. The MALDI-TOF analysis also appeared to indicate that rather than gallic-type hydrolysable tannins being present, the gallic residues appeared to be linked to the flavonoid in the form of esters at the alcohol C3 site of certain flavonoid units.

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