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Abstract

Pistacia lentiscus L. is a shrub of the Anacardiaceae family whose fruits are used in Tunisian and Algerian diets. The phenolic composition at 5 different physiological stages of the fruit was investigated using two different targeted methodologies: ultra-high-performance liquid chromatography–UV/visible detection (UHPLC-UV/vis) for anthocyanins and UHPLC coupled with tandem mass spectrometry (UHPLC-MS/MS) for the other polyphenols. For the specific analysis of anthocyanins, compound identification was confirmed by UHPLC-MS/MS and LC-NMR analysis. This study revealed the identification of 30 phenolic compounds including 9 anthocyanins, 7 flavanols, 7 flavonols, 2 phenolic acids, 1 stilbene, 2 flavanones, 1 flavanonol and 1 dihydrochalcone. Quantification showed significant qualitative and quantitative variation in phenolic content during the ripening of *P. lentiscus* fruits, flavonols being the main compounds for the unripe berries and anthocyanins for ripe berries. To the best of our knowledge, our study reports the presence of piceid and protocatechuic acid in *P. lentiscus* L. fruits, as well as several anthocyanins in *Pistacia*, for the first time. The results indicate potential applications of *P. lentiscus* L. fruits as a source of phenolic compounds to be used as nutraceuticals and as food colorants.

Key words: targeted metabolomics, *Pistacia lentiscus* L.fruits, ripening, HPLC-MS/MS, NMR

1. Introduction

The genus *Pistacia,* which belongs to the Anacardiaceae family, is divided into eleven species and is largely distributed and cultivated from the Mediterranean basin to Central Asia (Milia et al., 2021). Among these species, *P. lentiscus* is a wild and cultivated species, known for its aromatic natural resin (Pachi et al., 2020), traditionally used as food by the population of the Mediterranean region. The resin is used as a mastic gum and also as flavouring in bread preparation and rice dough (Burešová et al., 2017). Fruits are commonly eaten, either raw or roasted. Moreover, the fruit oil represents a source of vegetable oils traditionally consumed in Tunisian and Algerian diets (Trabelsi et al., 2012; Mezni et al., 2016; Yosr et al., 2018; Milia et al., 2021). In addition, fruits and other aerial parts of *P. lentiscus* are used in folk medicine (Bozorgi et al., 2013; Milia et al., 2021). Interesting pharmacological properties of the plant including antioxidant (Atmani et al., 2009), anti-diabetic (Mehenni et al., 2016), anti-tumor (Remila et al., 2015), and anti-microbial (Mezni et al., 2015) effects have been described in different parts of the plant such as leaves and fruit extracts (Milia et al., 2021).

Even if the majority of studies have been focused on fruit oil (Mezni et al., 2016; Trabelsi et al., 2012) and essential oil composition (Ben Khedir et al., 2016; Yosr et al., 2018), a few investigations indicated that *P. lentiscus* hydro-alcoholic fruit extracts constitute a rich source of phenolic compounds including phenolic acids, flavanols, flavonols and anthocyanins (Elez Garofulić et al., 2020; Longo et al., 2007).

The chemical and biological properties of *P. lentiscus* fruits can be affected by the development stage, as reported for many other berries (Benbouguerra et al., 2021b). In fact, the influence of fruit maturity on polyphenol and tocopherol constituents of *P. vera* kernels, a closely related species to *P. lentiscus,* has already been reported (Ballistreri et al., 2009). In addition, changes in the sterol and lipid composition with the ripening of fruits has been reported (Trabelsi et al., 2012). Therefore, harvesting time could be an important factor affecting food quality and the potential use of the fruits. To our knowledge, studies describing the evolution of the phenolic composition of *P. lentiscus* fruits during ripening are scarce.

Hence, the present study was focused on the characterization of the phenolic composition of *P. lentiscus* fruits during the developing stages. Phenolic content was analysed by a combination of LC-NMR and UHPLC-MS experiments to provide a detailed polyphenols description in the fruit, which could assist in the identification of potential nutraceutical applications from *P. lentiscus* fruits and extracts.

2. Materials and methods

2.1 Chemicals and reagents

Methanol (laboratory and UHPLC grades), Hexane (laboratory grade), Formic acid LC-MS grade (> 99%) were purchased from Fisher Scientific (France). Acetonitrile (HPLC and UHPLC grades) was obtained from VWR Chemicals (United Kingdom). 4-hydroxybenzoic acid and protocatechuic acid were purchased from Sigma-Aldrich (France). Quercetin, quercetin-3-*O*-glucoside, quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoronide, quercetin 3-*O*-rhamnoside, quercetin 3-*O*-rutinoside, myricetin, catechin, epicatechin gallate, gallocatechin, procyanidin B1, naringenin, naringenin 7-*O*-glucoside, taxifolin, *trans*-piceid, epigallocatechin gallate, delphinidin 3-*O*-glucoside and cyanidin 3-*O*-glucoside were purchased from Extrasynthese (France). MilliQ water was obtained with a Millipore system.

2.2 Plant material and extract preparation

P. lentiscus fruits were harvested from the forest of Tizi Neftah province of Amizour, Bejaia, Algeria (GPS coordinates 36.644°N and 4.921°E). The botanical identification was confirmed with support from the Laboratory of Botany, University of Bejaia (Algeria), according to a voucher herbarium specimen (N° 970704) deposited at the National Institute of Agronomy, Algiers, Algeria. The stage of maturity was determined by fruit colour and harvest month. The fruits were air-dried at 30°C in the dark and then crushed with an electric grinder and finally, defatted thrice with hexane (5 g: 40 mL). The defatted powder was then mixed with 100% methanol (5 g: 40 mL). Five extraction cycles were carried out in an ultrasonic bath for 10 min. The extracts were dried in a rotatory evaporator and solubilized in methanol/water (1:1, v/v) to obtain a final concentration equivalent to 5 g of dried fruit in 10 ml of solvent, and then they were stored at – 20°C until analysis.

2.3 Individual compound analysis

2.3.1 Anthocyanins analysis

Individual anthocyanin identification was performed by a combination of HPLC-MS and HPLC-NMR analysis following the method developed by Acevedo et al. (2012). For anthocyanin identification, the extract reconstituted in water was passed through an XAD column, cleaned with water, recovered using methanol as mobile phase and dried in a rotary vacuum evaporator. HPLC-NMR experiments were performed on a BRUKER AVANCE III

600 MHz spectrometer (Wissembourg, France) equipped with a $^{1}H–^{13}C$ inverse-detection flow probe. ^{1}H-NMR spectra were obtained in stopped-flow mode. For 2D-NMR experiments, individual anthocyanins were collected after on-flow ^{1}H-LC–NMR analysis onto a FOXY collector from Teledyne ISCO (Lincoln, USA), lyophilized and analyzed by using classical COSY and NOESY 2D-NMR experiments (Acevedo De la Cruz, Alexander et al., 2012).

Individual anthocyanin quantification was performed on a UHPLC-MWL-MS/MS system (Thermo Scientific, France) composed of an Accela 1250 system coupled to a TSQ Quantum Access Max triple quadrupole equipped with an H-ESI ion source. Anthocyanins were separated on a Zorbax SB-C18 column (2.1 x 100 mm, 1.8 µm, Agilent) with 1 µL of volume injection. Mobile phase A was deionized water containing 2.5% formic acid (v/v) and mobile phase B was methanol (UHPLC grade) containing 2.5% formic acid (v/v). The elution gradient was for solvent B: 2.5% (0-2 min); 15% (7 min); 23% (9.3 min); 55% (10 min); 95% (11-12,5 min); 2.5% (13-15 min). The temperature and the flow were fixed to 35°C and 0.4 mL/min, respectively. The mass detector operated in the positive mode while the source parameters were as follows: Sheath gas 60 a.u; Auxiliary Gas 20 a.u., Voltage 1500V, Vaporizer Temperature 450°C, Capillary Temperature 150 °C. All compounds were quantified using the MWL detector at 520 nm using a calibration curve built with Dp3glu (range 0.97-1000 mg/L). Results were expressed as mg delphinidin 3-*O*-glucoside (Dp3glu) equivalents per 100 g of dry weight fruits (mg DGE/100g DW). The limits of detection (LOD = 0.08 mg DGE/100g DW) and quantification (LOQ = 0,23 mg DGE/100g DW) were calculated as LOD=3.3 x s/b and LOQ=10 x s/b, where “b” is the slope of the curve and “s” the standard deviation of the signal determined as the residual standard deviation of the calibration line in the LOD region (Kruve et al., 2015).

2.3.2 UHPLC-QqQ-MS/MS analysis of other phenolic compounds

Individual phenolic acids and flavonoid compounds were quantified by a targeted UHPLC-QqQ-MS/MS approach according to a previously developed methodology (Loupit et al., 2020; Djemaa-Landri et al., 2020) using an Infinity UHPLC 1260 system coupled to a 6430 triple quadrupole (QqQ) mass spectrometer (Agilent Technologies, France). Separation was achieved with an Agilent Poroshell 120 EC-C18 column (150 x 2.1 mm, 2.7 µm) thermostated at 40°C. Acidified water and acidified acetonitrile (both containing formic acid 0.1%, v/v) were used as solvents A and B, respectively. The elution parameters consisted of a 0.4

mL/min flow and a solvent B gradient as follows: 1-10% (0-4 min); 20% (12 min); 33% (13-16 min); 40% (21 min); 95% (22-24 min); 95% (25 min); 1% (28 min). Measurements were based on Multiple Reaction Monitoring (MRM) in positive or negative mode depending on the compounds (Supplementary Figure 1). Collision energies applied are shown in Table 2. Quantification was done by comparison with a calibration curve with standards in the range of 0.03 to 15.4 mg/L. All compounds were quantified with their corresponding standard except procyanidin B3 and an unknown trimer, expressed as procyanidin B1 and C1, respectively. Calibration parameters can be found in Supplementary Table 1.

2.4 Statistical analysis

All results were expressed as mean ± standard deviation ($n \geq 3$). One-way analysis of variance (ANOVA) tests was performed using Origin.

3. Results and discussion

Pistacia lentiscus fruits were collected at 5 different stages of maturity (Figure 1): September 2017 (less red unripe fruits, stage 1), October 2017 (red unripe fruits, stage 2), November 2017 (Red unripe fruits, stage 3), December 2017 (majority black ripe fruits, stage 4), January 2018 (black fully ripe fruits, stage 5).

3.1. Identification of phenolic compounds

3.1.1 Anthocyanins

An UHPLC-UV/vis-MS/MS methodology was used to obtain more details of the individual anthocyanin composition of the fruits. Classic methods of anthocyanin analysis usually require a very low acidic pH to assure the presence of the flavylium cationic form, which facilitates a good separation of anthocyanins and is easily detected at red wavelengths (520 nm). This low pH is commonly achieved by using a high percentage of formic acid (5%-10%) in the mobile phases. However, coupling with MS instruments usually requires lower concentrations of acid. After several tested gradients (data not shown) reducing the amount of acid, a method using 2.5% formic acid was established allowing a good separation leading to the detection of 9 major anthocyanin peaks (Figure 2). A series of different scan modes of tandem mass spectrometry was used to identify these compounds. First of all, a full MS scan

was applied to determine the molecular weight of the molecules. Afterwards, MS/MS fragmentation analysis was performed by using a product ion scan, which selectively isolated the parent ions in the first quadrupole, and fragmented them in the collision cell. The data obtained were consistent with cyanidin and delphinidin derivatives conjugated with sugars. The results were confirmed with a precursor ion scan, which screened the presence of precursors of cyanidin (*m/z*: 287) and delphinidin (*m/z*: 303), and by a neutral loss scan, which screened the presence of pentosides (-132 u loss), hexosides (-162 u loss), and dihexosides (-324 u loss). To complete the identification, compounds were analysed by ^{1}H-LC-NMR (Acevedo De la Cruz, Alexander et al., 2012). The identified compounds are listed in Table 1.

Peak 1 was identified as a delphinidin dihexoside. The full scan MS revealed a molecular ion at *m/z* 627, while the MS/MS product ion scan showed the characteristic *m/z* 303 of the delphinidin aglycon. The precursor ion scan was consistent with delphinidin and the neutral loss ion scan indicated the loss of two hexoses. Peak 2 was also identified as a delphinidin dihexoside. The full scan MS revealed a molecular ion at *m/z* 627, while the MS/MS product scan showed the delphinidin aglycon at *m/z* 303. For peaks 1 and 2, ^{1}H-LC-NMR data confirmed the identification of delphinidin dihexoside; nevertheless, signal complexity precluded complete annotation. MS and NMR data of peak 3 were consistent with delphinidin 3-*O*-galactoside (Table 1). MS spectra exhibited fragment ions at *m/z* 465 and 303. 2D-NMR NOESY spectrum confirmed the position of the galactose moiety. Similarly, peak 4 was identified as delphinidin 3-*O*-glucoside, with further confirmation by coinjection with the pure standard. Based on MS and NMR data, peak 5 was identified as cyanidin 3-*O*-galactoside. This compound presented a parent ion at *m/z* 449 and a fragment ion at 287 (cyanidin aglycon). The position of the galactose was confirmed by a NOESY experiment. Peak 6 was attributed to delphinidin pentoside, presenting a molecular ion at *m/z* 435 and a fragment ion at *m/z* 303. Using MS, NMR data, and comparison with injection of a standard, peak 7 was identified as cyanidin 3-*O*-glucoside (Table 1). Peak 8 and peak 9 were attributed to cyanidin and delphinidin pentoside (molecular ions at *m/z* 419 and 435, respectively), loss of pentoside moiety providing typical ions of cyanidin and delphinidin aglycones (*m/z* 287 and 303, respectively). Unfortunately, NMR spectra complexity precludes the complete identification of peaks 6, 8 and 9.

Regarding the growing interest in anthocyanins as natural dyes in foods, several studies have pointed out the richness of different plants in these compounds (Krga & Milenkovic, 2019).

However, few data have been reported for *P. lentiscus* fruits. So far, research carried out on anthocyanins in *P. lentiscus* fruits had unravelled the presence of just 3 compounds: delphinidin 3-*O*-glucoside, cyanidin 3-*O*-arabinoside and cyanidin 3-O-glucoside (Longo et al., 2007). Indeed, most studies on anthocyanins in the whole *Pistacia* genus only report 3 anthocyanins (El Bishbishy et al., 2020; Erşan et al., 2016; Ojeda-Amador et al., 2019). In our study, the presence of 9 anthocyanins was observed: 3 cyanidin derivatives (cyanidin 3-*O*-galactoside, 3-*O*-glucoside and pentoside) and 6 delphinidin derivatives (2 delphinidin dihexosides, 2 delphinidin pentosides, delphinidin 3-*O*-galactoside and 3-*O*-glucoside).

3.1.2 Other phenolic compounds

Besides the anthocyanin profile, the phenolic composition of *P. lentiscus* fruit was analysed by UHPLC-QqQ mass spectrometry. The approach was based on a multiple reaction monitoring (MRM) method previously developed for the analysis of phenolic compounds in natural extracts (Gabaston et al., 2020; Loupit et al., 2020). The identification relied on the comparison of the retention time with the pure standard and the presence of at least 2 fragment ions. To assure the good attribution of the chromatographic peaks, samples were also spiked with a solution of standards at a concentration of 2 mg/L. A total of 21 compounds were retained as being identified in the different stages of maturation (Table 2) including 2 phenolic acids, 1 stilbene, 7 flavanols, 7 flavonols, 2 flavanones, 1 flavanonol and 1 dihydrochalcone.

This study revealed the presence of 4-hydroxybenzoic acid and protocatechuic acid, the latter being detected for the first time in *P. lentiscus* fruit. Stilbenes, a family of polyphenols with interesting potential and found in several plants, particularly in grapevines (Benbouguerra et al., 2021a; Gabaston et al., 2020) were also screened using 9 different stilbene standards following the MRM methodology (Loupit et al., 2020), but only *trans*-piceid, a glucosidic derivative of *trans*-resveratrol, was detected unequivocally in the sample. Indeed, *trans*-piceid was detected for the first time in *P. lentiscus* fruit. Seven flavonols were detected, mainly quercetin derivatives (quercetin 3-*O*-rutinoside, quercetin 3-*O*-galactoside, quercetin 3-*O*-glucuronide, quercetin 3-*O*-glucoside, quercetin 3-*O*-rhamnoside, quercetin) and myricetin. Our screening did not indicate the presence of kaempferol derivatives in *P. lentiscus* fruits.

In the case of flavanols, 7 compounds were detected including catechin, gallocatechin, procyanidin B1, epigallocatechin gallate and epicatechin gallate, procyanidin B3 and an unidentified trimer. As previously commented, all polyphenols were identified by the MS/MS

227 data and further confirmed by spiking with pure compounds, but because of lack of standards
228 two compounds (procyanidin B3 and the unknown trimer) were tentatively identified by their
229 MS/MS signal and retention time. Finally, 4 other phenolic compounds were detected
230 including 2 flavanones (naringenin 7-*O*-glucoside and naringenin), 1 flavanonol (taxifolin)
231 and 1 dihydrochalcone (phloretin).
232
233 **3.2 Phenolic content**
234 The individual content of the identified phenolic compounds in each maturity stage is
235 reported in Table 3. A significant variation in phenolic content is observed during the ripening
236 of *P. lentiscus* fruits, both in terms of quality and quantity. While the anthocyanin content
237 increased during ripening, most other phenolic compounds presented the highest content at
238 stage 1 (less red unripe fruits), followed by an overall decrease by half in the rest of the stages
239 of maturity.
240 The presence of anthocyanins was observed at all stages but their content was significant only
241 for the two last stages (majority black ripe and black fully ripe fruits). At these stages 4 and 5,
242 anthocyanins reached levels of 1373 and 1273 mg/100 g DW respectively, becoming the main
243 polyphenol constituents of the extract (53 and 59% of the total polyphenols content,
244 respectively). The delphinidin derivatives were predominant in all cases reaching 1038 ± 30
245 (stage 4) and 1116 ± 20 mg/100 g DW (stage 5), which represented 76 and 88% of total
246 anthocyanins, respectively. In contrast to *P. lentiscus*, only cyanidin derivatives had been
247 reported in *P. vera* (Bellomo & Fallico, 2007), with a similar increase of anthocyanin content
248 during maturity (Ballistreri et al., 2009). Based on the results obtained in this study and those
249 found in the literature, we can say that the ripe fruits of *P. lentiscus* represent a rich and
250 interesting source of anthocyanins, in general, and in delphinidin derivatives in particular.
251 While anthocyanins are the most abundant polyphenols in the ripe fruit, flavonols constitute
252 the main phenolic family at the early stages of maturity (until stage 3), with levels of 216 ± 23
253 mg/100 g DW (representing 58% of the total polyphenol content) at stage 1 and 108 ±5
254 mg/100 g DW (61% of the total polyphenols) at stage 3. Afterwards, their content decreased
255 to just 34 ± 2 mg/100 g DW at stage 5. Among the 9 flavonols determined, quercetin-*O*-
256 galactoside was the predominant flavonol at all stages of maturity (except in stage 5), but
257 decreasing from 93 ± 5 mg/100 g DW (stage 1) to 10 ± 1 mg/100 g DW. It was followed in
258 terms of abundance by quercetin-*O*-glucoside, varying from 64 ± 8 mg/100 g DW (stage 1) to
259 11 ± 1 mg/100 g DW (stage 5). Significant amounts of quercetin 3-*O*-glucuronide and

quercetin 3-*O*-rutinoside were also observed. The presence of quercetin derivatives had previously been reported in *P. lentiscus* fruits (Mehenni et al., 2016) and other *Pistacia* species such as *P. vera* (Romani et al., 2002) and *P. atlantica* (Khallouki et al., 2017). In agreement with our results, flavonol glycosides are considered the most abundant phenolic family in *P. lentiscus* fruits at the early fruiting stage (Elez Garofulić et al., 2020).

In the case of flavanols, gallocatechin was the main compound detected, which is in agreement with other studies on *P. vera* (Ojeda-Amador et al., 2019). Levels of this monomer decreased substantially between stages 1 and 2, but then increased during fruit ripening to recover the initial values (over 100 mg/100 g DW). The monomer catechin was the second most abundant flavanol in all cases, varying from 37 ± 1 mg/100 g DW (stage 1) to 18 ± 1 mg/100 g DW (stage 5). A phytochemical study carried out on *P. lentiscus* ripe fruits had also shown a high concentration of catechin (Mehenni et al., 2016). The amount of catechin decreases during ripening similar to the rest of flavanols, appearing to be stable or increase slightly at the latest stages. A study carried out on *P. vera* also showed a decrease of catechin concentration with fruit maturation (Kelebek et al., 2020), as observed in other fruits such grape berry (Benbouguerra et al., 2021b). Interestingly, levels of epicatechin and epigallocatechin were much lower than their isomers catechin and gallocatechin. Epicatechin was under the LOD for the three first stages and under the LOQ of the method (0.04 mg/100 g) at the 4th and 5th stages. Epigallocatechin was under the LOD (0.02 mg/100 g) for all cases. The main procyanidin dimer was tentatively identified as B3, which is a catechin dimer (catechin-(4α→8)-catechin), which seems to confirm the prevalence of catechin forms in *P. lentiscus* fruits over epicatechin forms. In fact, the other dimer identified, procyanidin B1, is an epicatechin-catechin dimer (epicatechin-(4β→8)-catechin), but it accounted for much lower amounts than procyanidin B3 and could not be quantified at the ripe stages. Levels of the epicatechin dimer B2 (epicatechin-(4β→8)-epicatechin) were not quantifiable at any stage (under the LOD of 0.1 mg/100 g), which is again in agreement with the low abundance of epicatechin forms over catechin in the fruit. Other flavanols such as epicatechin gallate and epigallocatechin gallate were observed in small amounts regardless of the stage of maturity. Previous observation of the prevalence of catechin forms seems to indicate that it cannot be ruled out that these minor forms are actually catechin gallate and gallocatechin gallate, since catechin gallate and epicatechin gallate share the same pattern of MS/MS fragmentation, which is also the case between gallocatechin gallate and epigallocatechin gallate.

Three other flavonoids were detected and quantified in *P. lentiscus* fruits: naringenin 7-*O*-glucoside and small amounts of naringenin and taxifolin. Previous studies have shown the presence of taxifolin in high quantities in the leaves of *P. lentiscus* (Vaya & Mahmood, 2006). Naringenin was also detected in minor quantities in *P. vera* hulls (Barreca et al., 2016).
The present investigation also revealed the presence of 2 phenolic acids: 4-hydroxybenzoic acid and protocatechuic acid. The latter was detected for the first time as the main phenolic acid in *P. lentiscus* fruits. The highest concentration was observed at stage 1 (49 ± 4 mg/100 g DW) and reached its lowest level at stage 5 (2 ± 1 mg/100 g DW). In addition to phenolic acids, 2 other non-flavonoid compounds were identified and quantified: phloretin and piceid. Significant amounts of phloretin were observed in *P. lentiscus* fruits up to 47 ± 5 mg/100 g DW at stage 1. Piceid was observed in small amounts in all stages. The presence of stilbenes in *Pistacia* genus was previously reported in *P. vera*: *trans*-resveratrol (Ballistreri et al., 2009; Tokuşoglu et al., 2005) and *trans*-piceid (*trans*-resveratrol-3-*O*-β-glucoside) were found in *P. vera* peanuts (Grippi et al., 2008). However, to the best of our knowledge, this is the first time that piceid has been reported in *P. lentiscus*.

The variation in the content of phenolic compounds during maturation implies variations in therapeutic potential, nutritional benefits and industrial interest of *P. lentiscus* L. fruits. Several studies have previously highlighted the involvement of these fruits in the protection and/or prevention of several pathologies, indicating a correlation between total polyphenols and the antioxidant capacity of *P. lentiscus* fruits (Remila et al., 2015; Yemmen et al., 2017). Antidiabetic potential of ripe fruits of *P. lentiscus* via α-amylase inhibitory capacities has also shown a positive correlation with the concentration of total phenolic compounds (Mehenni et al., 2016). However, most of these studies are based on colorimetric tests for determining the polyphenol content, which cannot be enough to fully understand the potential uses of *P. lentiscus* fruits.

Our results show that the black ripe fruits represent an excellent source of anthocyanins. This class of polyphenols have shown human health benefits linked to a decrease of inflammation and oxidative stress biomarkers and improved cardiovascular risk and metabolic diseases incidence (Ockermann et al., 2021). They have also shown neuroprotective potential effects (Ullah et al., 2019; Zhang et al., 2021), and daily consumption of anthocyanins improved the condition of subjects with diabetes (Fallah et al., 2020). Moreover, anthocyanins are widely exploited in the food industry as natural colorants and preservatives due to their antioxidant

potential (Echegaray et al., 2020). Our results indicate levels of anthocyanins around 1200-1300 mg/100 g DW for *P. lentiscus* fruits, which are in the same range of other fruits widely recognised as sources of these flavonoids. For example, in a study comparing the concentrations of anthocyanins in common foods by a similar HPLC-DAD methodology, blackberries reached levels of 300 mg/100 g FW, wild blueberries had 486 mg/100 g FW, black currant 476 mg/100 g FW, red grapes 120 mg/100 g FW, and strawberries 21 mg/100 g FW (Wu et al., 2006).

On the other hand, unripe *P. lentiscus* fruits can be a valuable source of other flavonoids, namely flavonols, whose remarkable health benefits are associated with their promising anticancer, antioxidant, antimicrobial and antiviral properties (Barreca et al., 2021). Our study reveals maximum amounts of 200 mg/100 g DW at the 1st stage of maturity. For comparison, yellow onions contain 27–119 mg of flavonols per 100 g FW, whereas red onions contain 41–192 mg of flavonols per 100 g FW (Slimestad et al., 2007). Comparing these values, the fruits of *P. lentiscus* could be an interesting source of these flavonoids. Unripe stages could also provide a significant amount of flavanols, mostly in the form of catechin gallate, although other sources such as tea leaves can provide a higher amount of these compounds. In fact, some tea species can reach in some cases over 200 mg total catechins per gram of tea leaves (DW), with epicatechin gallate as the major compound (Deka et al., 2021). Anyway, unripe *P. lentiscus* fruits could be used as an alternative source of flavonols and flavanols, but in that case, they should be better considered as sources for extraction of flavonoids to be formulated in nutraceuticals rather than being directly consumed as foods. Such a strategy has also been proposed for other unripe fruits whose green stages contain more polyphenols than the mature ones, and which are considered a very good raw material for the production of polyphenol-based nutraceuticals with high antioxidant potential (Wojdyło & Oszmiański, 2020). It is worth mentioning that in the case of preparing extracts for nutraceutical purposes, green *P. lentiscus* fruits could also be combined with other parts of the *P. lentiscus* plant such as leaves, which have also proven to contain high amounts of flavonols showing health benefits (Azib et al., 2019)**.**

4. Conclusion

Chemical analyses of the various extracts of *P. lentiscus* fruits during ripening led to the identification of 30 compounds, including 9 anthocyanins, detected and identified by a combination of UHPLC-UV/vis, UHPLC-MS/MS and LC-NMR methodologies. To the best of our knowledge, this is the most detailed study on the polyphenolic composition in *P. lentiscus* fruits, especially concerning the anthocyanin composition, indicating the presence of 3 cyanidin derivatives and 6 delphinidin derivatives in the ripe stages of the fruit, with different glycoside moeieties (glucosides, galactosides, dihexosides and pentosides). Among anthocyanins, delphinidin galactoside was the main compound, while quercetin galactoside and quercetin glucoside were the main flavonols detected and gallocatechin was the main flavanol. Protocatechuic acid and *trans*-piceid were quantified for the first time in *P. lentiscus* fruits.

The contents of each family of polyphenols were highly influenced by the fruit maturation stage. Flavonols were the main polyphenols at the beginning of the fruit ripening, but their levels decreased during the maturation. Flavanols followed a similar pattern, except for gallocatechin. On the opposite side, anthocyanins increased enormously in the last stages of ripening to become the main polyphenols in the mature fruit, followed by gallocatechin. The increase in anthocyanin content during ripening is relevant for using ripe *P. lentiscus* fruits as an important source of this kind of molecules in the diet or anthocyanin-based nutraceuticals and for food applications such as dye additives. Unripe fruits would be interesting as a source of other flavonoids to produce natural extracts enriched in flavonols and flavanols.

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Figure captions

Figure 1. Images of *Pistacia lentiscus* L. fruits collected at different dates corresponding to different stages of maturity

Figure 2. UHPLC-DAD chromatogram of *Pistacia lentiscus* anthocyanins. **1**: Dp dihex (1); **2**: Dp dihex (2); **3**: Dp 3-O-gal; **4**: Dp 3-O-glu; **5**: Cy 3-O-gal; **6**: Dp pent; 7: Cy 3-O-glu; **8**: Cy pent; **9**: Dp pent. *Dp=dephinidin; Cy=Cyanidin; glu=glucoside; gal=galactoside; hex=hexoside; pent=pentoside; dihex=dihexoside*

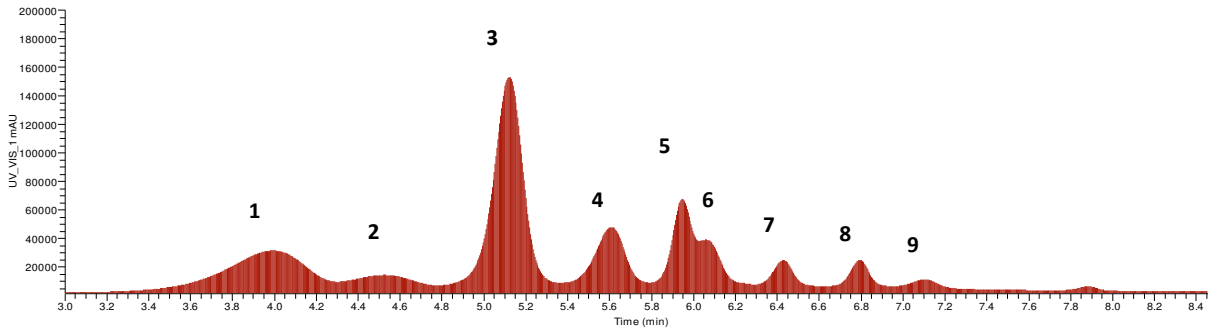
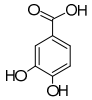
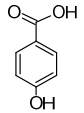
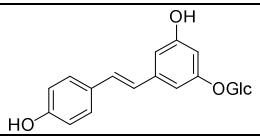
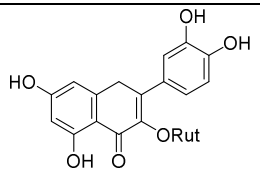
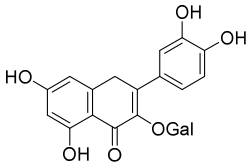
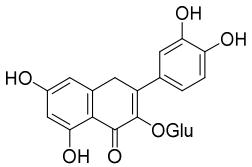
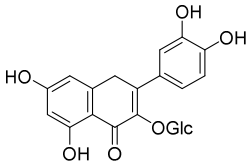
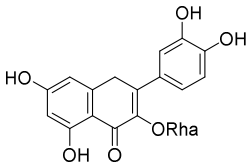
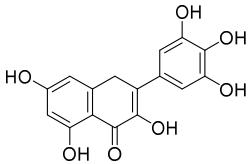
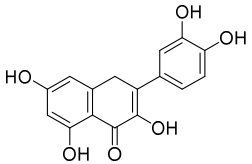
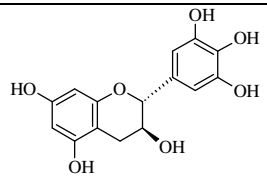


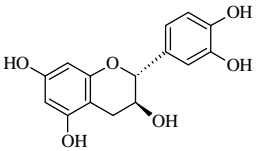
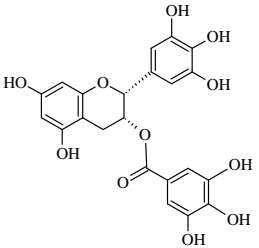
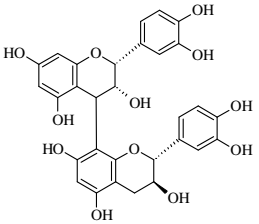
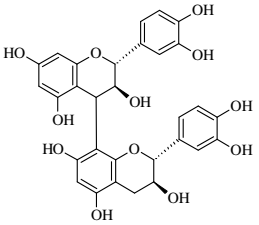
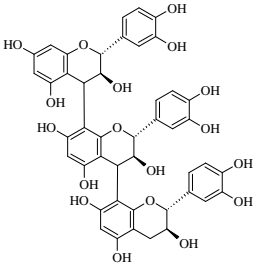
Table 1. UHPLC-QqQ-MS data (peak number, retention time, *m/z* values in positive mode) and LC-NMR data of *P. lentiscus* fruit anthocyanins.

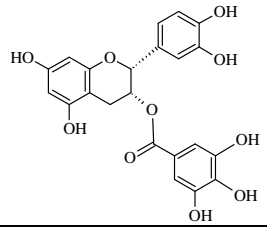
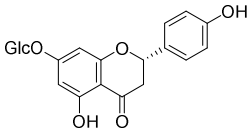
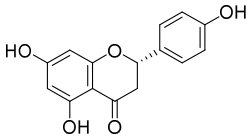
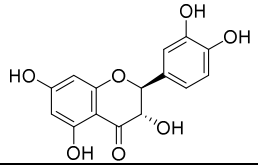
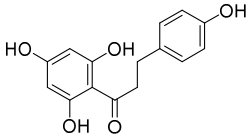
Peak	Compound	t_R (min)	MH^+	MS^2	^{1}H-NMR
1	delphinidin dihexoside 1	3.89	627	303	-
2	delphinidin dihexoside 2	4.48	627	303	-
3	delphinidin 3-*O*-galactoside	5.01	465	303	9.05 (1H, s, H-4), 7.87 (2H, s, H-2′/6′), 6.95 (1H, brs, H-8), 6.73 (1H, d, *J* = 2 Hz, H-6), 5.34 (1H, d, *J* = 8 Hz, H-1″), 4.10 (1H, dd, *J* = 8 and 10 Hz, H-2″), 4.05 (1H, brd, *J* = 3 Hz, H-4″), 3.90-3.84 (3H, H-5″, H-6a″, H-6b″), 3.78 (1H, dd, *J* = 3 and 10 Hz, H-3″)
4	delphinidin 3-*O*-glucoside	5.49	465	303	9.04 (1H, s, H-4), 7.85 (2H, s, H-2′/6′), 6.97 (1H, brs, H-8), 6.73 (1H, d, *J* = 2 Hz, H-6), 5.40 (1H, d, *J* = 8 Hz, H-1″), 3.98 (1H, dd, *J* = 2 and 12 Hz, H-6b″), 3.75 (1H, dd, *J* = 6 and 12 Hz, H-6a″), 3.70-3.50 (4H, H-2″, H-3″, H-4″, H-5″)
5	cyanidin 3-*O*-galactoside	5.84	449	287	9.03 (1H, s, H-4), 8.27 (1H, dd, *J* = 2 and 8 Hz, H-6′), 8.08 (1H, d, *J* = 2 Hz, H-2′), 7.02 (1H, d, *J* = 8 Hz, H-5′), 6.65 (1H, d, *J* = 2 Hz, H-6), 5.26 (1H, d, *J* = 8 Hz, H-1″), 3.98 (1H, dd, *J* = 8 and 10 Hz, H-2″), 3.94 (1H, brd, *J* = 3 Hz, H-4″), 3.85-3.75 (3H, H-5″, H-6a″, H-6b″), 3.77 (1H, dd, *J* = 3 and 10 Hz, H-3″)
6	delphinidin pentoside 1	6.00	435	303	-
7	cyanidin 3-*O*-glucoside	6.33	449	287	9.03 (1H, s, H-4), 8.27 (1H, dd, *J* = 2 and 8 Hz, H-6′), 8.08 (1H, d, *J* = 2 Hz, H-2′), 7.02 (1H, d, *J* = 8 Hz, H-5′), 6.65 (1H, d, *J* = 2 Hz, H-6), 5.40 (1H, d, *J* = 8 Hz, H-1″), 3.98 (1H, dd, *J* = 2 and 12 Hz, H-6b″), 3.75 (1H, dd, *J* = 6 and 12 Hz, H-6a″), 3.70-3.50 (4H, H-2″, H-3″, H-4″, H-5″)
8	cyanidin pentoside	6.69	419	287	-
9	delphinidin pentoside 2	7.00	435	303	-

Table 2. Compound, structure, retention time, MRM-MS condition for the phenolic compounds identified in *P. lentiscus* fruits (Rut = rutinoside; Gal = galactoside; Glucur = glucuronide; Glu = glucoside; Rha = rhamnoside).

Compounds	Structure	t_R (min)	Ion mode (+/–)	Precursor ion	Product ions[a]	Collision energies (V)	Fragmentor (V)
Phenolic acids							
protocatechuic acid		5.32	–	153	**109** 93 65	12 12 24	84
4-hydroxibenzoic acid		13.98	–	137	**93**	12	84
Stilbenes							
trans-piceid		13.01	–	389	**227** 143	4 44	150
Flavonols							
quercetin 3-*O*-rutinoside		13.62	–	609	**301** 271 255	28 60 60	150
quercetin 3-*O*-galactoside		13.69	+	463	**301** 271 255	16 44 40	150

quercetin 3-*O*-glucuronide		13.87	–	477	**301** 151	16 40	150
quercetin 3-*O*-glucoside		14.01	–	463	**301** 271 255	16 44 40	150
quercetin 3-*O*-rhamnoside		15.63	–	447	**301** 271 255	16 40 40	150
myricetin		16.01	–	317	**151** 137	16 20	125
quercetin		17.25	–	301	**151** 121	12 20	175
Flavanols							
gallocatechin		5.91	-	305	**125** 137	12 12	150

catechin		8.30	–	289	**109** 123	29 25	100
epigallocatechin gallate		10.32	–	457	**305** 169 125	12 8 40	118
procyanidin B1		7.90	–	577	**289** 425 407	8 15 15	160
procyanidin B3		8.17	–	577	**289** 425 407	8 15 15	160
Unknown trimer (quantified as procyanidin C1)		8.9 (11.07)	–	865	**125** 407 289	40 40 56	186

Epicatechin gallate		13.36	–	441	**289** 169 125		
Flavanones							
naringenin 7-*O*-glucoside		15.59	+	435	**273** 135	10 20	118
naringenin		18.40	–	271	**151** 119	8 20	118
Flavanonols							
taxifolin		13.13	–	303	**125** 285 57	4 12 40	118
Dihydrochalcone							
phloretin		16.12	+	275	**107**	25	118

[a]Value in bold denote quantification ion.

Table 3: Quantitative analysis of phenolic compounds in *Pistacia lentiscus* fruits (in mg/100g DW).

Compounds	Stage 1[a]	Stage 2	Stage 3	Stage 4	Stage 5
Anthocyanins					
delphinidin dihexosise 1	<LOD	<LOQ	<LOQ	122 ± 3	248 ± 6
delphinidin dihexoside 2	<LOD	<LOD	<LOD	30 ± 4	10 ± 1
delphinidin galactoside	0.8±0.1	1.0±0.2	3 ± 1	513 ± 16	646 ± 8
delphinidin glucoside	0.7±0.1	0.7±0.1	0.8±0.1	319 ± 4	71 ± 1
cyanidin galactoside	0.7±0.2	0.8±0.3	5 ± 1	201 ± 9	103 ± 3
delphinidin pentoside 1	<LOD	<LOD	<LOD	37 ± 2	123 ± 3
cyanidin glucoside	0.8±0.1	0.8±0.1	1.1±0.2	93 ± 5	20 ± 1
cyanidin pentoside	0.7±0.1	0.8±0.1	3 ± 1	42 ± 2	35 ± 5
delphinidin pentoside 2	0.7±0.1	0.7±0.1	0.7±0.1	17 ± 1	19 ± 1
Total anthocyanins	**4.4**±0.7	**4.8**±0.9	**13.6 ± 3.4**	**1373 ± 45**	**1273 ± 27**
Phenolic acids					
protocatechuic acid	49 ± 4	39 ± 2	22 ± 2	7 ± 1	2 ± 1
4-hydroxybenzoic acid	<LOD	0.20 ±0.05	<LOD	<LOD	<LOD
Total phenolic acids	**49 ± 4**	**39 ± 2**	**22 ± 2**	**7 ± 1**	**2 ± 1**
Stilbenes					
trans-piceid	0.21 ± 0.01	0.10 ± 0.05	0.22 ± 0.04	0.11 ± 0.04	0.10 ± 0.04
Flavonols					
quercetin rutinoside	23 ± 3	5.1 ± 0.2	20 ± 1	4.0 ± 0.4	5.0 ± 0.3
quercetin galactoside	93 ± 5	29 ± 3	36 ± 2	24 ± 2	10 ± 1
quercetin glucuronide	34 ± 7	11 ± 1	21 ± 1	13 ± 1	7 ± 1
quercetin glucoside	64 ± 8	20 ± 1	29 ± 1	23 ± 2	11 ± 1
quercetin rhamnoside	1.1± 0.1	0.4 ± 0. 1	0.4 ± 0. 1	0.6 ± 0. 1	0.3 ± 0.1
myricetin	0.5 ± 0.1	0.2 ± 0.1	1 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
quercetin	0.4 ± 0.1	0.2 ± 0. 1	1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
Total flavonols	**216 ± 23**	**66 ± 4**	**108 ± 5**	**65 ± 6**	**34 ± 2**
Flavanols					
catechin	37 ± 1	16 ± 3	17 ± 0.1	21 ± 1	18 ± 1
gallocatechin	104 ± 9	19 ± 0.7	34 ± 2	48 ± 2	104 ± 8
(epi)gallocatechin gallate	1.5 ± 0.1	0.4 ± 0.1	3.0 ± 0.1	2.0 ± 0.2	3.0 ± 0.2
procyanidin B1	0.6 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	<LOQ	<LOQ
procyanidin B3	2.0 ± 0.1	1.1 ± 0.1	1.1 ± 0. 1	0.4 ± 0.1	0.4 ± 0.1
procyanidin C1	2.0 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	0.5 ± 0.1	0.2 ± 0.1
(epi)catechin gallate	1 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	1 ± 0.1	1 ± 0.1
Total flavanols	**148 ± 10**	**38 ± 4**	**56 ± 2**	**72 ± 3**	**127 ±9**
Flavanones					
naringenin glucoside	15.1 ± 0.1	5.2 ± 0.1	3.3 ± 0.1	5.0 ± 0.2	7.0 ± 0.2
naringenin	0.13± 0.02	0.11± 0.02	<LOQ	0.14± 0.02	<LOD
Flavanonols					
taxifolin	0.7 ± 0.1	1.0 ± 0.1	0.3 ± 0.1	0.7 ± 0.1	0.6 ± 0.1
Dihydrochalcone					
Phloretin	47 ± 5	8 ± 1	20 ± 1	18 ± 1	22 ± 1