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1 **Analysis of individual anthocyanins, flavanols, flavonols and other**
2 **polyphenols in *Pistacia lentiscus* L. fruits during ripening**

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1 **Analysis of individual anthocyanins, flavanols, flavonols and other**
2 **polyphenols in *Pistacia lentiscus* L. fruits during ripening**

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4
5 **Abstract**

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

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

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

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

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

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

62 **2. Materials and methods**

63 **2.1 Chemicals and reagents**

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

73

74 **2.2 Plant material and extract preparation**

75 *P. lentiscus* fruits were harvested from the forest of Tizi Neftah province of Amizour, Bejaia,
76 Algeria (GPS coordinates 36.644°N and 4.921°E). The botanical identification was confirmed
77 with support from the Laboratory of Botany, University of Bejaia (Algeria), according to a
78 voucher herbarium specimen (N° 970704) deposited at the National Institute of Agronomy,
79 Algiers, Algeria. The stage of maturity was determined by fruit colour and harvest month.
80 The fruits were air-dried at 30°C in the dark and then crushed with an electric grinder and
81 finally, defatted thrice with hexane (5 g: 40 mL). The defatted powder was then mixed with
82 100% methanol (5 g: 40 mL). Five extraction cycles were carried out in an ultrasonic bath for
83 10 min. The extracts were dried in a rotatory evaporator and solubilized in methanol/water
84 (1:1, v/v) to obtain a final concentration equivalent to 5 g of dried fruit in 10 ml of solvent,
85 and then they were stored at – 20°C until analysis.

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87

88 **2.3 Individual compound analysis**

89 **2.3.1 Anthocyanins analysis**

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

95 600 MHz spectrometer (Wissembourg, France) equipped with a ^1H - ^{13}C inverse-detection
96 flow probe. ^1H -NMR spectra were obtained in stopped-flow mode. For 2D-NMR
97 experiments, individual anthocyanins were collected after on-flow ^1H -LC-NMR analysis onto
98 a FOXY collector from Teledyne ISCO (Lincoln, USA), lyophilized and analyzed by using
99 classical COSY and NOESY 2D-NMR experiments (Acevedo De la Cruz, Alexander et al.,
100 2012).

101 Individual anthocyanin quantification was performed on a UHPLC-MWL-MS/MS system
102 (Thermo Scientific, France) composed of an Accela 1250 system coupled to a TSQ Quantum
103 Access Max triple quadrupole equipped with an H-ESI ion source. Anthocyanins were
104 separated on a Zorbax SB-C18 column (2.1 x 100 mm, 1.8 μm , Agilent) with 1 μL of volume
105 injection. Mobile phase A was deionized water containing 2.5% formic acid (v/v) and mobile
106 phase B was methanol (UHPLC grade) containing 2.5% formic acid (v/v). The elution
107 gradient was for solvent B: 2.5% (0-2 min); 15% (7 min); 23% (9.3 min); 55% (10 min); 95%
108 (11-12,5 min); 2.5% (13-15 min). The temperature and the flow were fixed to 35°C and 0.4
109 mL/min, respectively. The mass detector operated in the positive mode while the source
110 parameters were as follows: Sheath gas 60 a.u.; Auxiliary Gas 20 a.u., Voltage 1500V,
111 Vaporizer Temperature 450°C, Capillary Temperature 150 °C. All compounds were
112 quantified using the MWL detector at 520 nm using a calibration curve built with Dp3glu
113 (range 0.97-1000 mg/L). Results were expressed as mg delphinidin 3-*O*-glucoside (Dp3glu)
114 equivalents per 100 g of dry weight fruits (mg DGE/100g DW). The limits of detection (LOD
115 = 0.08 mg DGE/100g DW) and quantification (LOQ = 0,23 mg DGE/100g DW) were
116 calculated as $\text{LOD}=3.3 \times s/b$ and $\text{LOQ}=10 \times s/b$, where “b” is the slope of the curve and “s”
117 the standard deviation of the signal determined as the residual standard deviation of the
118 calibration line in the LOD region (Kruve et al., 2015).

119

120 **2.3.2 UHPLC-QqQ-MS/MS analysis of other phenolic compounds**

121 Individual phenolic acids and flavonoid compounds were quantified by a targeted UHPLC-
122 QqQ-MS/MS approach according to a previously developed methodology (Loupit et al.,
123 2020; Djemaa-Landri et al., 2020) using an Infinity UHPLC 1260 system coupled to a 6430
124 triple quadrupole (QqQ) mass spectrometer (Agilent Technologies, France). Separation was
125 achieved with an Agilent Poroshell 120 EC-C18 column (150 x 2.1 mm, 2.7 μm) thermostated
126 at 40°C. Acidified water and acidified acetonitrile (both containing formic acid 0.1%, v/v)
127 were used as solvents A and B, respectively. The elution parameters consisted of a 0.4

128 mL/min flow and a solvent B gradient as follows: 1-10% (0-4 min); 20% (12 min); 33% (13-
129 16 min); 40% (21 min); 95% (22-24 min); 95% (25 min); 1% (28 min). Measurements were
130 based on Multiple Reaction Monitoring (MRM) in positive or negative mode depending on
131 the compounds (Supplementary Figure 1). Collision energies applied are shown in Table 2.
132 Quantification was done by comparison with a calibration curve with standards in the range of
133 0.03 to 15.4 mg/L. All compounds were quantified with their corresponding standard except
134 procyanidin B3 and an unknown trimer, expressed as procyanidin B1 and C1, respectively.
135 Calibration parameters can be found in Supplementary Table 1.

136

137

138 **2.4 Statistical analysis**

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

141

142

143 **3. Results and discussion**

144 *Pistacia lentiscus* fruits were collected at 5 different stages of maturity (Figure 1): September
145 2017 (less red unripe fruits, stage 1), October 2017 (red unripe fruits, stage 2), November
146 2017 (Red unripe fruits, stage 3), December 2017 (majority black ripe fruits, stage 4), January
147 2018 (black fully ripe fruits, stage 5).

148

149 **3.1. Identification of phenolic compounds**

150 **3.1.1 Anthocyanins**

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

161 was applied to determine the molecular weight of the molecules. Afterwards, MS/MS
162 fragmentation analysis was performed by using a product ion scan, which selectively isolated
163 the parent ions in the first quadrupole, and fragmented them in the collision cell. The data
164 obtained were consistent with cyanidin and delphinidin derivatives conjugated with sugars.
165 The results were confirmed with a precursor ion scan, which screened the presence of
166 precursors of cyanidin (m/z : 287) and delphinidin (m/z : 303), and by a neutral loss scan,
167 which screened the presence of pentosides (-132 u loss), hexosides (-162 u loss), and
168 dihexosides (-324 u loss). To complete the identification, compounds were analysed by
169 $^1\text{H-LC-NMR}$ (Acevedo De la Cruz, Alexander et al., 2012). The identified compounds are
170 listed in Table 1.

171 Peak 1 was identified as a delphinidin dihexoside. The full scan MS revealed a molecular ion
172 at m/z 627, while the MS/MS product ion scan showed the characteristic m/z 303 of the
173 delphinidin aglycon. The precursor ion scan was consistent with delphinidin and the neutral
174 loss ion scan indicated the loss of two hexoses. Peak 2 was also identified as a delphinidin
175 dihexoside. The full scan MS revealed a molecular ion at m/z 627, while the MS/MS product
176 scan showed the delphinidin aglycon at m/z 303. For peaks 1 and 2, $^1\text{H-LC-NMR}$ data
177 confirmed the identification of delphinidin dihexoside; nevertheless, signal complexity
178 precluded complete annotation. MS and NMR data of peak 3 were consistent with delphinidin
179 3-*O*-galactoside (Table 1). MS spectra exhibited fragment ions at m/z 465 and 303. 2D-NMR
180 NOESY spectrum confirmed the position of the galactose moiety. Similarly, peak 4 was
181 identified as delphinidin 3-*O*-glucoside, with further confirmation by coinjection with the
182 pure standard. Based on MS and NMR data, peak 5 was identified as cyanidin 3-*O*-
183 galactoside. This compound presented a parent ion at m/z 449 and a fragment ion at 287
184 (cyanidin aglycon). The position of the galactose was confirmed by a NOESY experiment.
185 Peak 6 was attributed to delphinidin pentoside, presenting a molecular ion at m/z 435 and a
186 fragment ion at m/z 303. Using MS, NMR data, and comparison with injection of a standard,
187 peak 7 was identified as cyanidin 3-*O*-glucoside (Table 1). Peak 8 and peak 9 were attributed
188 to cyanidin and delphinidin pentoside (molecular ions at m/z 419 and 435, respectively), loss
189 of pentoside moiety providing typical ions of cyanidin and delphinidin aglycones (m/z 287
190 and 303, respectively). Unfortunately, NMR spectra complexity precludes the complete
191 identification of peaks 6, 8 and 9.

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

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

202

203 **3.1.2 Other phenolic compounds**

204 Besides the anthocyanin profile, the phenolic composition of *P. lentiscus* fruit was analysed
205 by UHPLC-QqQ mass spectrometry. The approach was based on a multiple reaction
206 monitoring (MRM) method previously developed for the analysis of phenolic compounds in
207 natural extracts (Gabaston et al., 2020; Loupit et al., 2020). The identification relied on the
208 comparison of the retention time with the pure standard and the presence of at least 2
209 fragment ions. To assure the good attribution of the chromatographic peaks, samples were
210 also spiked with a solution of standards at a concentration of 2 mg/L. A total of 21
211 compounds were retained as being identified in the different stages of maturation (Table 2)
212 including 2 phenolic acids, 1 stilbene, 7 flavanols, 7 flavonols, 2 flavanones, 1 flavanonol and
213 1 dihydrochalcone.

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

224 In the case of flavanols, 7 compounds were detected including catechin, gallic acid, gallocatechin,
225 procyanidin B1, epigallocatechin gallate and epicatechin gallate, procyanidin B3 and an
226 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

260 quercetin 3-*O*-rutinoside were also observed. The presence of quercetin derivatives had
261 previously been reported in *P. lentiscus* fruits (Mehenni et al., 2016) and other *Pistacia*
262 species such as *P. vera* (Romani et al., 2002) and *P. atlantica* (Khallouki et al., 2017). In
263 agreement with our results, flavonol glycosides are considered the most abundant phenolic
264 family in *P. lentiscus* fruits at the early fruiting stage (Elez Garofulić et al., 2020).
265 In the case of flavanols, galliccatechin was the main compound detected, which is in
266 agreement with other studies on *P. vera* (Ojeda-Amador et al., 2019). Levels of this monomer
267 decreased substantially between stages 1 and 2, but then increased during fruit ripening to
268 recover the initial values (over 100 mg/100 g DW). The monomer catechin was the second
269 most abundant flavanol in all cases, varying from 37 ± 1 mg/100 g DW (stage 1) to
270 18 ± 1 mg/100 g DW (stage 5). A phytochemical study carried out on *P. lentiscus* ripe fruits
271 had also shown a high concentration of catechin (Mehenni et al., 2016). The amount of
272 catechin decreases during ripening similar to the rest of flavanols, appearing to be stable or
273 increase slightly at the latest stages. A study carried out on *P. vera* also showed a decrease of
274 catechin concentration with fruit maturation (Kelebek et al., 2020), as observed in other fruits
275 such grape berry (Benbouguerra et al., 2021b). Interestingly, levels of epicatechin and
276 epigallocatechin were much lower than their isomers catechin and galliccatechin. Epicatechin
277 was under the LOD for the three first stages and under the LOQ of the method (0.04 mg/100
278 g) at the 4th and 5th stages. Epigallocatechin was under the LOD (0.02 mg/100 g) for all
279 cases. The main procyanidin dimer was tentatively identified as B3, which is a catechin dimer
280 (catechin-(4 α →8)-catechin), which seems to confirm the prevalence of catechin forms in *P.*
281 *lentiscus* fruits over epicatechin forms. In fact, the other dimer identified, procyanidin B1, is
282 an epicatechin-catechin dimer (epicatechin-(4 β →8)-catechin), but it accounted for much
283 lower amounts than procyanidin B3 and could not be quantified at the ripe stages. Levels of
284 the epicatechin dimer B2 (epicatechin-(4 β →8)-epicatechin) were not quantifiable at any stage
285 (under the LOD of 0.1 mg/100 g), which is again in agreement with the low abundance of
286 epicatechin forms over catechin in the fruit. Other flavanols such as epicatechin gallate and
287 epigallocatechin gallate were observed in small amounts regardless of the stage of maturity.
288 Previous observation of the prevalence of catechin forms seems to indicate that it cannot be
289 ruled out that these minor forms are actually catechin gallate and galliccatechin gallate, since
290 catechin gallate and epicatechin gallate share the same pattern of MS/MS fragmentation,
291 which is also the case between galliccatechin gallate and epigallocatechin gallate.

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

307

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

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

325 potential (Echegaray et al., 2020). Our results indicate levels of anthocyanins around 1200-
326 1300 mg/100 g DW for *P. lentiscus* fruits, which are in the same range of other fruits widely
327 recognised as sources of these flavonoids. For example, in a study comparing the
328 concentrations of anthocyanins in common foods by a similar HPLC-DAD methodology,
329 blackberries reached levels of 300 mg/100 g FW, wild blueberries had 486 mg/100 g FW,
330 black currant 476 mg/100 g FW, red grapes 120 mg/100 g FW, and strawberries 21 mg/100 g
331 FW (Wu et al., 2006).

332 On the other hand, unripe *P. lentiscus* fruits can be a valuable source of other flavonoids,
333 namely flavonols, whose remarkable health benefits are associated with their promising
334 anticancer, antioxidant, antimicrobial and antiviral properties (Barreca et al., 2021). Our study
335 reveals maximum amounts of 200 mg/100 g DW at the 1st stage of maturity. For comparison,
336 yellow onions contain 27–119 mg of flavonols per 100 g FW, whereas red onions contain 41–
337 192 mg of flavonols per 100 g FW (Slimestad et al., 2007). Comparing these values, the fruits
338 of *P. lentiscus* could be an interesting source of these flavonoids. Unripe stages could also
339 provide a significant amount of flavanols, mostly in the form of catechin gallate, although
340 other sources such as tea leaves can provide a higher amount of these compounds. In fact,
341 some tea species can reach in some cases over 200 mg total catechins per gram of tea leaves
342 (DW), with epicatechin gallate as the major compound (Deka et al., 2021). Anyway, unripe *P.*
343 *lentiscus* fruits could be used as an alternative source of flavonols and flavanols, but in that
344 case, they should be better considered as sources for extraction of flavonoids to be formulated
345 in nutraceuticals rather than being directly consumed as foods. Such a strategy has also been
346 proposed for other unripe fruits whose green stages contain more polyphenols than the mature
347 ones, and which are considered a very good raw material for the production of polyphenol-
348 based nutraceuticals with high antioxidant potential (Wojdyło & Oszmiański, 2020). It is
349 worth mentioning that in the case of preparing extracts for nutraceutical purposes, green *P.*
350 *lentiscus* fruits could also be combined with other parts of the *P. lentiscus* plant such as
351 leaves, which have also proven to contain high amounts of flavonols showing health benefits
352 (Azib et al., 2019).

353

354

355 **4. Conclusion**

356 Chemical analyses of the various extracts of *P. lentiscus* fruits during ripening led to the
357 identification of 30 compounds, including 9 anthocyanins, detected and identified by a
358 combination of UHPLC-UV/vis, UHPLC-MS/MS and LC-NMR methodologies. To the best
359 of our knowledge, this is the most detailed study on the polyphenolic composition in *P.*
360 *lentiscus* fruits, especially concerning the anthocyanin composition, indicating the presence of
361 3 cyanidin derivatives and 6 delphinidin derivatives in the ripe stages of the fruit, with
362 different glycoside moieties (glucosides, galactosides, dihexosides and pentosides). Among
363 anthocyanins, delphinidin galactoside was the main compound, while quercetin galactoside
364 and quercetin glucoside were the main flavonols detected and gallocatechin was the main
365 flavanol. Protocatechuic acid and *trans*-piceid were quantified for the first time in *P. lentiscus*
366 fruits.

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

376

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383 **5. Bibliography**

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527

528

529 **Figure captions**

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

532



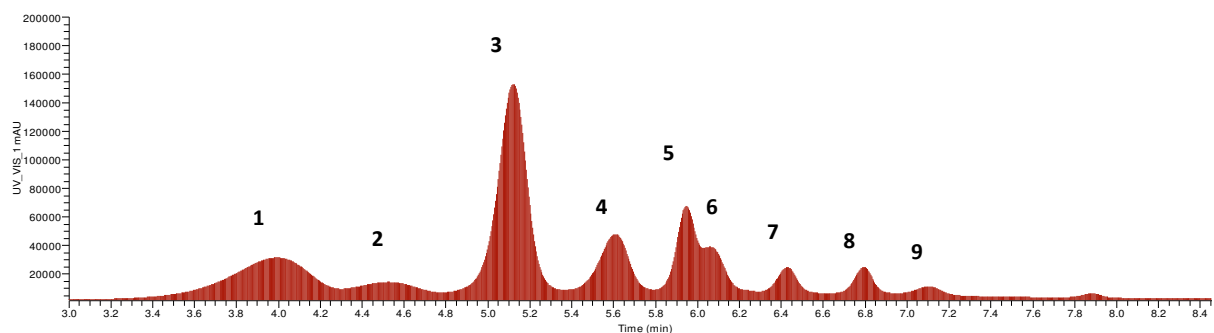
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536 **Figure 2.** UHPLC-DAD chromatogram of *Pistacia lentiscus* anthocyanins. **1:** Dp dihex (1); **2:**
537 Dp dihex (2); **3:** Dp 3-O-gal; **4:** Dp 3-O-glu; **5:** Cy 3-O-gal; **6:** Dp pent; **7:** Cy 3-O-glu; **8:** Cy
538 pent; **9:** Dp pent.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

540




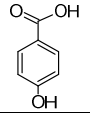
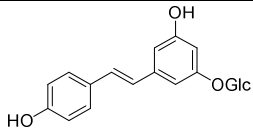
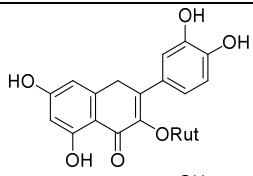
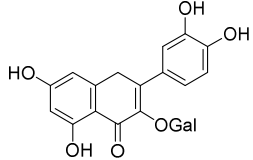
1 **Table 1.** UHPLC-QqQ-MS data (peak number, retention time, m/z values in positive mode) and LC-NMR data of *P. lentiscus* fruit anthocyanins.

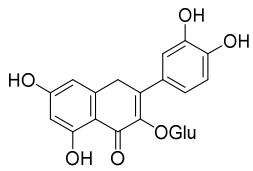
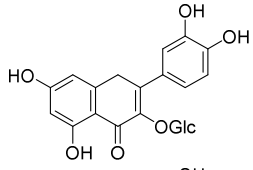
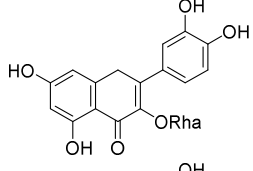
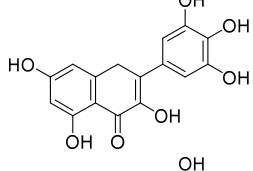
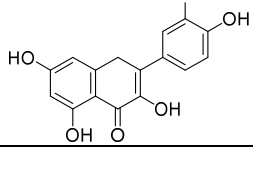
Peak	Compound	t_R (min)	MH^+	MS^2	1H -NMR
1	delphinidin dihexoside 1	3.89	627	303	-
2	delphinidin dihexoside 2	4.48	627	303	-
3	delphinidin 3- <i>O</i> -galactoside	5.01	465	303	9.05 (1H, s, H-4), 7.87 (2H, s, H-2'/6'), 6.95 (1H, brs, H-8), 6.73 (1H, d, $J = 2$ Hz, H-6), 5.34 (1H, d, $J = 8$ Hz, H-1''), 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- <i>O</i> -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- <i>O</i> -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- <i>O</i> -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	-

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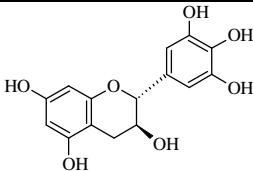
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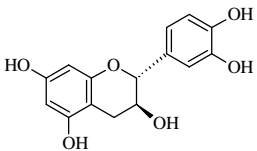
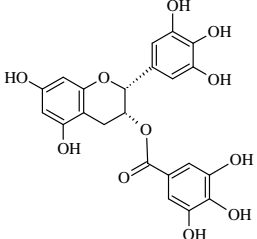
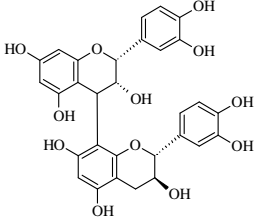
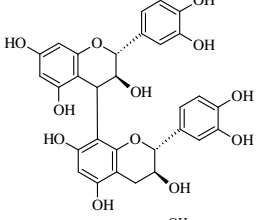
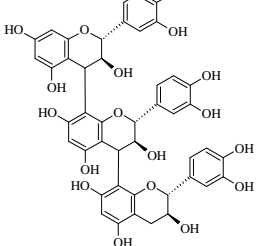
- 4 **Table 2.** Compound, structure, retention time, MRM-MS condition for the phenolic compounds identified in *P. lentiscus* fruits (Rut = rutinose;
 5 Gal = galactoside; Glucur = glucuronide; Glu = glucoside; Rha = rhamnoside).

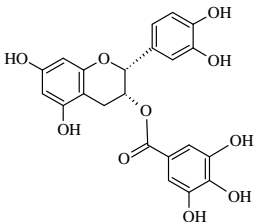
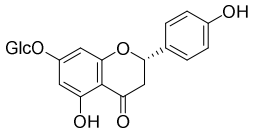
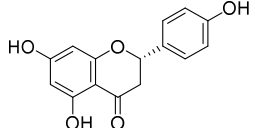
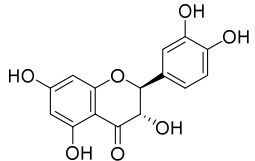
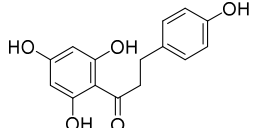
Compounds	Structure	t_R (min)	Ion mode (+/-)	Precursor ion	Product ions ^a	Collision energies (V)	Fragmentor (V)
Phenolic acids							
protocatechuic acid		5.32	-	153	109 93 65	12 12 24	84
4-hydroxybenzoic acid		13.98	-	137	93	12	84
Stilbenes							
<i>trans</i> -piceid		13.01	-	389	227 143	4 44	150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

quercetin 3- <i>O</i> -glucuronide		13.87	-	477	301 151	16 40	150
quercetin 3- <i>O</i> -glucoside		14.01	-	463	301 271 255	16 44 40	150
quercetin 3- <i>O</i> -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
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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	

6 ^aValue in bold denote quantification ion.

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8 **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 dihexoside 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					
<i>trans</i> -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

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