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This paper must be cited as:

Melgar-Castañeda, B.; Dias, MI.; Ciric, A.; Sokovic, M.; Garcia-Castello, EM.; Rodríguez López, AD.; Barros, L.... (01-2). Bioactive characterization of Persea americana Mill. by-products: A rich source of inherent antioxidants. Industrial Crops and Products. 111:212-218. https://doi.org/10.1016/j.indcrop.2017.10.024

The final publication is available at

https://doi.org/10.1016/j.indcrop.2017.10.024

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Additional Information

1 # Characterization of natural bioactive compounds in *Persea* 2 *americana* Mill. by-products

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4 Bruno Melgar[a,b], Maria Inês Dias[a], Marina Sokovic[c], Esperanza M. Garcia-Castello[b], 5 Antonio D. Rodriguez-Lopez[d], Lillian Barros[a], Isabel C.F.R. Ferreira[a,*]

6

7 [a]*Centro de Investigação de Montanha (CIMO), ESA, Instituto Politécnico de Bragança,* 8 *Campus de Santa Apolónia, 5300-253 Bragança, Portugal.*

9 [b]*Institute of Food Engineering for Development, Universitat Politècnica de València,* 10 *Camino de Vera, s/n CP, 46022 Valencia, Spain.*

11 [c]*University of Belgrade, Department of Plant Physiology, Institute for Biological* 12 *Research "Siniša Stanković", Bulevar Despota Stefana 142, 11000 Belgrade, Serbia.*

13 [d]*Institute for Industrial, Radiophysical and Environmental Safety (ISIRYM), Universitat* 14 *Politècnica de València, Camino de Vera, s/n CP, 46022 Valencia, Spain.*

15

16 *Corresponding author. Tel.+351 273 303219; fax +351 273 325405. E-mail address: 17 iferreira@ipb.pt (I.C.F.R. Ferreira)

18

Abstract

Avocado (*Persea americana* Mill.) is a worldwide consumed fruit, with great interest for cosmetic and pharmaceutical industries; however, 30% of avocado fruits are bio-wastes (peels and kernels), converting them into potential source of bioactive compounds, such as phenolic compounds. Therefore, the hydroethanolic extracts of peels and kernels of *Persea america* Mill. var. Hass were analysed regarding their individual phenolic profile by HPLC-DAD/ESI-MS and correlated with their antioxidant, antimicrobial and cytotoxic activities. Avocado by-products presented a very distinct phenolic profile, presenting higher concentration in peels, mainly in (epi)catechin derivatives, followed by chlorogenic derivatives. Peels also presented the highest antioxidant potential, mainly due to the presence of phenolic compounds, and an overall better performance in the antibacterial assays. Further studies needs to be conducted to better understand the correlation between the presence of phenolic compounds and bioactivities, however, the main objective is to implement these biocompounds in different products and industries.

Keywords: Avocado; *Persea americana* Mill. var. Hass; by-products; phenolic compounds; antioxidant activity; antimicrobial activity.

1. Introduction

The world population is increasingly higher each year, which can reach 9.7 billion by the year 2050, leading to many concerns about food security issues, especially food inequity in undeveloped countries (UN DESA, 2015). The numbers are alarming, demonstrating great differences between countries, in this sense the Food and Agriculture Organisation report 795 millions of undernourishment people in the world (FAO, IFAD, & WFP, 2015) and the World Health Organisation published data where more than 1,9 billion adults are overweight (WHO, 2014).

Currently, some food crops are being exploited with purposes other than human feed, like the ones used to produce biofuels (Naik, Goud, Rout, & Dalai, 2010), broadening the food scarcity. Fortunately, food industries and academia have started to play special attention to food by-products in order of their revalorization, keeping a better control of waste management and finding alternatives to the usage of food crops in other applications (Ayala-Zavala et al., 2011; M. Librán, Mayor, M. Garcia-Castello, & Vidal-Brotons, 2013; Sharma, Mahato, Cho, & Lee, 2017).

Avocado (*Persea americana* Mill.) is a dicotyledoneous plant from the *Lauraceae* family, native from south central Mexico, but with global consumption. It is manly consumed as a fresh fruit, although, food, cosmetic and pharmaceutical industries process its pulp in order to increase commercialisation and give a higher added value to avocado (FAO, 2004). There are several varieties within the *P. americana* species such as Bacon, Hass, Fuerte, Gwen, among many others, being Hass one of the most commercialise varieties. Hass avocados has around 14% and 16% of total fruit weight in its peels and kernel, respectively, accounting around 30% of its weight on by-products (Calderón-Oliver et al., 2016). The peels and pulps of many fruits, including avocado, contain a large amount of antioxidants that are responsible for the plant

62 defence system, against different types of stresses, such as temperature and light
63 (Ghasemzadeh & Ghasemzadeh, 2011; Manach, Scalbert, Morand, Rémésy, & Jiménez,
64 2004). They are also known to have effects against some human degenerative diseases
65 (Ghasemzadeh & Ghasemzadeh, 2011; Šiler et al., 2014; Vinha et al., 2014; Kaur Kala,
66 Mehta, Tandey, Sen, & Mandal, 2016).
67 The recovery of bioactive compounds from different waste materials has been the main
68 focus of many scientific studies, since the agro-industries valorise these by-products,
69 generating a large amount of phytochemicals, that can be applied as functional food
70 compounds or as food ingredients, (e.g. colorants, emulsifiers, thickeners, antioxidants,
71 among others) (Azeredo, 2009; Dias, Sousa, Alves, & Ferreira, 2016; Gong & Bassi,
72 2016). Due to the high volume of by-products generated from the avocado industries
73 and the possibility of extracting functional biomolecules, the main focus of this study
74 was to analyse the waste materials (peels and kernels) of *Persea america* Mill. var.
75 Hass, concerning their individual phenolic profile, and also evaluate their antioxidant,
76 antimicrobial and cytotoxic potential, in order to acquire better understanding of their
77 intrinsic compounds and functions, which would allow to find an adequate use of these
78 by-products.
79

80 **2. Material and Methods**

81 *2.1. Samples preparation*

82 Ripen avocado (*Persea americana* Mill. var. Hass) were purchased from a local market
83 in Bragança, Portugal and stored at 4 °C until further used. Within 24 hours, fruits were
84 cut, peeled and separated in three fractions (peel, kernel and pulp). Pulp was discarded,
85 peels were carefully cleaned and kernels were frozen. Afterwards, kernels and peels
86 were lyophilized (LabConco, Frezone -105 °C, 4.5 L Cascade Benchtop Freeze Dry

87 System, Kansas, MO, USA), grounded (~20 mesh), and stored at -20 °C in a cool and
88 dry place until further use.
89
90 *2.2. Extraction procedure*
91 Hydroethanolic extraction (ethanol: water, 80:20 *v/v*) was performed with 1 g of
92 lyophilized peels and kernels, by magnetic stirring with 25 mL of aqueous ethanol (25
93 ºC at 150 rpm) for 1 h and subsequently filtered through the Whatman no. 4 paper.
94 Supernatants were collected and tissues were re-extracted one more time with the same
95 conditions. The obtained extracts were combined, and the ethanol was evaporated
96 (rotary evaporator Büchi R-210, Flawil, Switzerland), the remaining aqueous phase was
97 frozen and subsequently lyophilized.
98 The obtained dry extracts were re-dissolved in order to prepare stock solutions in (i)
99 aqueous ethanol solution 80%, for antioxidant activity evaluation (final concentration
100 40 mg/mL) and for phenolic characterization (final concentration 5 mg/mL); (ii) water
101 (final concentration 8 mg/mL) for cytotoxicity evaluation, and (iii) 5% DMSO in
102 distilled water (final concentration 10 mg/mL) for antimicrobial properties. Stock
103 solutions were further diluted to different concentration for the evaluation of distinct *in*
104 *vitro* bioactivity assays.
105
106 *2.3. Phenolic compounds*
107 For phenolic profile characterization, LC-DAD–ESI/MSn analyses were performed
108 using a Dionex Ultimate 3000 UPLC instrument (ThermoScientific, San Jose, CA,
109 USA), coupled to a diode-array detector (using several wavelengths, 280 nm, 330 nm
110 and 370 nm) and to a mass detector (Linear Ion Trap LTQ XL mass spectrometer,
111 equipped with an ESI source, ThermoFinnigan, San Jose, CA, USA). The elution

112 gradient, chromatographic and mass spectrometer conditions were performed according
113 to the previously described by Bessada, Barreira, Barros, Ferreira, & Oliveira, (2016).
114 Identification was performed by comparing their fragmentation pattern, retention times
115 and UV–vis spectra with authentic standards, when available, or by comparing the
116 obtained information with available data from literature. The quantification was
117 performed using calibration curves of phenolic standards. When a standard was not
118 available to quantify a phenolic compound, a similar compound of the same phenolic
119 group was used. Phenolic compounds quantification results are present in mg/g of
120 extract.

121

122 *2.4. Bioactive properties evaluation*

123 *2.4.1. Antioxidant activity assays*

124 Stock solution from *P. americana* by-products were successively diluted and submitted
125 to different *in vitro* assays to evaluate the antioxidant activity of the samples, following
126 the previously described procedure by Vieira, Barros, Martins, & Ferreira, (2016). The
127 results were expressed as EC_{50} values (sample concentration providing 50% of
128 antioxidant activity or 0.5 of absorbance in the reducing power assay) for antioxidant
129 activity. Trolox was used as positive control.

130

131 *2.4.2. Antimicrobial activity assays*

132 Antibacterial activity was assayed using the following, four gram-positive bacteria:
133 *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (clinical isolate), *Micrococcus*
134 *flavus* (ATCC10240), and *Listeria monocytogenes* (NCTC7973) and four gram-
135 negative bacteria: *Escherichia coli* (ATCC 35210), *Pseudomonas aeruginosa* (ATCC
136 27853), *Salmonella typhimurium* (ATCC 13311), and *Enterobacter cloacae* (ATCC

137 35030). For the antifungal assays, seven microfungi were used: *Aspergillus fumigatus*
138 (ATCC1022), *Aspergillus ochraceus* (ATCC12066), *Aspergillus versicolor*
139 (ATCC11730), *Aspergillus niger* (ATCC6275), *Penicillium funiculosum* (ATCC
140 36839), *Penicillium ochrochloron* (ATCC9112), *Penicillium verrucosum* var.
141 *cyclopium* (food isolate) and *Trichoderma viride* (IAM 5061).
142 The minimum inhibitory (MIC), minimum bactericidal concentrations (MBC) and
143 minimum fungicidal concentrations (MFC) were determined by methodologies,
144 described by Chahdoura et al. (2016). Bacterial and fungal organisms were obtained
145 from the Mycological Laboratory, Department of Plant Physiology, Institute for
146 Biological Research "Sinisa Stanković", University of Belgrade, Serbia and the results
147 were expressed in mg/mL. Standard drugs, namely streptomycin, ampicillin, bifonazole
148 and ketoconazole were used as positive controls. Samples were tested in duplicate and
149 the experiments were repeated three times.
150
151 *2.5. Statistical analysis*
152 All the extractions and assays were performed in triplicate and results were expressed as
153 mean values and standard deviation (SD). Results were analysed using a Student´s t-
154 test, in order to determine the significant difference between the two samples, with p =
155 0.05. The treatment was carried out using IBM SPSS Statistics for Windows, version
156 23.0. (IBM Corp., Armonk, New York, USA).
157
158 **3. Results and discussion**
159 *3.1 Phenolic profile of* P. americana *by-products*
160 **Table 1** presents the peak characteristics (retention time, λ_{max} in the visible region, mass
161 spectral data), tentative identifications and quantification of phenolic compounds in the

162 hydroethanolic extracts from pulp and kernel of *P. americana*. Twenty-nine phenolic
163 compounds were identified, fourteen flavan-3-ols ((epi)catechin derivatives), nine
164 flavonoids (quercetin, kaempferol and isorhamnetin glycoside derivatives) and six
165 phenolic acids (chlorogenic and coumaric acid derivatives). Peels and kernel present a
166 very distinct profile being the only common compounds, peaks 10 and 12. The phenolic
167 profile of avocado has been previously described by other authors in pulp (Hurtado-
168 Fernández et al., 2013; Hurtado-Fernández, Pacchiarotta, Mayboroda, Fernández-
169 Gutiérrez, & Carrasco-Pancorbo, 2014), peels (Kosińska et al., 2012) and seeds
170 (Kosińska et al., 2012; Ramos-Jerz, Villanueva, Jerz, Winterhalter, & Deters, 2013).
171 However, many of the identified compounds were identified for the first time in
172 avocado by-products. A representative chromatogram of peels and kernel are presented
173 in **Figure 1**.

174 The main family of phenolic compounds found in avocado peels and kernels were
175 (epi)catechin derivatives. Peaks 7 and 10 were positively identified as (+)-catechin and
176 (-)-epicatechin, respectively, by comparison with commercial standards taking into
177 account also their retention time, mass and UV-vis characteristics. Peak 7 (catechin)
178 was the major compound found in kernel sample, while epicatechin (peak 10) was the
179 major compound in peel samples. Peaks 3, 9 and 21 presented a pseudomolecular ion at
180 *m/z* 577 and MS^2 fragments at *m/z* 451 (-126 mu), 425 (-152 mu) and 407 (-152-18 mu)
181 and also *m/z* 289 and 287, coherent with the loss of two (epi)catechin units, being for
182 that manner tentatively identified as B-type (epi)catechin dimers. Similarly, peaks 11
183 and 12 ($[M-H]^-$ at *m/z* 865), peaks 13 and 14 ($[M-H]^-$ at *m/z* 1153), peaks 15 and 16
184 ($[M-H]^-$ at *m/z* 1441) and peaks 18, 19 and 20 ($[M-H]^-$ at *m/z* 1729) were assigned as B-
185 type (epi)catechin trimers, tetramers, pentamers and hexamers (Barros et al., 2015;

186 Peláez-Cid, Velázquez-Ugalde, Herrera-González, & García-Serrano, 2013; Rached et
187 al., 2016).
188 The second major family of compounds found in avocado peels samples were
189 flavonoids, mainly quercetin derivatives. Peak 24 was identified as quercetin-3-*O*-
190 glucoside by comparison of its UV spectrum (λ_{max} 352 nm) and retention time with a
191 commercial standard. Peaks 17, 22, 23, 25, 26 and 28 presented a pseudomolecular ion
192 $[M-H]^-$ at *m/z* 625, 595, 477, 463, 609 and 579, respectively, and an unique MS^2
193 fragment at *m/z* 301, being tentatively identified as quercetin-dihexoside, quercetin-
194 pentoside-hexoside, quercetin-glucuronide, quercetin-hexoside, quercetin-rhamnoside-
195 hexoside and quercetin-rhamnoside-pentoside, respectively. Peaks 27 ($[M-H]^-$ at *m/z*
196 461) and 28 ($[M-H]^-$ at *m/z* 491) presented a unique MS^2 fragments at *m/z* 285 and 315,
197 respectively, corresponding to the loss of a glucuronide unit (-176 mu), being
198 tentatively identified as kaempferol- and isorhamnetin-glucuronide, respectively.
199 Regarding the phenolic acids, caffeoylquinic acids and *p*-coumaroyl quinic acid isomers
200 were the only compounds found in both samples, being the majority of them found in
201 kernel samples, being peaks 8 and 6 the only phenolic acids found in peels samples.
202 Peaks 1, 2, 6 and 8 were identified as caffeoylquinic acid derivatives according to their
203 UV spectra and pseudomolecular ions, being previously found by Kosińska et al. (2012)
204 and Ramos-Jerz et al. (2013) in peels and seeds of *P. americana*. Peak assignments of
205 the different caffeoylquinic acids and *p*-coumaroyl quinic acid isomers were made using
206 the recommended IUPAC numbering system (IUPAC, 1976) as also the hierarchical
207 keys previously developed by (Clifford, Johnston, Knight, & Kuhnert, 2003; Clifford,
208 Knight, & Kuhnert, 2005). By comparison its UV spectrum (λ_{max} 326 nm) and retention
209 time with a commercial standard, peak 8 was identified as 5-*O*-caffeoylquinic acid.
210 Peaks 1 and 2 ($[M-H]^-$ at *m/z* 353) were identified as *cis* 3-*O*-caffeoylquinic acid and

211 *trans* 3-*O*-caffeoylquinic acid, respectively, according to their elution order and also
212 yielding a base peak at *m/z* 191 and the ion at *m/z* 179 with an intensity >70% base
213 peak, characteristic of 3-acylchlorogenic acids (Clifford et al., 2003, 2005). After UV
214 irradiation (366 nm, 24 h) of hydroxycinnamic acids in our laboratory, it was possible to
215 observe that the hydroxycinnamoyl *cis* derivatives elute before the corresponding *trans*
216 ones and therefore the assignment of cis and trans forms for peaks 1 and 2. Compound 6
217 was tentatively identified as 4-*O*-caffeoylquinic acid ($[M-H]^-$ at *m/z* 353) according to
218 the fragmentation pattern yielding a base peak at *m/z* 173 [quinic acid-H-$H_2O]^-$,
219 accompanied by a secondary fragment ion at *m/z* 179 (~75% abundance), thus being
220 distinct from the other two isomers (Clifford et al., 2003, 2005).
221 Taking into account the same findings, peaks 4 and 5 were tentatively identified as *cis*
222 and *trans* 3-*p*-coumarouylquinic acids. Kosińska et al. (2012) have previously
223 identified these compounds in avocado peels.
224

225 *3.2. Antioxidant capacity of* P. americana *by-products*

226 Polyphenolic content in plants and fruits have been extensively studied due to the good
227 impact these substances has shown as a potential health benefits arising from their
228 biological activity as hepatoprotective, antiinflammatory, antiviral, antimicrobial and
229 antioxidant activities (Ambigaipalan, 2015; Carocho & Ferreira, 2013; Dias et al., 2016;
230 Kaur Kala et al., 2016). From the 29 different compounds found on *P. americana* by-
231 products, 23 of them were found in peels (227.9 mg/g of extract) and only 8 in kernels
232 (72.5 mg/g of extract), this represent around 3-fold higher polyphenols content in peels,
233 these results obtained proved to be consistent with the other reports (Kosińska et al.,
234 2012; Rodríguez-Carpena, Morcuende, & Estévez, 2011). It is also important here to
235 stress, the higher phenolic content in *P. americana* by-products compared with the

236 edible pulp (Wang, Bostic, & Gu, 2010). This greater polyphenolic content has also
237 displayed higher antioxidant capacity, and as expected *P. americana* peels proof to
238 excel the kernels activity in around 1.5-fold higher in all the antioxidant tests performed
239 and shown in **Table 2.** Although the differences in the total phenolic content between
240 peels and kernels is 3 times higher, the antioxidant activity observed it is only around
241 half (1.5) greater. This could be explained due to the different degrees of polymerization
242 of the polyphenolic compounds of *P. american* peels. As it was mention before,
243 Epicatechin was the most abundant compound in both by-products, peels are also
244 majoritarian in different sort of B-type epicatechin dimer, trimer, tetramers and flavonol
245 glycosides. On one hand Hollman et al. (1999) reports higher antioxidant activity on
246 aglycones compared with glycosides, while Mishra, et al. (2013) point out the
247 difference between dimers, trimers, tetramers, heptamers and hexamers specificity on
248 their properties as an antioxidants. These and other interaction could explain the
249 differences obtained in the antioxidant activity between *P. americana* by-products, but
250 in order to obtain better comprehension on this phenomena, more analysis have to be
251 performed.
252

253 *3.3 Antimicrobial activity of* P. americana *by-products*

254 Flavonoids are known to be synthesized by plants in response to microbial infections,
255 thus, it is not surprising this sort of compounds exhibit antimicrobial activity in many *in*
256 *vitro* assays. Many flavonoid rich plant and fruits from different species have been
257 reported with antimicrobial activity (Balouiri, Sadiki, & Ibnsouda, 2016; Rodrigo &
258 Martínez-l, 2015; Sansano, Rivas, Pina-Pérez, Martinez, & Rodrigo, 2017; Šiler et al.,
259 2014; Vieira, Fernandes, et al., 2016). *P. americana* by-products have shown great
260 antibacterial and moderate antifungal activity against the strains tasted **Table 3**. The

261 bactericidal effect of the samples proved to outstand from the controls employed in 7
262 out of 8 different gram positive and negative strains, although, in this particular assay
263 extracts from kernels displayed better MCB in 6 out of 8 strains, same power in *B.*
264 *cereus* strain, and only worst power in *E. clocae* strain. The results here exposed shown
265 better performance when compared with the results reported by Raymond Chia &
266 Dykes. (2010). Contrary to bactericidal effect, fungicidal effect only was shown in 2
267 strains but only with kernel extracts, from which, the better fungicidal effect was against
268 *Trhichoderma viridely*. Comparing the fungistatic effect, both by-product extract were
269 effective against all 8 strains, but only kernel extract did not shown effectivity against
270 P. funiculosum. Both peels and kernels extract performed better at fungistatic level in 3
271 strains (*A. ochraceus, T. viridely* and *P. ochrochloron*) when compared with
272 ketokonazole antifungal commertial drug and kernel extract display better fungistastic
273 effect in strain vs bifonazole (stronger control used). Antibacterial flavonoids might be
274 having multiple cellular targets, rather than one specific site of action. Tsuchiya &
275 Iinuma. (2000) suggested an alteration of membrane fluidity in hydrophilic and
276 hydrophobic regions in this way flavonoids might reduce the fluidity of outer and inner
277 layers of membranes. Mishra, et al. (2009) propose that one of the molecular actions of
278 flavonoids is to form complex with proteins through nonspecific forces such as
279 hydrogen bonding and hydrophobic effects, as well as by covalent bond formation, this
280 interactions have the ability to inactivate microbial adhesins, enzymes, cell envelope
281 transport proteins, and so forth.
282
283 **4. Conclusion**
284 One of the main goals of this research was to revalorize *P. americana* by-products by
285 characterizing their main nutritional and functional properties. With these assays we

286 were able to detect new phenolic compounds in avocado by-products, quantify them and
287 test their antioxidant capacity which shown to be superior to the capacity reported by
288 other authors in the edible pulp. In the same manner, previous reports about
289 antimicrobial activity were revised and confronted with the data obtained, the results
290 obtained exhibit good capacity against certain bacterial and fungal strains. All the data
291 recollected is just the begging of a series of experiments that have to be design in order
292 to implement these functional molecules in different products and industries.

293

294 **Acknowledgements**

295 The authors are grateful to the Foundation for Science and Technology (FCT, Portugal)
296 and FEDER under Programme PT2020 for financial support to CIMO
297 (UID/AGR/00690/2013) and L. Barros grant (SFRH/BPD/107855/2015). B. Melgar
298 thanks CONACyT for his grant (No. 329930).

299

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