

Proanthocyanidins from Averrhoa bilimbi fruits and leaves

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1 **Manuscript title: Procyanidins from *Averrhoa bilimbi* fruits and**
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16 **Procyanidins from *Averrhoa bilimbi* fruits and leaves**

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38 **Abstract**

39 Proanthocyanidins from *Averrhoa bilimbi* fruits and leaves were analysed by thiolysis with
40 benzyl mercaptan and high performance liquid chromatography - mass spectrometry and
41 consisted of pure B-type procyanidins. These tannins consisted of almost pure homopolymers,
42 with epicatechin accounting for most of the monomeric subunits in fruits (97%) and leaves
43 (99%). Leaves contained more procyanidins (4.5 vs 2.2 g/100 g dry weight) with a higher
44 mean degree of polymerisation (9 vs 6) than fruits. This study thus contributes information on
45 the proanthocyanidins of a traditional food that can make an important contribution to the
46 intake of compounds with antioxidant and health benefits. The fruits are prized for culinary
47 purposes and the leaves are used in traditional medicine.

48

49 **Keywords:** *Averrhoa bilimbi*, cucumber tree, food analysis, proanthocyanidins, condensed
50 tannins, gel-NMR, thiolysis, benzyl mercaptan

51

52 **1. Introduction**

53 Investigation into the phytochemical profiles of underutilized and/or wild foods is becoming
54 increasingly important in the context of food security and tree foods are of particular interest,
55 as trees are generally more resilient to periodic droughts and unseasonal weather events than
56 crops. Underutilised foods can be especially valuable when staple foods are in short supply.
57 Information on the contents of non-nutrients is needed to explore their bioactivities and
58 dietary health benefits (Rush, 2001). Therefore, knowledge of the phytochemical composition
59 of wild foods will allow local populations to better exploit local resources and their benefits
60 (Scoones et al. 1992).

61 *Averrhoa bilimbi* (L.), commonly called the cucumber tree (Figure 1), belongs to the family
62 of Oxalidaceae and grows in tropical regions (Central America, Asia and Caribbean Islands).
63 The fruits are consumed locally in culinary preparations (fresh in salad or pickled) or as juice.
64 The juice can also be used as a remedy to treat dental disorders, sore throats and stomach
65 problems (Ariharan et al. 2012). *Averrhoa bilimbi* fruits have shown anti-obesity properties or
66 anti-cholesterolemic activity (Ambili et al. 2009) and also antibacterial and antioxidant
67 activities (Ashok Kumar et al. 2013). However, their high acidity (pH = 4) and high oxalate
68 concentration (Morton et al. 1987) has led to renal failure after prolonged consumption of the
69 juice in humans (Bakul et al. 2013). In terms of phytochemical compounds, the fruits are a
70 good source of vitamin C (Ariharan et al. 2012) and various flavonoids (myricetin, luteolin,
71 quercetin and apigenin) have been quantified (Koo Hui & Suhaila, 2001). Although the
72 presence of tannins has been mentioned in the fruits (Ashkok Kumar et al. 2013,
73 Hasanuzzaman et al. 2013), to our knowledge, proanthocyanidins have not previously been
74 detected or characterised in *A. bilimbi* fruits or leaves. The leaves are traditionally used as a
75 paste made with water for dermatological issues (skin rashes, itches, shingles, eczema,
76 pimples) and against rheumatism (Ariharan et al. 2012). This information will be useful for

77 probing the health benefits of *A. bilimbi* fruits and leaves, for expanding food databases on
78 proanthocyanidins (websites 1 and 2) and for enabling intake calculations, especially for
79 populations consuming wild tropical and underutilised fruits and vegetables.

80

81 **2. Materials and methods**

82 *2.1. General*

83 Acetone (analytical reagent grade), acetonitrile (HPLC grade), dichloromethane (HPLC
84 grade) and hydrochloric acid (37%, analytical reagent grade), were purchased from
85 ThermoFisher Scientific Ltd (Loughborough, U.K.); (\pm)-taxifolin (98%); benzyl mercaptan
86 (99%), epicatechin (EC) and catechin (C) (\geq 99% HPLC) were purchased from Sigma-Aldrich
87 (Poole, U.K.). Deionised water was obtained from a Milli-Q System (Millipore, Watford,
88 U.K.).

89

90 *2.2. Samples*

91 *Averrhoa bilimbi* leaves and fruits were harvested in December 2013 in a private botanical
92 garden in Trois-Rivières, Guadeloupe, France. Any excess humidity was removed with
93 kitchen paper, air-dried for a few hours, protected from direct light and immediately packed in
94 an air-tight glass container and sent to Reading, U.K. by airplane (1-3 days). Upon arrival,
95 leaves and fruits were freeze-dried and finely ground in an impeller SM1 cutting mill (Retsch,
96 Haan, Germany) to pass a 1 mm sieve. The ground plant material was stored in the dark at
97 room temperature.

98

99 *2.3. Extraction and purification*

100 *2.3.1. Extractable proanthocyanidins*

101 Finely ground fruits (5.3 g) and leaves (5.5 g) were extracted using magnetic stirring for 1 h
102 with acetone/water (125 mL; 7:3, v/v) and the solution was separated from the residue after
103 filtration through a Büchner funnel. Acetone was removed under vacuum at 30 °C; the
104 remaining aqueous solution was centrifuged for 3 min at 2045 x g and freeze-dried to give the
105 extract (fruits = 1.6 g, yield = 31%; leaves = 0.8 g, yield = 14%). Acetone was allowed to
106 evaporate from the plant residue in the fume cupboard overnight and protected from direct
107 light before freeze-drying; these residues were used for the analysis of unextractable
108 proanthocyanidins.

109

110 2.4. *Proanthocyanidin analysis*

111 2.4.1. *Thiolysis of extractable proanthocyanidins*

112 Acetone-water extracts (8 mg) were weighed in triplicates into screw cap glass tubes with a
113 stirring magnet. Methanol (1.5 mL) was added followed by methanol acidified with
114 concentrated HCl (3.3%; 500 µL) and benzyl mercaptan (50 µL). Tubes were capped and
115 placed into a water bath at 40 °C for 1 h under vigorous stirring. The reaction was stopped by
116 placing the tube in an ice bath for 5 min. Distilled water (2.5 mL) and the internal standard,
117 taxifolin in methanol (500 µL; 0.1 mg/mL), were added and thoroughly mixed. The mixture
118 was transferred into a 800 µL vial, closed with a crimp top and analyzed by HPLC-MS within
119 12 h (Ramsay et al. 2015).

120

121 2.4.2. *Thiolysis of in situ and unextractable proanthocyanidins*

122 Whole freeze-dried fruits and leaves or the plant residues (200 mg), which remained after the
123 aqueous acetone extraction, were reacted with the thiolysis reagent (2 mL methanol, 1 mL of
124 3.3% HCl in methanol, and 100 µL benzyl mercaptan) in triplicates as above. After the
125 reaction, methanol (1 mL) was added to the mixture. The sample was mixed and centrifuged

126 at 2727 x g for 3 min and supernatant (1 mL) was transferred into another screw cap glass
127 tube. Distilled water (9 mL) and internal standard, taxifolin in methanol (500 µL; 0.1 mg/mL),
128 were added and thoroughly mixed. The mixture was transferred into a vial, closed with a
129 crimp top and analysed by HPLC-MS as soon as possible or within the next 12 h.

130

131 2.5. Liquid chromatography-mass spectrometry (HPLC-MS) analysis

132 LC-MS was used to check for the presence of free flavan-3-ols in the plant materials and
133 extract and to confirm the identity of terminal and extension units using an Agilent 1100
134 Series HPLC system and an API-ES instrument Hewlett Packard 1100 MSD detector (Agilent
135 Technologies, Waldbronn, Germany). Samples (20 µl) were injected into the HPLC
136 connected to an ACE C₁₈ column (3 µm; 250 x 4.6 mm; Hichrom Ltd, Theale, U.K.), which
137 was fitted with a corresponding ACE guard column, at room temperature. The HPLC system
138 consisted of a G1379A degasser, G1312A binary pump, G1313A ALS autoinjector, and
139 G1314A VWD UV detector. Data were acquired with ChemStation software (version A 10.01
140 Rev. B.01.03). The flow rate was 0.75 ml/min using 1% acetic acid in water (solvent A) and
141 HPLC-grade acetonitrile (solvent B). The following gradient programme was employed: 0-35
142 min, 36% B; 35-40 min, 36-50% B; 40-45 min, 50-100% B; 45-55 min, 100-0% B; 55-60
143 min, 0% B. Eluting compounds were recorded at 280 nm. Mass spectra were recorded in the
144 negative ionisation scan mode between *m/z* 100 and 1000 using the following conditions:
145 capillary voltage, -3000 V; nebuliser gas pressure, 35 psi; drying gas, 12 ml/min; and dry
146 heater temperature, 350 °C (Ramsay & Mueller-Harvey, 2015). Flavan-3-ols and their benzyl
147 mercaptan adducts were identified by their retention times and characteristic UV-VIS spectra
148 between 220 and 595 nm. Peak areas of flavan-3-ols at 280 nm were integrated and quantified
149 using molar response factors relative to taxifolin: 0.30 for catechin and epicatechin; 0.26 for
150 their benzyl mercaptan adducts (Gea et al. 2011). This provided information on the

151 proanthocyanidin composition in terms of % terminal and % extension flavan-3-ol units (i.e.
152 molar percentages). It also allowed calculation of the mean degree of polymerisation (mDP),
153 % procyanidins (PC) and % *cis*- and *trans*-flavan-3-ols (molar percentages) (Gea et al. 2011).

154

155 2.6. Gel-NMR analysis

156 Samples were prepared as previously described (Grabber et al. 2013). Briefly, finely milled
157 plant material (50 mg) was mixed in DMSO-*d*6 (400 µL) and pyridine-*d*5 (100 µL) and
158 transferred to a 5 mm NMR tube. ¹H-¹³C correlation 2D NMR (HSQC) spectra were recorded
159 at 27 °C on a Bruker Avance III 500 instrument equipped with TopSpin 2.4 software and a 5-
160 mm BBI ¹H/¹³C gradient probe (Bruker, Coventry, U.K.). Spectral resonances were
161 referenced to the residual signals of DMSO-*d*6 (2.49 ppm for ¹H and 39.5 ppm for ¹³C
162 spectra) using 128 scans.

163

164 3. Results and Discussion

165 *Averrhoa bilimbi* fruits and leaves were analysed by thiolytic degradation with benzyl
166 mercaptan for proanthocyanidin content and composition directly using the ground plant
167 materials (i.e. *in situ* analysis) and also the aqueous acetone extracts and plant residues that
168 remained from the solvent extractions. The thiolysis reaction released proanthocyanidin
169 terminal units as flavan-3-ols and extension units as benzyl mercaptan derivatives, which
170 were analysed by reverse-phase HPLC-MS (Ramsay & Mueller-Harvey, 2015). The
171 proanthocyanidin contents and compositions are described in Table 1 for both fruits and
172 leaves. Figures 3 and 4 illustrate the HPLC chromatograms of fruit and leaf
173 proanthocyanidins after thiolysis. The total proanthocyanidin content in fruits is lower than in
174 leaves (2.2 vs 4.5 g/100 g of dry weight). The average proanthocyanidin polymer size in fruits
175 was also lower (mDP of 6 vs 9) than in leaves [*Note: no free flavan-3-ols could be detected in*

176 *the plant materials or extract before thiolysis*]. The key finding is that *A. bilimbi* fruits and
177 leaves contained only pure procyanidins (PC) (Figure 2).
178 Epicatechin accounted for 97% of the flavan-3-ol units in fruit proanthocyanidins and for
179 99% of the leaf proanthocyanidins, with catechin accounting for the rest. Catechin and
180 epicatechin occurred as terminal units in fruits and leaves, but epicatechin was the only
181 extension unit. Catechin and epicatechin were assigned to peaks 1 and 2, respectively, at
182 retention times of 23 min and 27 min (Figures 3 and 4), with ion fragments at m/z 289.3 [$M -$
183 H] $^-$. The epicatechin-benzyl mercaptan adduct was assigned to peak 3 at a retention time of
184 43 min and generated ion fragments at m/z 411.3 [$M - H$] $^-$ and, after loss of the benzyl
185 mercaptan molecule ($- 124$ amu) at m/z 287.2.
186 Unextractable proanthocyanidins were also investigated as they are often overlooked (Gea et
187 al. 2011), yet their proportion can exceed extractable proanthocyanidins in foods and may
188 thus represent a substantial amount of the dietary polyphenol intake (Pérez-Jiménez & Torres,
189 2011). In fact, there were higher amounts of unextractable than extractable proanthocyanidins
190 (fruits: 1.3 vs 0.8, leaves: 3.2 vs 1.3). The mDP values were also higher in the unextractable
191 than the extractable proanthocyanidins (fruits: 6.7 vs 4.6, leaves: 13.7 vs 6.5) and agrees with
192 our previous findings (Gea et al. 2011; Mechineni et al. 2014, Wang et al. 2015).
193 A gel-NMR analysis ($^1H - ^{13}C$ HSQC) was also applied directly to the milled leaves and
194 fruits in order to verify the results from thiolysis. This analysis revealed distinct signals for
195 procyanidins: signals at 6.7 and 120 ppm could be assigned to H/C-2'/5'/6' and signals at 6.0
196 and 95 ppm were assigned to H/C-6 and H/C-8 (Figure 2). This confirmed that these
197 proanthocyanidins were procyanidins and B-type linkages. The presence of A-type
198 proanthocyanidins would have been indicated by signals at approximately H/C-3 (4.0/66
199 ppm) and H/C-3 (4.5/27.9 ppm) but it was not detected (Appeldoorn et al. 2009).

200 Although proanthocyanidins have limited bioavailability and are relatively stable in the
201 gastrointestinal tract (Serra et al. 2010), some evidence exists for their depolymerisation by
202 intestinal microorganisms (Pérez-Maldonado & Norton et al. 1996; Touriño et al. 2009).
203 Studies have also shown that procyanidins with lower mDP (< 4) are most likely absorbed in
204 the colon after metabolisation by the gut microbiota and their metabolites could be detected in
205 the plasma (Kerimi & Williamson, 2015). Proanthocyanidins and their metabolites can act as
206 antioxidants *in vivo* (López-Andrés et al. 2013) and modulate key biological pathways *in vivo*
207 (Nantz et al. 2013; Vertraetan et al. 2013).

208

209 **4. Conclusion**

210 This study revealed the presence of pure procyanidins in *A. bilimbi* fruits and leaves with a
211 moderate average proanthocyanidin size. Epicatechin accounted for 94% to 97% of the
212 flavan-3-ol subunits and these polymers had mean degrees of polymerisation that ranged from
213 5 to 14. Pure proanthocyanidins are not so common, especially in edible fruits. Therefore *A.*
214 *bilimbi* fruits and leaves are potentially valuable sources for proanthocyanidins that could be
215 used for future research into their nutritional and health benefits.

216

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220

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224

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322

323 **Figure captions**

324

325 Fig. 1. *Averrhoa bilimbi* fruits.

326

327 **Fig. 2.** Structure of a procyanidin dimer (catechin-(4→8)-epicatechin).

328

329 **Fig. 3.** HPLC chromatogram at 280 nm after *in situ* thiolysis of proanthocyanidins from

330 *Averrhoa bilimbi* fruits: **1**, catechin; **2**, epicatechin; **3**, epicatechin-benzyl mercaptan.

331

332 **Fig. 4.** HPLC chromatogram at 280 nm after *in situ* thiolysis of proanthocyanidins from

333 *Averrhoa bilimbi* leaves: **1**, catechin; **2**, epicatechin; **3**, epicatechin-benzyl mercaptan.

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347 **Table 1**348 Content and composition of *in situ*, extractable and unextractable proanthocyanidins in *Averrhoa bilimbi* fruits and leaves (n= 3).

Proanthocyanidins	Content (g/100 g DW)	mDP	PC (%)	<i>cis</i> (%)	<i>trans</i> (%)	Terminal units		Extension units
						(%)		(%)
						C	EC	EC
Fruits								
<i>In situ</i>	2.2 (0.1)	6.1 (0.1)	100	96.8 (0.2)	3.2 (0.2)	3.1 (0.2)	13.5 (0.5)	83.4 (0.2)
Extractable	0.8 (0.1)	4.6 (0.1)	100	94.4 (0.1)	5.6 (0.1)	5.7 (0.1)	15.9 (0.1)	78.4 (0.1)
Unextractable	1.3 (0.1)	6.7 (0.6)	100	96.9 (1.5)	3.1 (1.6)	3.2 (1.6)	11.8 (0.2)	85.0 (1.3)
Leaves								
<i>In situ</i>	4.5 (0.2)	9.2 (0.1)	100	99.5 (0.1)	0.5 (0.1)	0.5 (0.1)	10.4 (0.1)	89.1 (0.1)
Extractable	1.3 (0.1)	6.5 (0.3)	100	98.5 (0.3)	1.5 (0.3)	1.5 (0.3)	13.8 (0.5)	84.7 (0.8)
Unextractable	3.2 (0.1)	13.7 (0.1)	100	99.6 (0.1)	0.4 (0.1)	0.4 (0.1)	6.9 (0.2)	92.7 (0.1)

349

350 DW: dry weight; mDP: mean degree of polymerisation; PC: procyanidins; C: catechin (a 2,3-*trans* flavan-3-ol); EC: epicatechin (a 2,3-*cis*

351 flavan-3-ol); % represents relative molar percentages.

352