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Abstract

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

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

1. Introduction

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

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

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

2. Materials and methods

2.1. General

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

2.2. Samples

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

2.3. Extraction and purification

2.3.1. Extractable proanthocyanidins

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

2.4. Proanthocyanidin analysis

2.4.1. Thiolysis of extractable proanthocyanidins

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

2.4.2. Thiolysis of in situ and unextractable proanthocyanidins

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

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

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

LC-MS was used to check for the presence of free flavan-3-ols in the plant materials and extract and to confirm the identity of terminal and extension units using an Agilent 1100 Series HPLC system and an API-ES instrument Hewlett Packard 1100 MSD detector (Agilent Technologies, Waldbronn, Germany). Samples (20 µl) were injected into the HPLC connected to an ACE C_{18} column (3 µm; 250 x 4.6 mm; Hichrom Ltd, Theale, U.K.), which was fitted with a corresponding ACE guard column, at room temperature. The HPLC system consisted of a G1379A degasser, G1312A binary pump, G1313A ALS autoinjector, and G1314A VWD UV detector. Data were acquired with ChemStation software (version A 10.01 Rev. B.01.03). The flow rate was 0.75 ml/min using 1% acetic acid in water (solvent A) and HPLC-grade acetonitrile (solvent B). The following gradient programme was employed: 0-35 min, 36% B; 35-40 min, 36-50% B; 40-45 min, 50-100% B; 45-55 min, 100-0% B; 55-60 min, 0% B. Eluting compounds were recorded at 280 nm. Mass spectra were recorded in the negative ionisation scan mode between *m/z* 100 and 1000 using the following conditions: capillary voltage, -3000 V; nebuliser gas pressure, 35 psi; drying gas, 12 ml/min; and dry heater temperature, 350 °C (Ramsay & Mueller-Harvey, 2015). Flavan-3-ols and their benzyl mercaptan adducts were identified by their retention times and characteristic UV-VIS spectra between 220 and 595 nm. Peak areas of flavan-3-ols at 280 nm were integrated and quantified using molar response factors relative to taxifolin: 0.30 for catechin and epicatechin; 0.26 for their benzyl mercaptan adducts (Gea et al. 2011). This provided information on the

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

2.6. Gel-NMR analysis

Samples were prepared as previously described (Grabber et al. 2013). Briefly, finely milled plant material (50 mg) was mixed in DMSO-*d6* (400 μL) and pyridine-*d5* (100 μL) and transferred to a 5 mm NMR tube. ^{1}H-^{13}C correlation 2D NMR (HSQC) spectra were recorded at 27 °C on a Bruker Avance III 500 instrument equipped with TopSpin 2.4 software and a 5-mm BBI $^{1}H/^{13}C$ gradient probe (Bruker, Coventry, U.K.). Spectral resonances were referenced to the residual signals of DMSO-*d6* (2.49 ppm for ^{1}H and 39.5 ppm for ^{13}C spectra) using 128 scans.

3. Results and Discussion

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

the plant materials or extract before thiolysis]. The key finding is that *A. bilimbi* fruits and leaves contained only pure procyanidins (PC) (Figure 2).

Epicatechin accounted for 97% of the flavan-3-ol units in fruit proanthocyanidins and for 99% of the leaf proanthocyanidins, with catechin accounting for the rest. Catechin and epicatechin occurred as terminal units in fruits and leaves, but epicatechin was the only extension unit. Catechin and epicatechin were assigned to peaks 1 and 2, respectively, at retention times of 23 min and 27 min (Figures 3 and 4), with ion fragments at *m/z* 289.3 $[M - H]^-$. The epicatechin-benzyl mercaptan adduct was assigned to peak 3 at a retention time of 43 min and generated ion fragments at *m/z* 411.3 $[M - H]^-$ and, after loss of the benzyl mercaptan molecule (– 124 amu) at *m/z* 287.2.

Unextractable proanthocyanidins were also investigated as they are often overlooked (Gea et al. 2011), yet their proportion can exceed extractable proanthocyanidins in foods and may thus represent a substantial amount of the dietary polyphenol intake (Pérez-Jiménez & Torres, 2011). In fact, there were higher amounts of unextractable than extractable proanthocyanidins (fruits: 1.3 *vs* 0.8, leaves: 3.2 *vs* 1.3). The mDP values were also higher in the unextractable than the extractable proanthocyanidins (fruits: 6.7 *vs* 4.6, leaves: 13.7 *vs* 6.5) and agrees with our previous findings (Gea et al. 2011; Mechineni et al. 2014, Wang et al. 2015).

A gel-NMR analysis ($^1H - ^{13}C$ HSQC) was also applied directly to the milled leaves and fruits in order to verify the results from thiolysis. This analysis revealed distinct signals for procyanidins: signals at 6.7 and 120 ppm could be assigned to H/C-2'/5'/6' and signals at 6.0 and 95 ppm were assigned to H/C-6 and H/C-8 (Figure 2). This confirmed that these proanthocyanidins were procyanidins and B-type linkages. The presence of A-type proanthocyanidins would have been indicated by signals at approximately H/C-3 (4.0/66 ppm) and H/C-3 (4.5/27.9 ppm) but it was not detected (Appeldoorn et al. 2009).

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

4. Conclusion

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

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Figure captions

Fig. 1. *Averrhoa bilimbi* fruits.

Fig. 2. Structure of a procyanidin dimer (catechin–(4→8)–epicatechin).

Fig. 3. HPLC chromatogram at 280 nm after *in situ* thiolysis of proanthocyanidins from *Averrhoa bilimbi* fruits: **1**, catechin; **2**, epicatechin; **3**, epicatechin-benzyl mercaptan.

Fig. 4. HPLC chromatogram at 280 nm after *in situ* thiolysis of proanthocyanidins from *Averrhoa bilimbi* leaves: **1**, catechin; **2**, epicatechin; **3**, epicatechin-benzyl mercaptan.

Table 1

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 (%)	*cis* (%)	*trans* (%)	Terminal units (%)		Extension units (%)
						C	EC	EC
Fruits								
In situ	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								
In situ	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)

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