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Short communication

Procyanidins from *Averrhoa bilimbi* fruits and leaves

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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.

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 (Fig. 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). *A. bilimbi* fruits have shown anti-obesity properties or anti-cholesterolemic activity (Ambili et al., 2009) and also antibacterial and antioxidant activities (Kumar et al., 2013). However, their high acidity (pH 4) and high oxalate concentration (Morton, 1987) have 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 and Suhaila, 2001). Although the presence of tannins has been mentioned in the fruits (Kumar et al., 2013; Hasanuzzaman et al., 2013), to our knowledge, proanthocyanidins (Fig. 2) 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 (Website 1, 2015; Website 2, 2015) 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)

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Fig. 1. *Averrhoa bilimbi* fruits.

were purchased from Sigma–Aldrich (Poole, U.K.). Deionised water was obtained from a Milli-Q System (Millipore, Watford, U.K.).

2.2. Samples

A. 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.

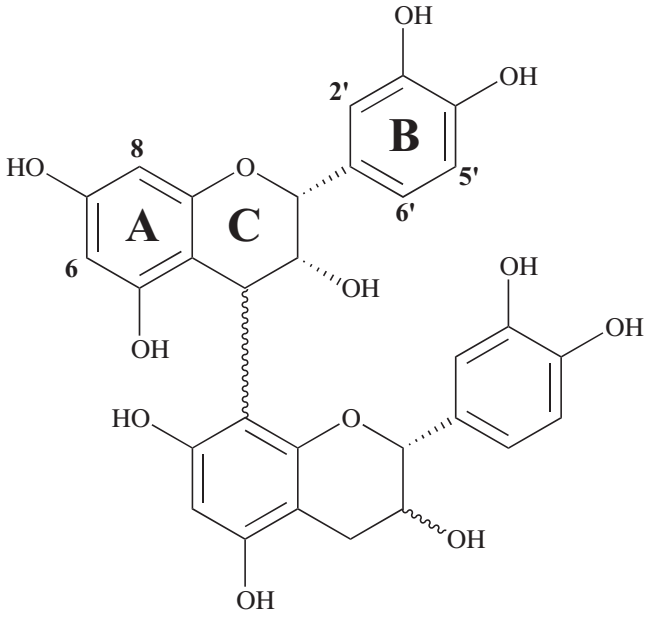

Fig. 2. Structure of a procyanidin dimer (epicatechin – (4 → 8) – epicatechin or epicatechin).

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 × *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 and Mueller-Harvey, 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 × *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 (Ramsay and Mueller-Harvey, 2015).

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 × 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