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ABSTRACT

The genus *Dioscorea* hosts a large number of yam species used medicinally and as a staple food by millions of people in tropical countries. All yams share a high antioxidant activity that correlates with their total phenolics contents. However, knowledge of edible species antioxidant phenolics remains incomplete. Bioassay-guided fractionation of the tubers of a yam species with high total phenolics content, *D. nummularia*, led to the isolation of two flavonols, caryatin and 3′-O-methyl caryatin, contributing over 92% of a stable methanol extract of the tuber in this species. Their structures were determined by high resolution mass spectrometry (HRMS), nuclear magnetic resonance (NMR) and a synthesized authentic sample. They displayed no bacterial toxicity. Their content was estimated in the tubers and bulbils of 411 landraces and hybrids belonging to eight food yam species using high performance-thin layer chromatography (HP-TLC). These were found to be highly variable at the intra- and interspecific levels to reach up to 1 030 and 457 μg/g DW respectively and added up to contents of already described antioxidant phenolics. This study provides additional understanding of the health benefits of a yam diet and constitutes the first phytochemical analysis of *D. nummularia*.

1. Introduction

With approximately 640 species, the genus *Dioscorea* L. (*Dioscoreaceae*, Dioscoreales) is incredibly diverse (Govaerts et al., 2007) and economically important. It hosts a large panel of edible yam species that constitute the major staple food of millions of people in tropical regions of Africa, Caribbean, South-East Asia and the numerous small islands of Oceania (Asiedu and Sartie, 2010). The most important edible species are *D. alata* L., *D. bulbifera* L., *D. cayenensis* Lam., *D. dumetorum* (Kunth) Pax, *D. esculenta* (Lour.) Burkill, *D. japonica* Thunb., *D. nummularia* Lam., *D. oppositifolia* L., *D. pentaphylla* L., *D. rotundata* Poir., and *D. trifida* L. About 98 % of the 70 Mt of yam produced in 2018 on 9 Mha originated from West Africa (FAOSTAT, 2020), and mainly from the two Guinea yams (*D. rotundata* and *D. cayenensis*). However, several species from Asia-Oceania exhibit attractive agronomic performances, high yields and interesting organoleptic qualities. Among them *D. alata* has the most ancient domestication history, is cultivated on the largest geographical scale and is gaining in importance in West Africa (Lebot, 2020).

A large body of work has stressed the numerous health benefits of a yam diet. Besides starch, all cultivated yams are a good source of proteins, fibers, potassium, carotenoids, polyphenols and vitamins C and E (Champagne et al., 2010, 2011; Chandrasekara and Kumar, 2016; Lebot et al., 2019). More specific health benefits also exist as a yam diet has been shown to reduce cognitive deterioration and brain lipid peroxidation in mice (Chan et al., 2004) as well as type-2 diabetes mellitus induced by a high fat diet (Ma et al., 2020). Many wild yams are used medicinally (Dutta, 2015; Mohan et al., 2011; Salehi et al., 2019) and their bioactive substances are as diverse as the afflictions they cure. These include allantoin, an hydantoin with stomach-protecting, antitumoral and anti-diabetic properties (Go et al., 2015; Lee et al., 2011; Liu et al., 2016; Ma et al., 2020). Steroidal saponins such as dioscin and diosgenin contribute to breast cancer protection (Aumsuwan et al., 2016), and to the antioxidative, hypolipidemic (Son et al., 2007), anticholesterolemia (Cayen and Dvornik, 1979), antifungal (Cho et al.,

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2013) and renal injury protection (Zhang et al., 2017) properties of yam extracts. All analyzed yam species contain significant quantities of bioactive phenolic compounds (Champagne et al., 2011; Chandrasekara and Kumar, 2016). For example, kaempferol-3,5-dimethyl ester was shown to contribute to the anti-tumoral activity of ethanol extracts of the Chinese medicinal yam, *D. bulbifera* (Gao et al., 2002). Compared to other medicinal plant species, medicinal yams are a noted source of antioxidants that have been suggested to contribute to many of their therapeutic benefits (Adeniran et al., 2020; Gao et al., 2002; Liu et al., 2016; Song et al., 2010). Following the screening of 56 selected Chinese medicinal plants, *D. bulbifera* ranked among the four best sources of antioxidants (Song et al., 2010).

Staple food yams have received less attention with regard to their phytochemical content although some species such as *D. bulbifera* may be medicinal predicaments and food contributors (Guan et al., 2017; Kundu et al., 2020). It has nevertheless been established that food and medicinal yams share a high antioxidant activity and that this sought-after property is associated with the health benefits of a yam diet (Chandrasekara and Kumar, 2016). Food yams such as *D. alata* (Chen and Lin, 2007; Cornago et al., 2011; Dilworth et al., 2012), *D. esculenta* (Cornago et al., 2011) and *D. japonica* (Hsu et al., 2011) possess high 1,1-diphenyl-2-picryllhydrazyl (DPPH) radical scavenging activity. These studies reported good correlations between the radical scavenging activity and the total phenolics contents of medicinal and food yam extracts, in agreement with the current knowledge on the high antioxidant activity of plant phenols and polyphenols (Shahidi and Ambigaipalan, 2015). However, knowledge on the phenolic compounds of yams remains incomplete.

An early exploration of yam phenolic compounds by HPLC revealed that food yams accumulate a rich blend of substances that belong to several chemical classes such as anthocyanins, simple phenolics, flavones and flavonols (Champagne et al., 2011). More recently, a survey of over 500 landraces and hybrids (covering eight species) of staple food yams using high-performance thin-layer chromatography (HP-TLC) was able to identify and quantify many phenolic substances (Lebot et al., 2019). These included allantoin, allantoic acid, chlorogenic acids, gallic acids and minor quantities of catechins (both catechin and epicatechin). Among them, chlorogenic acids, gallic acids and catechins are known antioxidant substances (Brand-Williams et al., 1995; Sang et al., 2002). However, despite the large range of authentic standards that was used, one substance present in many species that appeared yellow under white light could not be identified. Interestingly, this was the main phenolic substance of the tubers of some *D. nummularia* landraces which also accumulated the largest content of total phenolics (average of 9.55 mg/g).

The objective of this study was therefore to expand our knowledge of the major phenolics/polyphenolics substances of food yams. The major unknown phenolic/polyphenolic substance was purified from a *D. nummularia* landrace where its content was high. Its structure was determined by mass spectrometry and NMR. Its radical scavenging capacity and potential bacterial toxicity were then estimated. A similar process was applied to a minor, and structurally-related, substance. Finally, using the pure substances as standards, their content was estimated by HP-TLC in a panel of 411 yam landraces covering eight staple food species.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma-Aldrich (Darmstadt, Germany) unless otherwise stated. Solvents were of analytical grade and purchased from VWR International (Fontenay-sous-bois, France).

2.2. Plant material

All yam accessions (landraces and hybrids) were grown in a common field (Vanuatu Agricultural Research and Technical Centre – VARTC – located on Santo Island 80 m above sea level in Vanuatu – 15°23′S and 166°51′E) in August 2016 to minimize variations due to environmental factors. For each accession, four seed tubers were planted in a 1 × 1 m spacing and stacked together. The experimental setup contained 422 samples deriving from 411 accessions (acc.) of eight *Dioscorea* species. These included tubers of 273 *D. alata* acc. (216 acc. from Vanuatu, 57 acc. from India), 15 *D. bulbifera* acc., 22 *D. cayenensis-rotundata* acc., 2 *D. dumetorum* acc., 46 *D. esculenta* acc., 32 *D. nummularia* acc., 2 *D. pentaphylla* acc. and 4 *D. trifida* acc. and the aerial bulbils of 26 *D. bulbifera* acc. (complete list of samples and data in Supplementary data, Table A1). Healthy tubers were harvested at full maturity 10 months after planting.

2.3. Sample preparation

Sample preparation was similar to (Lebot et al., 2019). In short, freshly harvested tubers were washed, peeled and cut into 1 × 1 cm section French fries. Central fries were swiftly surface-dried with a clean towel to remove excessive mucilage and fully dried in a ventilated oven at 60−70 °C until constant weight. Dried chips were ground with a Forplex F00 1218 hammer mill (Boulogne, France) to less than 2 mm particle size powder. Powders were stored away from light at room temperature in Ziplock bags within plastic boxes containing silica gel.

Powders were either used for phenolic compound purification or were extracted with methanol for HP-TLC analyses.

2.4. Caryatin and 3′-O-methycaryatin purification

The high starch and fat content of yam tubers complicates secondary metabolite purification by generating unwanted precipitates and by slowing solubilisation steps. This was particularly the case for *D. nummularia* landraces because of the small nature of their starch grains (Lebot et al., 2017). The following protocol was therefore designed to circumvent these difficulties.

Dried tuber powder of *D. nummularia* landrace acc. DN2010 ("*nasghan iwé*" from the island of Vanua Lava, Banks archipelago) was first extracted with hexane (20 mL/g – 15 min incubation at 19 °C in a bath sonicator). The dried hexane extract yielded a white amorphous powder (3.1 mg/g dry tuber powder). The hexane-extracted powder was further extracted with methanol (20 mL/g dry initial tuber powder). Once dried, the methanol extract yielded a red/purple amorphous paste (13 mg/g dry initial tuber powder) which was re-dissolved in methanol (0.4 mL/g dry initial tuber powder) and left to precipitate at 4 °C for 12 h to remove excess polymeric sugars. The final supernatant was mixed with $CHCl_3$ and water to obtain a 28:8:1 chloroform:methanol:water mix. After discarding the upper purple phase and precipitates, the lower yellow phase was dried and dissolved in methanol. Less than 10 % of the two substances of interest was lost during these solvent extractions and cold precipitation steps. The final crude (but stable) methanol extract was injected in an HPLC-DAD (Agilent 1200, Santa Clara, USA) equipped with a RP-18 Nucleodur Sphinx column (250 mm x 10 mm, 5 µm bead diameter- Macherey Nagel, Hoerdt, France) and eluted with a mix of solvents A (0,4% formic acid in water) and B (acetonitrile). Linear elution gradient conditions were: 0 min 1% B, 3 min 1% B, 18 min 15 % B, 36 min 45 % B, 42 min 100 % B, 46 min 100 % B, 47 min 1% B, 54 min 1% B. The flow rate was 3.5 mL/min and the column was at room temperature. Peak detection wavelengths were set at 203 nm, 254 nm, 280 nm, 350 nm, and 450 nm. Peaks IV and VII (Fig. 1) were collected *via* a fraction collector (G1364C Agilent 1100, Santa Clara, USA). Injections were repeated until sufficient of each substance was obtained for NMR analysis. Purity of

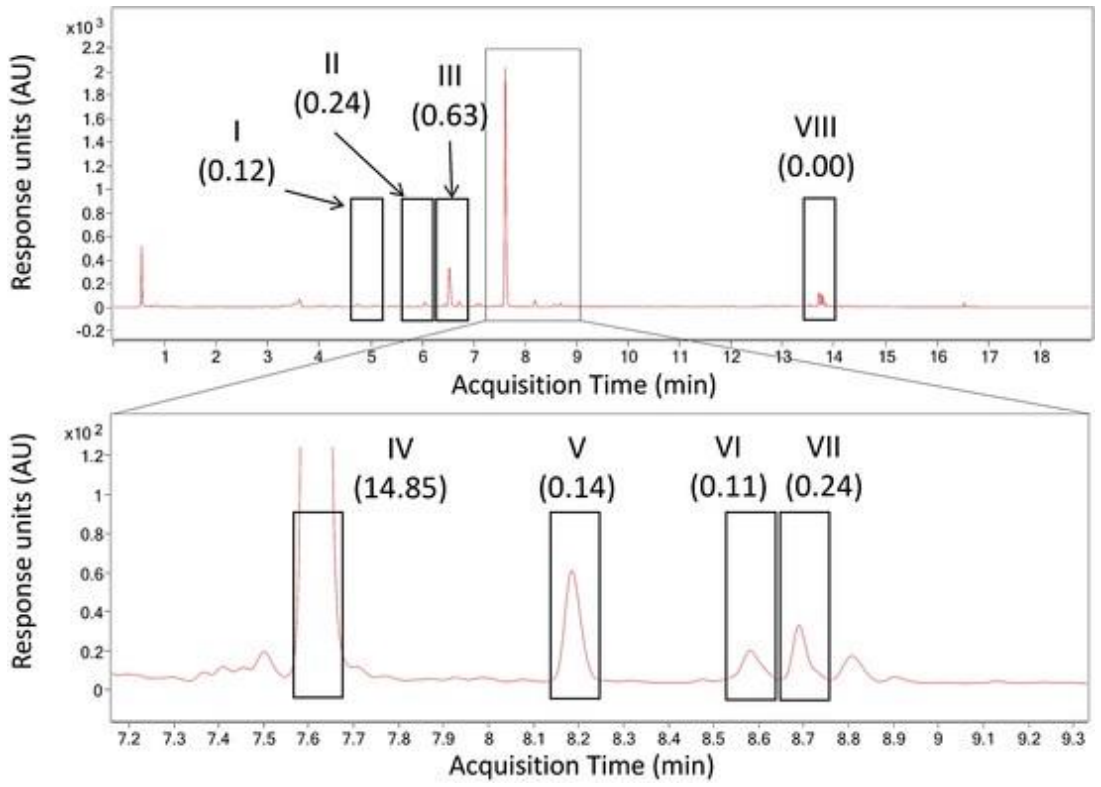

Fig. 1. HPLC-DAD analysis of a crude methanol extract of *D. nummularia* tuber. A typical chromatogram obtained with a detection set at 280 nm is shown. Numbers I-VIII refer to the collected fractions. Numbers in parenthesis represent the TEAC values (x10^3 mmol eq. trolox/fraction) of the fractions.

the collected substances was determined by UPLC-DAD-ESI-MS/MS (as in Section 2.5). If insufficiently pure (<95 % total ion count – TIC -- trace area), fractions IV and VII were re-chromatographed and fractions collection as described above.

2.5. UPLC-DAD-ESI-MS/MS analysis

UHPLC-DAD-ESI/QTOF analyses were performed on an Agilent Infinity® 1290 system (Agilent Technologies, Santa Clara, USA) coupled to a UV-vis DAD detector and a QTOF 6530 detector (Agilent Technologies, Santa Clara, USA) controlled by MassHunter® software (Agilent Technologies, Santa Clara, USA). Analytic separation was carried out on a Poroshell® 120 EC-C18 column (100 mm x 3.0 mm, 2.7 μm) protected by a guard column (Poroshell® 20 EC-C18, 5 mm x 3.0 mm, 2.7 μm). A gradient of 0.4 % formic acid in water (A) and acetonitrile (B) was applied with the following characteristics: 0 min, 1% B; 1.5 min, 1% B; 6 min, 10 % B; 12 min, 35 % B; 14 min, 100 % B; 16 min, 100 % B. The flow rate and column temperature were respectively set at 1.0 mL min^{-1} and 60 °C. A total of 2.0 μL of sample extract was injected. The ESI source operating conditions for positive ionization modes (in "Auto MSMS" acquisition mode) were: scan spectra from *m/z* 50-2000, capillary voltage 3.5 kV, nozzle voltage 2000 V, fragmentor 110 V, fixed collision-induced dissociation (CID) energy at 20 eV. Nitrogen was used as the nebulizing gas with a flow rate of 12 L min^{-1} and a temperature of 310 °C at 40 psi.

Spectroscopic and spectrometric characteristics of **1**: UV-vis spectrum: 346, 250, 260 (sh), 306 (sh); Pseudomolecular ion $[M+H]^+$: 331.0803 Da; MS/MS fragments (% relative abundance) : 316.0565 Da (78); 298.0463 Da (77); 270.0516 Da (100).

Spectroscopic and spectrometric characteristics of **2**: UV-vis spectrum: 344, 250, 258 (sh), 309 (sh); Pseudomolecular ion $[M+H]^+$: 345.0945 Da; MS/MS fragments (% relative abundance) : 330.0717 Da (100) ; 312.0624 Da (68); 284.0668 Da (86).

2.6. NMR analysis

NMR experiments were run at 25 ± 0.1 °C on a Bruker Avance III 500 spectrometer with BBFO probe for standards, or on a Bruker Avance III 1 GHz spectrometer with a TXI helium cooled cryo probe. NMR experiments were taken from Bruker's Topspin pulse program library. Relaxation delay was 2 s for all the experiments, a 30 degrees pulse was used for ^{13}C experiments, and 90 degrees for ^{1}H. Continuous wave water presaturation was used for COSY and NOESY experiments. All compounds were dissolved in deuterated methanol purchased from Eurisotop. Chemical shifts were referenced to solvent signal.

2.7. Synthesis of 3′-O-methylcaryatin

Compound **2** was synthesized according to the scheme shown on Fig. 2. The synthesis of the intermediate 4′,7-di-O-benzyl quercetin (compound **A**) was as reported by Ren et al. (2011). Compounds **B** and **2** were synthesized following procedures reported by Shi et al. (2012) with the following modifications.

Compound **B**: To a stirring mixture of dibenzylether **A** (69 mg, 0.143 mmol) and K_2CO_3 (138 mg, 1 mmol, 7 eq) in 6 mL dry DMF, methyl iodide (62 μL, 1 mmol, 7 eq) was added at room temperature. After 12 h, the resulting mixture was diluted with water (20 mL) and extracted with ethyl acetate (20 mL). The organic layer was washed with brine, dried over $MgSO_4$, filtered and concentrated. The crude material was purified by column chromatography (30 % AcOEt in petroleum ether) to give **B** as an oily product (52 mg, 70 % yield). ^{1}H NMR ($CDCl_3$, 500 MHz): 3.81 (s, 3H, OCH_3, 11-H), 3.90 (s, 3H, OCH_3, 12-H), 3.92 (s, 3H, OCH_3, 7′-H), 5.09 (s, 2H, OCH_2Ph, 7-H), 5.19 (s, 2H, OCH_2Ph, 4′-H), 6.38 (d, $J = 2.3$ Hz, 1H, 6-H), 6.54 (d, $J = 2.3$ Hz, 1H, 8-H), 6.94 (d, $J = 8.5$ Hz, 1H, 5′-H), 7.28-7.43 (m, 10H, OCH_2Ph), 7.58 (dd, $J = 8.5$ Hz, 2.1 Hz, 1H, 6′-H), 7.68 (d, $J = 2.1$ Hz, 1H, 2′-H); ^{13}C NMR ($CDCl_3$, 125 MHz): 56.2, 56.5, 60.0, 70.6, 70.8, 93.5, 96.4, 109.5, 111.8, 113.1, 121.5, 123.6, 127.3, 127.7, 128.1, 128.5, 128.7,

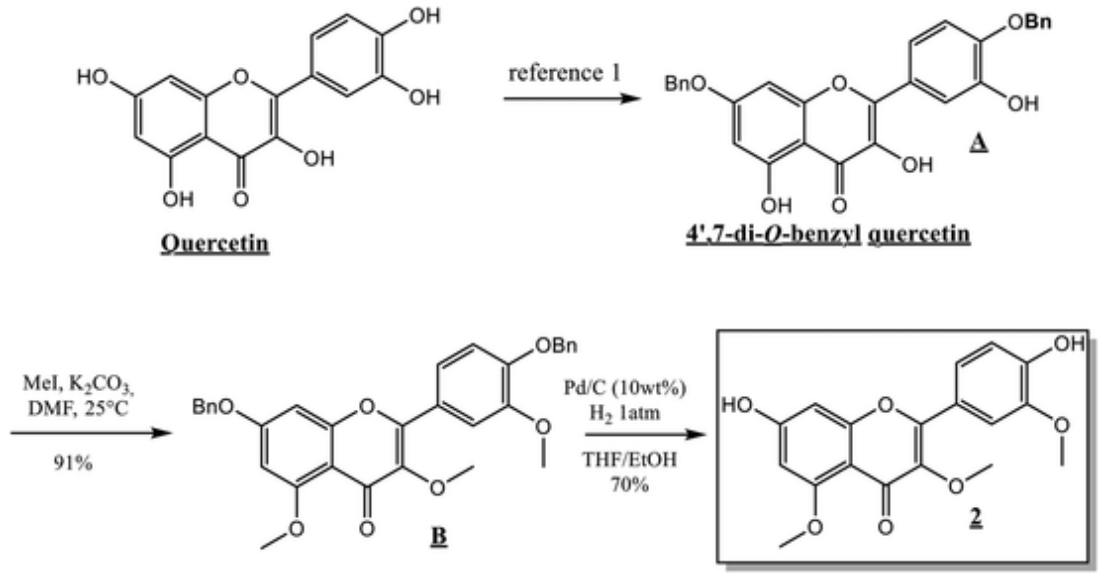

Fig. 2. Synthesis of compound **2** starting from quercetin.

128.8, 135.7, 136.6, 141.2, 149.2, 150.0, 152.7, 158.7, 161.0, 163.0, 174.1.

Compound **2**: To a solution of **B** (29 mg, 0.055 mmol) dissolved in ethanol (1.5 mL) and THF (1.5 mL), 5% Pd/C (10 mg) was added under vigorous stirring. The reaction vessel was evacuated and the atmosphere replaced by hydrogen. After 12 h, the reaction mixture was filtered through celite, concentrated and purified by flash chromatography (50 % AcOEt in petroleum ether) to yield **2** (9 mg, 50 %). ^{1}H NMR (CD_3OD, 500 MHz): 3.77 (s, 3H, OCH_3, 11-H), 3.90 (s, 3H, OCH_3, 12-H), 3.94 (s, 3H, OCH_3, 7′-H), 6.40 (d, J = 2.1 Hz, 1H, 6-H), 6.51 (d, J = 2.1 Hz, 1H, 8-H), 6.94 (d, J = 8.5 Hz, 1H, 5′-H), 7.61 (dd, J = 8.5 Hz, 2.1 Hz, 1H, 6′-H), 7.71 (d, J = 2.1 Hz, 1H, 2′-H); ^{13}C NMR (CD_3OD, 125 MHz): 56.4, 56.5, 60.3, 96.1, 97.1, 108.7, 112.8, 116.4, 123.1, 123.3, 141.5, 148.9, 150.7, 155.3, 160.2, 162.5, 164.8, 176.1.

C numbering followed accepted rules for flavonoids and is recalled on Fig. 3.

2.8. Antioxidant assay

Substances **1**, **2** and quercetin were dried and dissolved in methanol:water (80:20) at a final concentration of 0.1 mg/mL. They were then cascade-diluted by a factor of 3 in water. Diluted fractions (100 µL) were mixed with 1 mL of 12 h old 2,2′-azino-bis(3-éthylbenzothiazoline-6-sulphonic) acid (ABTS) solution (19.2 mg ABTS dissolved in 5 mL of water, mixed with 3.312 mg potassium persulfate and diluted to 400 mL with ethanol). After 30 min of incubation at room temperature, the absorbance was read at 734 nm and the antioxidant activity estimated against a standard curve of trolox. The remaining fraction of oxidized ABTS (expressed as % of initially oxidized ABTS = OD_{734} assay tube/OD_{734} blank tube containing 1 mL of oxidized ABTS and 100 µL of water) was then calculated for each assay tube and plotted against the quantities of substances **1**, **2**, quercetin or trolox (expressed in mmol) or volume of collected fractions I-VIII (expressed in l). TEAC indices were finally calculated for each purified substance as:

$$TEAC = (IC_{50}\ \text{Trolox}) / (IC_{50}\ \text{substance})$$

IC_{50}: quantity of pure substance (mmol) or collected fraction (l) yielding 50 % reduction of the initially oxidized ABTS.

The measurements were made in triplicate and the results averaged.

2.9. Anti-bacterial assay

Aliquots of −80 °C glycerol stocks of *Escherichia coli* and *Bacillus megaterium* were plated on solid nutrient agar. Single colonies were transferred to 10 mL of liquid nutrient agar and grown at 28 °C under gentle stirring (160 rpm) for 18 h. Proportionalities between the number of colony forming units (CFU) and OD_{600} were respectively determined to be 1 OD_{600} = 2.6 10^{10} CFU for *E. coli* and 1 OD_{600} = 1.2 10^{7} CFU for *B. megaterium*.

Dried substances **1**, **2** or tetracyclin (320 µg) were dissolved in 80 µL dimethyl sulfoxide (DMSO) and mixed with 720 µL sterile Mueller-Hinton (MH) liquid medium. They were then cascade-diluted by a factor of 2 directly in the wells of a 96 microwell plate (final vol-

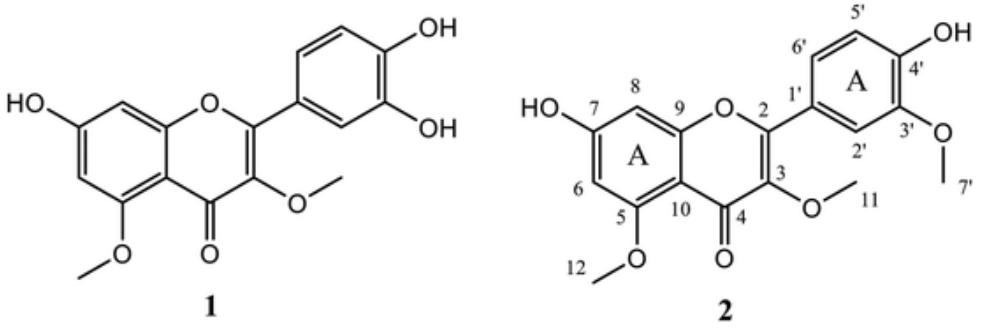

Fig. 3. Structure of the two substances purified in this study. **1**: caryatin; **2**: 3′-O-methylcaryatin. C-numbering and aromatic rings names are indicated on the structure of **2**.

ume of 50 µL). Sterile MH medium (100 µL) and 18 h old bacterial culture (50 µL) were then added. In parallel, a positive control (PCT) well was constructed that contained MH medium instead of the purified substances. Two types of negative control wells were also constructed. One only contained MH medium (NCT1 - to check for microbial contaminants) and one contained MH medium instead of the bacterial cultures (NCT2 - to subtract the absorbance of the diluted substances from the corresponding test samples). Each well was constructed in duplicate and average values were calculated. Plates were incubated at 28 °C for 24 h before being read at OD_{600}. The anti-bacterial activity (BT_{test}) was estimated with the following formula:

$$BT_{test} = ((OD_{600}\ test - OD_{600} - NCT2)*100/(OD_{600} + PCT - OD_{600} - NCT1))-100$$

and plotted against the substance concentration. Concentrations leading to 50 % inhibition of bacterial growth (IC_{50}) were then determined.

2.10. HP-TLC analyses

Methanol extraction (10 g powder and 20 mL of methanol) was carried in a bath sonicator (Lab Companion UC-02, Cole Parmer, Vernon Hills, IL, USA) for 10 min. Part of the supernatant was transferred to a 1 mL amber sample vial (Chromacol™, Thermo Fisher™, USA) and stored at 4 °C in the dark until analysis.

Yam tuber methanolic extracts (5.0 µL) were loaded on HP-TLC silica gel 60 F254 plates (Merk, Darmstadt, Germany, 20 × 10 cm glass plates, Ref 1.05642.0001) as straight 8.0 mm wide bands using an automatic sample loading unit (Camag, Muttenz, Switzerland). In parallel, increasing concentrations of purified caryatin and synthesized 3′-0-methylcaryatin were loaded to cover a concentration range of 1.0, 2.0, 4.0, 6.0, 8.0 and 10.0 µg. Plates were run for a total distance of 8 cm with 10 mL of toluene:ethyl acetate:formic acid (4:6:1 v/v) as mobile phase. Plates were air-dried and stained with NP reagent before being scanned at 366 nm. All machines were controlled online with winCATS™ software (Camag, Muttenz, Switzerland).

Caryatin and 3′-O-methylcaryatin were identified in the yam extracts on the basis of their color when visualized at 366 nm, their UV-vis absorption spectrum, their Rf values and a comparison with pure standards. Their quantity in the extracts was estimated from their peak areas (scan at 366 nm) that were plotted against standard curves constructed with the peak areas of the pure standards. Peak separation was good for the two substances in all extracts at the scanning wavelength. The repeatability of the measurements was determined to be greater than 95 % for both substances during two parallel analyses of a yam sample. The standard curves that were constructed with the two substances had a Pearson correlation coefficient of 0.99.

3. Results and discussion

3.1. Phytochemicals purification

HPLC-DAD analysis of crude *D. nummularia* methanol extracts typically yielded a single major UV absorbing peak flanked by minor peaks (Fig. 1). This major peak also made most of the total ion count (TIC) trace area from UPLC-MS/MS analysis. This agrees with an HP-TLC analysis of yam phytochemicals where *D. nummularia* methanol extracts were characterized by a single major yellow band and several minor bands (Lebot et al., 2019). This unidentified reported yellow substance had a similar UV-vis absorption spectrum as the major peak seen on Fig. 1.

The eight most UV-absorbing peaks of the crude *D. nummularia* methanol extract chromatogram were collected separately after their LC separation (fractions I-VIII on Fig. 1). Fraction IV clearly contained most of the antioxidant activity of the extract since it made 91 % of the sum of the TEAC of the tested fractions (which was roughly equal to the one of the injected sample). Focus was therefore placed on fraction IV. Attention was also placed on fraction VII because it was also a significant contributor to the overall antioxidant activity and because it was a single major substance (more than 85 % of the total TIC trace area from UPLC-MS/MS analysis) unlike fractions II and III. After a second LC cleanup, the two purified substances, **1** and **2** (Fig. 3) respectively originating from fractions IV and VII, represented more than 95 % of the total TIC trace areas from UPLC-MS/MS analysis.

3.2. Structure determination

Compounds **1** and **2** shared a similar UV-vis spectrum that was characterized by two absorption maxima (250 & 346 nm and 250 & 344 nm respectively) flanked by a shoulder (260 & 306 nm and 258 & 309 nm respectively). Such a UV-vis spectrum is typical of flavonols, a class of flavonoids (Markham and Mabry, 1975). The fact that absorption maxima were in the low range for flavonols suggested that **1** and **2** could be methoxylated at positions 3, 5 and/or 4′ (Markham and Mabry, 1975). Their respective pseudomolecular ions at *m/z* 331.0803 Da and 345.0945 Da for $[M+H]^+$ under HRMS suggested molecular formulas of $C_{17}H_{14}O_7$ and $C_{18}H_{16}O_7$ respectively. These correspond to a trihydroxydimethoxy flavonol and a dihydroxyltrimethoxy flavonol respectively. Both compounds had a similar MS/MS fragmentation profile with ions of *m/z* 316.0565 Da (-15, •CH3), 298.0463 Da (-33, $\bullet CH_3 + H_2O$), 270.0516 Da (-61, $\bullet CH_3 + H_20 + CO$) for **1** and 330.0717 Da (-15, $\bullet CH_3$), 312.0624 Da (-33, $\bullet CH_3 + H_2O$), 284.0668 Da (-61, $\bullet CH_3 + H_20 + CO$) for **2**. This suggests that they share some basic structural characteristics that differ by a methylene group or bridge and that they both contain at least one methoxy group. To confirm the flavonoid core structure and determine the position of the hydroxyl and methoxy groups, NMR analyses were conducted (full NMR data are present on Supplementary data, Fig. A1).

Compound **1**. ^1H and COSY NMR spectra of compound **1** showed two aromatic spin systems: one with 2 protons attributed to ring A (6.52 and 6.42 ppm, d, *J*=1.5 Hz, 1H, H6 and H8) and the other one with 3 protons corresponding to ring B : H-2′(7.63, d $J = 2$ Hz, 1 H) H-6′ (7.53 pm, dd, $J = 2$ and 8.5 Hz, 1 H) and H-5′ (6.9 ppm, d $J = 8.5$ Hz, 1 H). Two methoxy groups were visible on the ^1H spectrum. One of them (3.9 ppm, s, 3 H) gave a long range correlation with the proton at 6.42 ppm, which is attributed to H-6. The other methoxy showed a NOE interaction with protons 2′ and 6′ which confirmed its position on carbon 3. HSQC analysis assigned the primary and tertiary carbons (C-6′ : 122.1 ppm; C-5′ : 116.5 ppm; C-2′ : 116.3 ; C-6 : 97.3 ppm; C-8 : 96.2 ppm : O-CH_3 (3) : 60.0 ppm; O-CH_3(5) : 56.6 ppm). C-4 was identified by its chemical shift (176.2 ppm). HMBC analysis assigned C-1′ and C-3′ (^3J coupling with H-5′: respectively 123.1 and 146.3 ppm), C-10 (^3J coupling with H-6 and H-8, 108.5 ppm), C-7 (^2J with H-6 and H-8, 165 ppm,), C-9 (^2J with H-8, 160.2).

C2 and C4 showed the same correlations, (3 J coupling with H-2′, and H-6′,^4J or ^2J with H-5′) and were assigned to the peaks at 155.5 ppm and 149.4 ppm respectively, with the help of MNova simulation software.

It was therefore concluded that **1** is caryatin (Fig. 3). Caryatin has been previously described in two unrelated woody species, the pecan tree, *Carya illinoinensis* (Abdallah et al., 2011; Xu et al., 2020), and *Rhododendron delavayi* (Song et al., 2009) and in the medicinal yam of Nigeria, *D. hirtiflora* (Adeniran et al., 2020) and China, *D. bulbifera* (Gao et al., 2002). This study makes nevertheless, the first description of caryatin in the flesh of a food yam tuber.

Compound **2.** Three methoxy groups were visible on the proton NMR spectrum of compound **2**, as suspected by Mass and UV spectroscopies. ^1H and COSY NMR spectra showed a 3 spins systems in the aromatic regions corresponding to the 2′, 5′, 6′ protons of ring B. The coupling constants confirmed the assignment to 2′ (7.71 ppm, d,

J = 2.1 Hz, 1 H) 5′ (6.94 ppm, d, J = 8.5 Hz, 1 H) and 6′ (7.61 ppm, dd, J = 8.5 Hz, 2.1 Hz, 1 H). A second 2 spins system corresponded to the H-8 and H-6 protons of the second aromatic ring. Long range COSY weaker signals, confirmed by NOESY between H-2′ and the methoxy at 3.94 ppm, and between H-6 and the methoxy at 3.90 ppm assigned them to H-7′ and H-12 respectively, and removed ambiguity between the protons H-6 (6.40 ppm, d, J = 2.1 Hz, 1 H) and H-8 (6.51 ppm d, J = 2.1 Hz, 1 H). A weak NOE interaction was observed between H-11 methoxy (3.77 s, 3H, OCH_3) and H-2′ and H-6′ protons.

HSQC analysis assigned the corresponding protonated carbons (C-12: 56.4 ppm; C-7′; 56.5 ppm, C-11 ; 60.3 ppm; C-8 ; 96.1 ppm, C-6 ; 97.1 ppm; C-2′: 112.8 ppm; C-5′: 116.4 ppm, C-6‘; 123.3 ppm). C-4 was identified by its chemical shift (176.1 ppm). On the HMBC spectrum, the ^{3}J correlations between methoxy groups and carbons at 141.5, 148.9 and 162.5 ppm confirmed their assignment C-3, C-3′ and C-5 respectively. HMBC analysis assigned C-1′ (^{3}J coupling with H-5′123.1 ppm), C-4′ (^{2}J coupling with H-5′, 150.7 ppm), C-2 (^{3}J coupling with H-2′ and H-6′, 155.3 ppm) C-9 (^{2}J coupling with C-8, 160.2). The other HMBC correlations were consistent with the proposed structure (3′-O-methylcaryatin). The 2 remaining carbons (C-10, 108.7 ppm and C7 164.8 ppm) were differentiated by their chemical shift values, through simulations with MNOVA and ACDLabs softwares.

The NMR analysis of **2** was consistent with the proposed structure, but did not confirm it, hence an authentic of 3′-O-methylcaryatin was *de novo* synthesized (Fig. 2). The retention times and mass fragmentation profiles of the synthetic sample matched those of the purified substances through UPLC-DAD-MS/M analysis. Moreover, ^{1}H and ^{13}C chemical shifts of the purified **2** matched with an excellent precision (< 0.1 ppm) those of the synthetized authentic compound (Supplementary data, Fig. A1). The minor peaks at 0.7-1.5 ppm in the proton spectrum, and around 160 ppm in the ^{13}C spectrum were therefore attributed to impurities accompanying purified **2**. It is therefore concluded that **2** is 3′-O-methylcaryatin (Fig. 3).

3′-O-methylcaryatin has only been previously described in *C. illinoinensis* (Abdallah et al., 2011; Xu et al., 2020) and is described here for the first time in *Dioscorea*.

3.3. Biological activities

1 and **2** are flavonols, a class of phenolic compounds with known antioxidant activity (Shahidi and Ambigaipalan, 2015). Measurements of their ABTS radical scavenging activity revealed that **1** had a trolox equivalent antioxidant capacity (TEAC) index of 0.74 and **2** of 0.43. These indices were respectively 4 and 6.8 times lower than for quercetin, a common flavonol with known antioxidant activity (Table 1).

The mechanism of the antioxidant activities of plant phenolic substances including flavonols such as **1** and **2** has been extensively studied (Dueñas et al., 2010; Letan, 1966; Shahidi and Ambigaipalan, 2015). Structure-function analyses of flavonoid antioxidant activity have concluded that 3′,4′-diOH and to a lesser extent 5-OH, 3-OH and a conjugation of the A and B rings *via* a 4-keto and C2-C3 double bond are the positive contributors of the activity (Shahidi and Ambigaipalan, 2015). As such, quercetin has the highest activity of all flavonoids (Shahidi and Ambigaipalan, 2015) with its methyl ethers being less active. **1** and **2** were respectively found to only retain 2% and 1% of the quercetin derived lipid peroxidation protection activity (Letan, 1966). Using the ABTS radical scavenging assay, minor losses of activity were induced by O-methyl groups since 3′-O-methylquercetin retained a third of the TEAC index of quercetin (Dueñas et al., 2010). In agreement with this later observation, **1** and **2** TEAC indices were found to be 25 % and 14 % of the one measured for quercetin (Table 1).

Table 1
Biological activities of caryatin (**1**), 3′-O-methylcaryatin (**2**) and quercetin.

Substances	TEAC[a]	IC_{50} *E. coli*	IC_{50} *B. megaterium*
1	0.74 +/- 0.01	> 50 µg/mL	> 50µg/mL
2	0.43 +/- 002	> 50 µg/mL	> 50µg/mL
quercetin	2.96 +/- 012	-	-

[a] Trolox equivalent antioxidant capacity expressed as mean +/- standard deviation (n = 3).

Nevertheless, the impact of flavonols on animal health cannot be predicted from their sole *in vitro* antioxidant capacity. Methylation of quercetin has indeed been shown to improve intestinal absorption and confer resistance to hepatic metabolism (Wen and Walle, 2006). These two features increase the *in vivo* efficacy of flavonols on non-intestinal targets. Upon ingestion, **2** was actually found to be a more effective antioxidant (in terms of internal reduced glutathione and malondialdehyde levels) than **1** (Abdallah et al., 2011). Flavonols also provide additional benefits to animal health with different structure-activity relationships than their antioxidant capacity. For example, kaempferol-3,5-dimethyl ether displayed an at least 5 times greater anti-tumoral activity than **1** and myricetin (Gao et al., 2002). **2** had a greater hypoglycemic effect than **1** (Abdallah et al., 2011). Additional animal studies are still needed to estimate the respective health contribution of the different flavonols present in food preparations.

Both **1** and **2** exhibited no measurable toxicity towards a Gram positive and a Gram negative bacteria (Table 1). Concentrations above 8 µg/mL even improved bacterial growth and a 20 % increases in the cellular densities was observed for both bacteria at 50 µg/mL of **1** and **2**, probably because they served as carbon food source.

3.4. Caryatin and 3′-O-methylcaryatin distribution in yams

HP-TLC is a robust, accurate and cost-efficient technology for the high-throughput quantitation of phytochemicals (Agatonovic-Kustrin et al., 2017). It has proved to be effective on starchy produce such as yam tubers (Lebot et al., 2019)).

Using HP-TLC and standard curves constructed with pure substances, the contents of **1** and **2** were estimated in the mature tubers of a large panel of 411 landraces covering eight yam species (Supplementary data, Table A1; Fig. 4). Landraces of *D. nummularia* contained the highest contents of **1** and **2** with respective mean values of 310 and 91 µg/g of DW. Highest values were 1 030 and 457 µg/g DW respectively. Analyzed landraces of *D. bulbifera* also contained high contents of **1** and **2**, although both substances were of more similar abundance in *D. bulbifera* than in *D. nummularia*. Aerial bulbils contained roughly twice the quantities of **1** and **2** than tubers with respective mean contents of 216 and 174 µg/g DW. This agrees with the reported pharmacological use of this species (Guan et al., 2017; Kundu et al., 2020). With contents of **1** and **2** reaching up to 179 and 241 µg/g DW respectively, some landraces of *D. alata* also contained very important quantities of these substances. Nevertheless, most landraces accumulated no detectable amount of these substances. Within the analyzed panel of *D. alata* accessions, Indian landraces contained on average higher contents than those from Vanuatu with mean content values that were 4.1 and 13.2 times higher for **1** and **2** respectively. Only a few landraces of *D. esculenta* contained low amounts of **1** and **2** and **1** was only detectable in some landraces of *D. pentaphylla*. All analyzed landraces of *D. cayenensis-rotundata*, *D. dumetorum* and *D. trifida* contained no detectable amount of **1** and **2**.

One puzzling result of this study is the very disparate levels of accumulation of **1** and **2** which ranged from 0.1 % of dry weight content to nil in the analyzed sampling. Wild *D. nummularia* originates from The Philippines, Indonesia and Melanesia. It was domesticated on multiple, independent occasions in Melanesia during prehistoric times and quickly dispatched to neighboring Micronesia and Polynesia (Chaïr et al., 2016). This, and other Asian species such as the related to *D. alata* (Couto et al., 2018) and *D. bulbifera* accumulated high concentrations of **1** and **2**. However, accumulation of **1** and **2** was not important in all

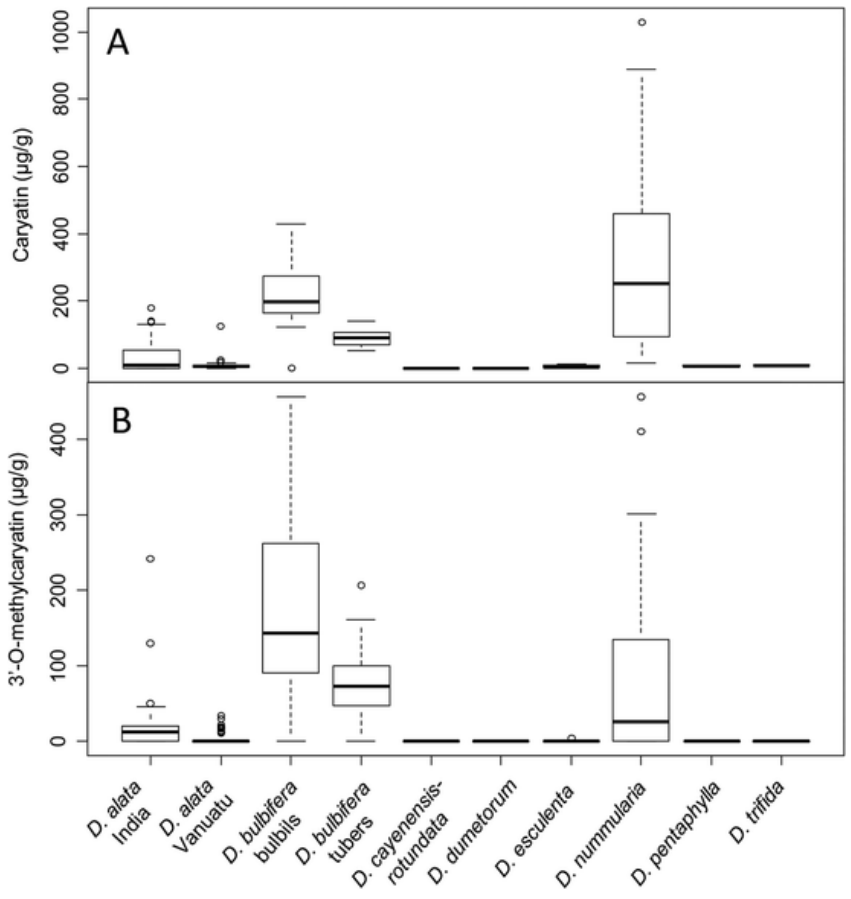

Fig. 4. Box plot analysis of caryatin (A) and 3′-O-methylcaryatin (B) contents in 411 accessions of yam spanning eight species.

Asian yams and may occur in non-Asian species such as the African yam, *D. hirtiflora*, also known to accumulate **1** (Adeniran et al., 2020). This study also found that intraspecific variations of **1** and **2** concentrations are as important as interspecific differences and may reach 2-3 orders of magnitude. This may be the result of a process of selection since all analyzed landraces are actively cultivated to serve a diversity of uses including daily food and ceremonial occasions. At its higher accumulation levels, **1** will provide a yellow tinge to the tuber flesh that can be used as a selection character. Inversely, the quest for white ceremonial varieties may explain the existence of landraces with barely undetectable amounts of **1**.

One major contribution of this work is that it reveals that **1** and **2** are significant contributors of the antioxidant activity of the tubers of many landraces of *D. bulbifera*, *D. alata* and *D. nummularia*. In the case of the *D. nummularia* landrace that was used for the purification of **1** and **2**, these substances were the main phenolics/polyphenolics (this study and Lebot et al., 2018) and logically the main contributors of its antioxidant activity. However, many food yams may accumulate additional phenolic substances with antioxidant activity such as chlorogenic acids, gallic acids and catechins (Lebot et al., 2018). These will be additional contributors of the tubers antioxidant power. Based on the results of this study and those of Lebot et al. (2018), the respective accumulation of all these phenolic substances will be very dependent on species and, to a similar extent, to the landrace that is selected. Although it has not yet been explored, growing conditions may also have an impact on the accumulation levels of individual phenolics.

This study raises interest in the marginally-cultivated South Pacific yam *D. nummularia*. Its tubers contained the highest quantities of **1** and **2** of the analyzed yam landrace panel. This species is the main staple crop of large parts of Papua New Guinea and Vanuatu (Walter and Lebot, 2003). It exhibits several superior agronomic characteristics to other cultivated staple yam species such as high yields (up to 40 t/ha), resistance to anthracnose (*Colletotrichum gloeosporioides*), development on many soils (incl. acidic) and production of tubers that are easier to harvest (less buried, no attachment point) and contain a high dry matter (31-36 %) and starch (78-90 %), contents that most likely explain their high food appreciation and long shelf life (Lebot et al., 2017).

4. Conclusion

This study confirms that several widely cultivated species of food yams contain caryatin (**1**) and 3′-O-methylcaryatin (**2**), two antioxidant substances with known benefits for human health. They were most abundant in *D. bulbifera*, that has a long record of food and medicinal use, *D. alata* and *D. nummularia* where caryatin alone explained over 90 % of the total antioxidant activity of a landrace tuber methanol extract. Measured intra- and interspecific variations in concentration were equally large. This suggests that there is ample room for biofortification of many yams with these substances *via* interspecific hybridization and intraspecific landrace improvement.

Author contributions

S.C., V.L. and L.L. conceived and designed the experiments. B.F., A.B., F.F., V.L. and L.L. performed the experiments. B.F., A.B., F.F., S.C., V.L. and L.L. analyzed the data. L.L. wrote the manuscript reviewed by all authors.

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Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jfca.2021.104010.

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